Attachment 1. II Copy Library Date 24.07.89

ETHICAL REVIEW COMMITTEE, ICDDR,B.

PROJECT COORDINATOR
~~Principal~~ Investigator S. TIPOKI

Co-Principal Investigator (if any) _____

Application No. 89-005

Supporting Agency (if Non-ICDDR,B) _____

Title of Study Establishment and application of virological techniques to study the epidemiology of rotaviruses and ~~other viral agents causing diarrhoea~~ in rural and urban Bangladesh.

Project status:
(✓) New Study
( ) Continuation with change
( ) No change (do not fill out rest of form)

Circle the appropriate answer to each of the following (If Not Applicable write NA).

1. Source of Population:
   (a) Ill subjects — Yes (No) — N/A
   (b) Non-ill subjects — Yes (No)
   (c) Minors or persons under guardianship — Yes No
2. Does the study involve:
   (a) Physical risks to the subjects — Yes (No) — N/A
   (b) Social Risks — Yes No
   (c) Psychological risks to subjects — Yes (No)
   (d) Discomfort to subjects — Yes (No)
   (e) Invasion of privacy — Yes (No)
   (f) Disclosure of information damaging to subject or others — Yes (No)
3. Does the study involve:
   (a) Use of records, (hospital, medical, death, birth or other) — Yes (No) — NO
   (b) Use of fetal tissue or abortus — Yes (No) — NO
   (c) Use of organs or body fluids — Yes No
4. Are subjects clearly informed about:
   (a) Nature and purposes of study — Yes (No) — N/A
   (b) Procedures to be followed including alternatives used — Yes No
   (c) Physical risks — Yes No
   (d) Sensitive questions — Yes No
   (e) Benefits to be derived — Yes No
   (f) Right to refuse to participate or to withdraw from study — Yes No
   (g) Confidential handling of data — Yes No
   (h) Compensation &/or treatment where there are risks or privacy is involved in any particular procedure — Yes No
5. Will signed consent form be required: — N/A
   (a) From subjects — Yes No
   (b) From parent or guardian (if subjects are minors) — Yes No
6. Will precautions be taken to protect anonymity of subjects — (Yes) No — N/A
7. Check documents being submitted herewith to Committee:
   ___ Umbrella proposal - Initially submit an overview (all other requirements will be submitted with individual studies).
   ___ Protocol (Required)
   ___ Abstract Summary (Required)
   ___ Statement given or read to subjects on nature of study, risks, types of questions to be asked, and right to refuse to participate or withdraw (Required)
   ___ Informed consent form for subjects
   ___ Informed consent form for parent or guardian
   ___ Procedure for maintaining confidentiality
   ___ Questionnaire or interview schedule *

* If the final instrument is not completed prior to review, the following information should be included in the abstract summary:
   1. A description of the areas to be covered in the questionnaire or interview which could be considered either sensitive or which would constitute an invasion of privacy.
   2. Examples of the type of specific questions to be asked in the sensitive areas.
   3. An indication as to when the questionnaire will be presented to the Cttee. for review.

AUG 15 1989

(PTO)

We agree to obtain approval of the Ethical Review Committee for any changes involving the rights and welfare of subjects before making such change.

S. TIPOKI
~~Principal Investigator~~
PROJECT COORDINATOR

Trainee

1. Investigator(s) : Dr. Fu Bingnan
Mr. J. Gomes
Ms. K. Khan
Ms. N. Nahar
Mr. B. Paul
Dr. G. Podder
Dr. N. Shahid
Ms. L. Unicomb

2. Title of Project : Establishment and application of virological techniques to study the epidemiology of rotaviruses and other viral agents causing diarrhoea in rural and urban Bangladesh.

4. Starting Date : June 1989

5. Date of Completion : June 1992

6. Total Budget Required : US$ 262,000

7. Funding Sources :

8. Programme Coordinator : Dr. Saul Tzipori

9. Aims of Program :

a. General Aim :

To establish a virology laboratory to monitor the role of enteric viruses in diarrhoeal disease in clinical and epidemiological investigations, and to assess the efficacy of viral vaccines in Bangladesh.

b. Specific Aims :

1. To develop standard virological techniques and diagnostic capabilities at ICDDR,B.

2. To detect and characterize group A rotavirus and identify the serotypes most predominant in rural and urban Bangladesh.

3. Use methods such as serotyping and electropherotyping, to follow the epidemiology of Group A rotavirus in the population over a protracted period to monitor the frequency of appearance and/or disappearance of viral strains.

4. To develop capabilities to measure the immune response (isotype and serotype specific systemic and secretory antibody) to group A rotavirus, to assess future rotavirus vaccine candidates.

5. To assess the relative contribution to diarrhoea of other enteric viruses (non-group A rotavirus, enteric adenovirus, astrovirus and Norwalk Agent) in Bangladesh, by developing appropriate diagnostic tests.

6. To determine the role of enteric viruses in persistent diarrhoea, in passive surveillance (cholera vaccine trial) and ICDDR,B surveillance programme-Dhaka.

7. To provide diagnostic virology (rotavirus group A and enteric adenovirus) service to patients admitted to the Clinical Research Centre with dehydrating diarrhoea below the age of 3 years.

c. Significance :

The establishment of virology at ICDDR,B is essential because of the significant role of enteric viruses (particularly group A rotavirus) in the aetiology of diarrhoea.

10. ETHICAL IMPLICATIONS :

The studies will utilize stool samples which have either been collected, or will be collected in the future. The studies will not directly involve human subjects.

11. BACKGROUND :

Group A Rotaviruses (RV)

Group A RV is the most common cause of dehydrating childhood diarrhoea in many parts of the world. Approximately 20% deaths from cases of diarrhoea in children under-five years of age are attributed to rotavirus[1]. Development of RV vaccines are in progress and some have undergone clinical and field trials[2,3]. It is likely that protection against RV disease is serotype specific. Therefore antigenic diversities of RV in the community needs to be determined before RV vaccine can be used.

The genome of RV consists of 11 segments of double-stranded RNA which can be separated by polyacrylamide gel electrophoresis. Electrophoresis therefore allows

comparisons between different strains by differences in segment mobilities. Two major electropherotypes have been described, designated long and short. In RV from humans, these patterns correlate with subgroup specificities, the long pattern corresponding to sub-group II and the short pattern corresponding to sub-group I[4]. Electropherotype diversity has been observed within and between communities[5], and have been used extensively to monitor the epidemiology of RV strains in a given population over time[6].

RVs are classified into several serotypes. The serotype antigens VP7 and to a lesser degree VP_3, are outer capsid polypeptides[7] where VP7 is the major serotype protein and has been shown to correlate with serotype specificity[20]. There are at least 2 subgroups and 9 serotypes of group A RV. Human RV serotypes are 1 to 4 and 8 and 9; serotypes 1 to 4 being the most commonly found.

A number of studies on the 4 major Group A human serotypes have revealed different serotype frequency[9,10], with serotype 1 currently being thought to be the most common world wide.

Non Group A Rotavirus :

Some RV were found to lack common group antigen and therefore do not react in conventional serological tests with Group A despite being morphologically identical. These

atypical RV which have been isolated from humans and animals are designated Group B and C[13,14]. Several reports have implicated group B[13] and C[14] in outbreaks and sporadic cases of diarrhoea in human. Their presence, prevalence and role in diarrhoea in Bangladesh is not known.

Adenovirus (AV) : Some AV have been known to produce diarrhoea. Forty two human types have been identified so far of which types 40 and 41 (Ad 40 and Ad 41) belonging to the subgenus F, produce diarrhoea[26]. AV are double stranded DNA viruses and replicate in cells of epithelial origin probably by attachment to cells via the fibre structure. Investigations worldwide, have shown that enteric AV are associated with between 7 and 17% of diarrhoea cases in children under 5 years of age[15].

Ad 40 and Ad 41 cause diarrhoea throughout the year and clinical features include watery stool, vomiting and moderately elevated temperature. Observation by Uhnoo et al. that 70% of sera from children with enteric adenovirus-associated diarrhoea displayed a type-specific seroconversion, measured by haemagglutination inhibition, supports the notion that enteric adenoviruses are causative agents of diarrhoea[16]. There are no reports on the occurrence of these viruses in Bangladesh.

Norwalk virus : Norwalk viruses cause a diarrhoeal disease of rapid onset and recovery often with mild systemic illness affecting all ages[17]. There are at least 3 serotypes known. Greenberg et al. studied the prevalance of antibody to Norwalk agent in children from many parts of the world. Children in U.S. and Yugoslavia acquired antibody more slowly than did children in Equador and Bangladesh[18]. The high prevalance of Norwalk antibody in children in developing countries may indicate that the Norwalk or an antigenically related virus infects children early in life. The importance of this virus in developing countries still remains to be determined.

Other Enteric Viruses

Astrovirus : Astroviruses were first described in 1975 in the faeces of newborn babies in Glasgow with and without diarrhoea[19]. The 28 nm particle was shed in large numbers, and has a star-like appearance. Astroviruses have been seen in two outbreaks of nosocomial diarrhoea in children. Rise in serum antibody to astrovirus was detected in 66% of volunteers who were fed stool filtrate containing the virus.

Calicivirus : Comprise a genus within the family Picornaviridae. They are small spherical viruses (30 - 40 nm) and infect mucous membranes of the respiratory and GI tracts and have been identified in diarrhoeal stools of

children[21]. At present the prevalence of such agents in developed and developing countries is not known.

Research Plan

1. Establishment of virological techniques and training of staff.

This will entail establishment of currently available ELISA tests, tissue culture techniques, serotyping tests and electrophoretic techniques, especially for group A RV and enteric AV. ELISA tests will be developed for the detection of group B RV, Norwalk agent, and astrovirus, and for antibody measurements particularly for group A and enteric AD. Tissue culture adaptation of epidemiologically significant or unusual strains of group A RV will also be carried out (see appendix for procedures)

2. Characterisation of Group A rotaviruses

The initial strategy for the detection of group A RV from a variety of studies would be :

1) Detection in stools by ELISA (commercial test initially, eventually a cheaper "in house" ELISA will be developed).

2) Extraction of ds RNA from stools that are RV positive for electropherotyping.

3) Serotyping of representative electropherotypes using;

i) ELISA incorporating serotype specific monoclonal antibodies (commercially obtained). (see appendix).

ii) Serotype specific, redioactively labelled synthetic oligonucleotide probes (available from the Armed Forces Research Institute and Medical Sciences (AFRIMS), Bangkok). This will be possible now with the establishment recently of DNA laboratory at the Centre. (see appendix).

A comparative study will be conducted to determine the relative merits of the two serotyping methods.

iii) adaptation to growth in cell culture of rotavirus strains that show unusual electrophoretic or serotypic pattern for further characterization.

3. Measurement of immune response to rotavirus.

A number of ELISA tests developed at The Royal Children's Hospital (RCH), in Melbourne for the measurement of isotype serospecific and group specific secretory and systemic antibodies are being adapted for use here. These tests include ELISAs for the detection of RV specific serum IgG, IgA, IgM, feacal IgG, IgA, IgM and salivary IgA antibodies. Likewise, tissue culture assays for neutralising antibodies, both in serum and faeces are being developed. (see appendix).

4. Diagnostic function

Diagnostic techniques for the detection of group A RV will be provided for patient care at the Clinial Research Centre. The service will be restricted to children younger than 3 years who are admitted with dehydrating watery diarrhoea.

It is expected that 20-30 specimens per day will be analysed for rotavirus, totaling approximately 8000 per year.

5. Collaborative studies which include virology

a) The effectiveness of Live Oral Polio Vaccine (TOVP) in children with Gastroenteritis.

With Urban Volunteer Programme and Clinical Sciences Division

b) Immune response in severely malnourished children following measles immunization.

With Clinical Sciences Divison

c) Cohort study on heterotypic and homotypic cross-protection among rotavirus serotypes, and shigella serotypes and serogroups, respectively.

(a major study which is currently being developed at LSD, it will include measurements of secretory and serum specific RV antibody in children and their mothers over a period of 3 years).

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12. Publications of Principal Investigators.

SCIENTIFIC PUBLICATIONS AS 1ST AUTHOR : (Paper)

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SCIENTIFIC PUBLICATIONS AS CO-AUTHOR : (Paper)

1. Samadi AR, Chowdhury AI, Huq MI, Shahid NS. Risk factors for mortality of complications for diarrhoea in Bangladesh. British Medical Journal. 290:1615-1617, 1985

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PUBLICATIONS : Leanne Unicomb

1. Rotavirus serotypes causing acute diarrhoea in hospitalized children in Jogjakarta, Indonesia.
Ruth F. Bishop, Leanne E. Unicomb and Graeme L. Barnes submitted for publication.

2. Epidemiology of rotavirus strains infecting children throughout Australia during 1986-1987 : A study of serotype and RNA electropherotype.
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G. Gerna, N. Passarani, M. Battaglia, L.E. Unicomb, M. Parea, A. Sarasini aand R.F. Bishop
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M. John Albert, Leanne E. Unicomb, Graeme L. Barnes and Ruth F. Bishop J. Clin. Microbiol. 25:1635-1640

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M. John Albert, Leanne E. Unicomb and Ruth F. Bishop
J. Clin. Microbiol. 25:183-185

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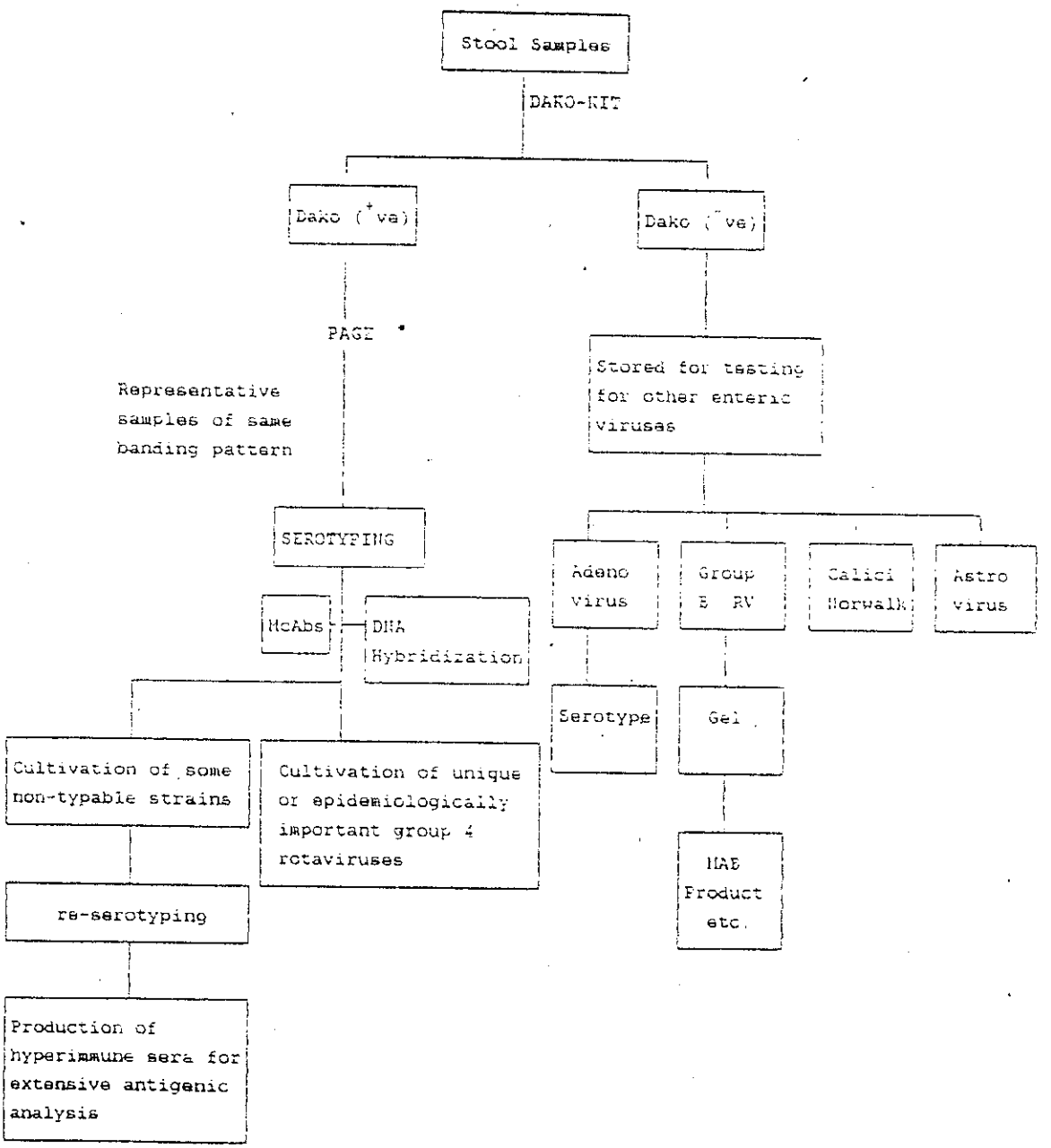
J. Clin Microbiol 24:1023-1028

13.

FLOW CHART 1

Working Plan - Study of Group A Rotaviruses and Other Enteric Viruses



FLOW CHART 2

Activities of Virology Laboratory - Years 1-2

1st year : * Gp A RV diagnostic — 8000 specimens

" hospital surveillance — 3000

" persistant diarrhoea study — 2053

" cholera vaccine trial — 5720

Second year: * Gp A RV diagnostic — 8300 specimens

hospital surveillance - * gp A RV

enteric Adenovirus+ (enteric AV) — 3200

Cholera Vaccine Trial - enteric AV

other enteric viruses++ — 5720

Persistent diarrhoea study - enteric AV

- other enteric viruses — 2053

Third year : Diagnostic - gp A RV*

- enteric AV+++

- other enteric viruses + — 8500

Hospital Surveillance - gp A RV

- enteric AV

- other enteric viruses — 3500

1. * detection of group A RV, RNA gel pattern, serotyping by ELISA and DNA hybridization
2. + enteric AV - detection of enteric AV, determination of serotype
3. ++ other enteric viruses - detection only.
4. +++ direct detection of enteric adenoviruses using serotype 40, 41 specific monoclonal antibodies

14. SPECIFIC TASKS OF EACH INVESTIGATOR

Dr. Fu Bingnan :

- Performance of rotavirus serotyping by hybridization of RV RNA with serotype specific oligonucleotide probes.

Ms. K.J. Khan : Ph.D. student.

- Development of ELISA test for detection of AV in faeces
- Examination of stools for presence of enteric AV
- Analysis of AV DNA for determination of serotype
- Optimization of cultivation of enteric AV in cell culture and investigation of mechanisms of AV adherence and penetration.
- Development of monoclonal antibodies to enteric AV.

Ms. Nahar :

- Performance of adapted ELISA tests, tissue culture assays and RNA gel electrophoresis.
- Quality control testing of reagents such as hyperimmune antisera, cell culture batches of group A rotaviruses and other viruses.

Mr. B. Paul :

- Maintenance of tissue culture reagents and equipment.
- Propagation of cell lines.
- Propagation of adapted group A rotaviruses.
- Adaptation of faecal Group A rotavirus to cell culture.

Dr. G. Podder :

- Performance of ELISA tests, tissue culture assays and RNA gel electrophoresis.
- Adaptation of previously developed ELISA test for use in the Virology Laboratory.
- Development of new ELISA tests such as development of an "in-house" ELISA for the detection of group A rotavirus in stool.

Dr. N.S. Shahid :

- Adaptation of ELISA tests for the detection of Norwalk agent in faecal samples.
- Adaptation of Norwalk virus to growth in cell culture.
- Development of a test to measure antibodies against the agent, either ELISA or neutralizing test.
- Adaptation of ELISA test for Calicivirus detection.
- Study of relatedness of Calici and Norwalk viruses by means of neutralization test.

Ms. L. Unicomb :

Coordination of above laboratory staff and Ph.D. student with the exception of Dr. Fu Bingnan.

- Collaboration with Dr. Shahid.
- Supervision of cell culture laoratory and facilities.
- Development of ELISA tests for non-group A rotaviruses and astrovirus.
- Adaptation of non-group A rotaviruses to cell culture
- Provide advice to all scientific staff at ICDDR,B on matters of virology and cell culture.

Appendix A:

Collection of stool specimens

Stool samples from the following protocols will be subjected to viral analysis.

1. Dhaka Treatment Centre's Surveillance Program

This has been an ongoing activity of the Dhaka ICDDR,B Treatment Centre since 1979. Every 25th patient coming to the Centre is entered into the program for indepth clinical microbiological and demographic work-up. Stool samples are requested from these patients and examined for various pathogens including Shigella, Salmonella enterotoxigenic E. coli (ETEC), V. cholerae and other vibrio species and C. jejuni by standard techniques. Rotavirus is currently identified by Dakopatt ELISA Kit. For the purpose of this project an aliquot of stool samples will be made separately in 1 drum vials and stored at - 70° for analysis. Approximately 3000 samples per year will be analysed for RV group A initially and other enteric viruses once techniques have been fully established.

2. Epidemiology of Persistent diarrhoea in Bangladesh children.

This community-based prospective study was planned to describe and quantify the problem of persistent diarrhoea in children under 5 years of age from rural Bangladesh and to identify important risk factors. A one year study was conducted whereby six hundred children aged 0-47 months of

age were enrolled and followed twice a week for the occurance of diarrhoea (acute and chronic). 2055 stool samples have been collected in this study which will be analysed for the presence of RV group A and subsequently for other enteric viruses.

3. Matlab passive surveillance (Cholera vaccine trial)

Stool samples from patients with diarrhoea coming from the DSS area who were admitted to all the three treatment centres of Matlab, Nayergaon and Kalirbazar since January 1985 were enrolled for "Passive" Surveillance. Rectal swabs were plated for salmonella/shigella and V. cholerae and E. coli. An aliquot of stool was stored at -20°C for future investigation. The study is ongoing and upto May 1, 1989, 12,800 samples have been collected. A total of 815 specimens have been sent to Dr. R. Ward of the Gamble Institute, USA, and 6265 specimens have been tested for rotaviruses so far. The outstanding 5720 specimens will be analyzed for the presence of group A rotavirus and for other enteric viruses. The 728 specimens remaining in Dhaka that are positive for group A rotavirus will be further studied (RNA electropherotyping, serotyping, cultivation).

APPENDIX B

RNA Electropherotyping : Double stranded RNA segments are extracted directly from faecal samples or from tissue culture supernatant and electrophoresed according to the method Herring et al (1982)[22].

1. Extraction of ds RNA

(a) Adjust pH to 5.6

i.e. if faecal homogenate is made in PBS or H_2O or if TC-SNT is used, add one-tenth of the vol. used of 1M sodium acetate pH 5.6 (i.e. if extracting 200 ul).

OR Make up faecal homogenate in 0.1M sodium acetate pH 5.6

(b) add an equal volume of the following extraction mixture to faecal homogenate/TC-SNT etc. (use a tube that fits in microfuge/centrifuge).

e.g. 200 ul faecal homogenate + 200 ul of the following extraction mixture.

3:2*(phenol-m-cresol-8 hydroxyquinoline - phenol mixture)
(chloroform-isoamyl alcohol 24:1)

For 10 ml

160 ul isoamyl alcohol + 3.9 ml chloroform + 6 ml phenol mixture.

recipe for phenol mixture:500g crystalline phenol + 70g

m-cresol + 0.5g 8.OH quinoline + 200g H_2O.

- this can be aliquotted in 20 ml lots and stored at -20°C.

(c) Vortex sample and extraction mixture for 1 min. and spin in a microfuge for 2 mins.10,000 rpm).

(d) retain top layer (this is the nucleic acid fraction) and discard the rest.

(e) ds RNA can be concentrated if required by adding 2 volumes of ethanol and freezing at -20°C for a few hours. After freezing, centrifuge in a microfuge and resuspend the pellet in an appropriate volume of distilled water or can be frozen, neat, at -20°C.

2. Electrophoresis. RNA samples are mixed with sample buffer containing bromophenol blue, and applied to 7.5mm thick, 10% polyacrylamide gels using a discontinuous buffer system, according to Laemmli[24], in the absence of SDS. Samples are electrophoresed for 16-18 hours at 15 mA set current.

3. Silver Staining of Polyacrylamide Gels : Gels are fixed and stained with silver according to the method of Dyall-Smith and Holmes[23].

(a) Cut stacking gel from the top of the gel and soak gel for a minimum of 30 mins. in fixer (10% ethanol,

0.5% acetic acid) - gels can be kept overnight in fixer if required.

N.B. : Make sure that there is an appropriate marker on the gel (such as a cut off corner) so that first and last samples etc. can be distinguished.

(b) Aspirate fixer and soak gel in 0.011M silver nitrate for a minimum of 30 minutes.

(c) Aspirate silver nitrate, rinse with distilled water and add a reducing solution of -

0.75M NaOH, 0.1M formaldehyde and

(d) aspirate reducing solution after the bands have been visible for 10 minutes, and store gels in 5% acetic acid until photographed.

N.B.: use 200 ml volumes.

APPENDIX C

ENZYME IMMUNOASSAY (EIA) FOR SEROTYPING GROUP A HUMAN ROTAVIRUSES USING MONOCLONAL ANTIBODIES - B (according to the method of Coulson et al 1987)[25].

1. Coat 96F NUNC tray with 100 ul/well of rabbit antisera to rotavirus serotype 1-4 diluted in phosphate buffered saline (PBS) pH 7.2 as follows:

Row	1	Anti-RV4 (serotype 1)	1:8,000
	2	Anti-RV5 (serotype 2)	1:6,000
	3	Anti-RV-3 (serotype 3)	1:6,000
	4	Anti-ST-3 (serotype 4)	1:8,000

2. Incubate tray at 37^{o} for 2 hours in a moist environment.
3. Wash x 3 with phosphate buffered saline pH 7.2 containing 0.05% Tween 20 (PBST).
4. Add 75 ul of skim milk powder solution (SMP) to each well, followed by 25 ul of 10% (W/V) faecal supernatant or 100 ul of control sample. Add each sample to all 4 wells comprising a column of the plate, e.g. sample No. 1 in column 1, sample No. 2 in column 2.
5. Incubate at 4^{o} for 16-20 hours.
6. Wash as in step 3.
7. Add 100 ul of mouse monoclonal antibodies to each well, diluted in SMP as follows:

Row	1	Mab RV-4:2	Protein A fraction	1:200
	2	Mab RV-5:3	Protein A fraction	1:2,000
	3	Mab RV-3:1	Protein A fraction	1:2,000
	4	Mab ST-3:1	Protein A fraction	1:1,000

or kit abs as above but diluted 1/200

8. Incubate at 37^{o}C for 2.5 hours in a moist environment.

9. Wash as in step 3.

10. Add 100 ul/well anti-mouse immunoglobulins conjugated to horseradish peroxidase at 1/800 (DAKO) diluted in SMP, or SILENUS brand (1/2,000).

11. Incubated at 37°C for 1.5 hours in a moist environment.

12. Wash as in step 3.

13. Add 100 ul TMB substrate to each well. Incubate at room temperature for 10 minutes and then stop the reaction with 50ul 2M H_2SO_4.

14. Read plates by eye or at 450 nm in a spectrophotometer.

APPENDIX D

DNA Hybridization for detection of serotypes of group A rotaviruses

Rotavirus RNA Extraction

1. Suspend 50-100 mg faecal sample in 0.5 ml of 0.1M sodium acetate, 1% SDS pH5.
2. Extract with phenol-chloroform mixture and keep the supernatant (dsRNA).

Blotting of RNA onto Nitrocellulose

1. Mix the RNA solution with one volume of 6.15M formaldehyde, 10X SSC.
2. Incubate at 65°C for 15 mins.
3. Spot on the nitrocellulose using Bio-dot apparatus.
4. Rinse with 10X SSC.
5. Air dried and bake at 80°C for 2 hrs.

Oligonucleotide probes labeling

1. 5-end labelled 8 pmol of probe with r-32p-ATP (3000 Ci/mmol).
2. Separate the labelled probe from unincorporated ATP by passing through Sephadex G-25 column.

Probe Hybridization

Buffer : 3X SSC, 0.5% BSA, 0.5% PVP, 1% SDS

Probe concentration : 10^6 cpm/mL hybridization buffer

Hybridization Temperature :

$38^{o}C$ for HuG2Ac and HuG3Ac

$42^{o}C$ for HuG1Ac, HuG4Ac, BoG6Ac and PoG5Ac

$45^{o}C$ for Hug8Ac

Washing condition :

1. 3 X SSC at Room temperature for 30 mins.
2. 3 X SSC at the hybridization temperature for 10 mins, twice.
3. 2 X SSC at room temperature for 1 hr.

APPENDIX E

Cultivation of tissue culture adapted rotaviruses

1. Activate virus stock with 10 ug\ml of porcine trypsin (Sigma -fraction IX) for 15 mins. to 30 mins. at 37^{o}C.
2. Wash confluent monolayer of MA104 cells x 2 with PBS.
3. Remove remaining PBS with pasteur pipette.
4. Add activated stock to monolayer (usually 1-2 ml/medium sized flask - 75 cm^{2}).
5. Incubate at 37^{o}C for 1h with gentle agitation every 15 mins.
6. Add Dulbecco Modified Eagle's Medium (DMM) containing 1 ug/ml porcine trypsin (20 ml/medium sized flask).
7. Incubate at 37^{o}C until CPE is advanced or for a maximum of 7 days.

APPENDIX - F

PROCEDURE FOR CULTIVATION OF HUMAN ROTAVIRUS FROM STOOL (According to the method of Albert & Bishop 1984)[11]

Selection of Stool Specimens :

Use preferably fresh stool specimens, or specimens stored at -70°C. These should be positive for rotavirus by ELISA using commercial kit.

Preparation of Sample for Inoculation :

Prepare a 20% homogenate of faeces in phosphate buffered saline (PBS, pH 7.0). Remove bacteria by passing though a 0.45 um membrane filter after prewetting filter with neat foetal calf serum (FCS). Activate with 10 ug/ml of trypsin (sigma type IX trypsin) for 30 minutes at 37°C.

Preparation of MA-104 Cells :

Cells are grown in Dulbecco's modified medium (DMM) with 12.5 ug/ml each of polymyxin B and neomycin sulphate to form confluent monolayers of cells in culture flask (75 cm^2 area, 200 ml capacity containing 20 ml of medium). Culture fluid is drained off and cells are removed with trypsin - EDTA (2 ml for each flask). When cells are stripped, 150 ml of DMM with 10% foetal calf serum is added (1:5 split ratio). Two ml each of diluted cells are seeded into screw-capped tubes and incubated at 37°C on a stationary rack until a confluent monolayer is obtained (3-4 days usually). These tubes are used for inoculation of activated faecal samples. Rinse the monolayer twice with PBS (containing phenol red indicator) just before inoculating the stool filtrate.

Inoculation of Trypsin Activated Filtrate :

0.2 to 0.5 ml of filtrate (or 1.0 ml whenever available) is inoculated into each of two tubes of monolayers and rolled in the drum for 1 hour at 37°C for virus adsorption. Excess fluid is drained from the tubes. Cells are rinsed once with PBS and inoculated with 2 ml of DMM containing 1 ug/ml of trypsin. The tubes are then incubated in the rolled drum at 37°C for up to 5 days, checking for evidence of CPE everyday.

Detection of Rotavirus in Inoculated Tubes :

After 3 freeze thaw cycles, supernatants can be tested in ELISA test.

Further Passages :

The duplicate tube is frozen and thawed three times, and the supernatant is treated with trypsin (as above). 1.0 ml of trypsin treated supernatant is passaged into each of two tubes containing fresh monolayers of cells as before. Tubes are not rinsed with PBS from passage 2 onwards after adsorption of virus. Cellular damage due to trypsin can occur. This does not seem to interfere with viral multiplication.

APPENDIX G

Concentration of rotavirus from tissue culture supernatant (ELISA antigen)

1. After one freeze-thaw cycle, cell debris is removed from pooled tissue culture supernatant by centrifugation at 5,000 g at $4^{o}C$ for 10 mins.
2. Supernatant is pooled and ultracentrifuged at 100,000g for 1 hour at $4^{o}C$.
3. The pellet is resuspended in 0.01M Tris-Hcl pH 7.5 with 10mM $CaCl_2$ and 15mM NaCl to give a 100 fold concentration of the original supernatant.

APPENDIX H

Virus Titration:

1. Wells of microtitre trays with MA104 monolayer are washed X2 with PBS.
2. Virus from a new batch is added in duplicate to wells in doubling dilutions, starting at a dilution of 1/10 (in DMM-1 ug/ml trypsin) for tissue culture supernant or 1/100 for concentrated ELISA antigen (50 ul volumes).
3. 50 ul of medium with 1 ug/ml trypsin to is added each well.
4. Incubate overnight 37^{o}C 5% CO_2.
5. Fix and stain with
   - antirotavirus antisera
   - FITC conjugated antisera.
6. Examine inverted microtitre plate under fluorescent microscope and count fluorescent cells.
7. Calculate titre as fluorescent cell forming units/ml (FFU/ml.).

APPENDIX I

Production of hyperimmune antisera

1) Seronegative animals are chosen (see appendix J)

2) Rabbits are immunized with 0.5ml of purified virus preparation (see appendix G) at a concentration of approximately 10^6 FFU/ml or greater - mixed with 0.5 ml of Freund's complete adjuvant and injected, subcutaneously, on 3 sites on the back.

3) Three weeks later rabbits are injected with 0.5 ml of virus preparation (as above) mixed with Freund's incomplete adjuvant and injected as above.

4) Three weeks later 0.5 ml of virus preparation only is injected as above.

5) Ten to 14 days later rabbits are kill bled, if ELISA antibody titre is greater than 1/20,000 (see appendix J) and/or neutralizing antibody titre is greater than 1/20,000.

Note 1 : Guinea pigs are immunized with volumes approximately half those given to rabbits.

Note 2 : Blood is taken at each time of immunization, if possible, to determine antibody levels.

APPENDIX J

ELISA for measuring serum antirotavirus antibodies in animals.

(1) Coat wells of microtitre trays with rotavirus ELISA antigen diluted in carbonate coating buffer and incubate overnight at 4°C (100 ul volumes).

(2) Wash X3 with PBS-T20

(3) Make doubling dilutions of serum in PBST-BSA or PBST with 2% skim milk powder (PBST-SMP) (100 ul volumes).
For pre-immune sera start at 1/20.
For postimmune sera start at 1/200 or greater.
Incubate 1 h 37°C humidified.

(4) Wash X 3 with PBST20

(5) Add conjugate (either antirabbit or anti-guineapig) diluted in PBST-BSA or PBST-SMP (100 ul volume) and incubate for 1 h at 37°C (humidified).

(6) Wash X 3 with PBST-20

(7) Add substrate.

APPENDIX K

DETECTION OF NEUTRALISING ANTIBODIES TO ROTAVIRUS (Fluorescent Focus Reduction Assay) :

1. Preparation of Cell Monolayers :

A cell suspension of MA104 cells (0.2 ml trypsinised cells/ml of medium) is made in DMEM (Dulbecco's modified Eagle's Medium with neomycin and polymixin (12.5 ug/ml of each) supplemented with 10% foetal calf serum is seeded (0.1 ml per well) into microtitre culture plates and incubated in a humidified incubator (5% CO_2, 95% O_2) at 37°C until monolayers are confluent.

2. Neutralising Antibody Assay :

(a) dilute serum in DMM + NP with 1 ug/ml Porcine trypsin type IX.

(b) If a titre is required serial dilutions can be made in a sterile microtitre tray in 50 ul volumes.

(c) Dilute virus in DMM containing 1 ug/ml trypsin. 50 ul is added to each well and plates incubated for 1 hour in a humidified incubator (5% CO_2, 95% O_2) at 37°.

N.B. The dilution of virus to be used should be determined by a titration. The dilution giving approximately 100 fluorescing cells per well should be used.

(d) Ten minutes prior to the completion of 1 hour incubation monolayers are washed twice with sterile PBS by:

(i) Gently aspirating medium from the wells (keep as sterile as possible);

(ii) add PBS to the top of wells with a pasteur pipette;

(iii) aspirate PBS;

(iv) repeat (ii) and (iii)

(e) Add 50 ul of virus-serum mixture to wells of the washed plate and spin plates at 1,000 rpm (200 g) for 30 mins.

N.B. Special centrifuge holders are required for microtitre plates. The spin is not essential but it appears to help the virus settle onto the monolayer.

(f) Add 50 ul of DMM with 1 ug/ml trypsin to each well.

(g) Incubate plate overnight (at 37°C 5% CO_2, 95% O_2) in humidified incubator.

3. Fluorescent Stain :

(a) Remove culture supernatant from each test well, and add 75% acetone and leave at room temp. for 5 mins.

(b) Aspirate gently and air dry the plate until no acetone smell can be detected.

(c) Add 50 ul of antiserum to rotavirus (e.g. rabbit anti-SA 11) diluted in PBS to an appropriate concentration.

(d) Incubate at 37°C for a minimum of 30 mins.

(e) Gently aspirate the antiserum and wash the wells three times with PBS by aspiration.

(f) Add 25 ul of a fluorescein isothiocyanate conjugated antibody directed against the appropriate animal immunoglobulins diluted in PBS.

(g) Incubate at 37°C for 45 mins.

(h) Aspirate and wash 3 times with PBS as above and allow plate to air dry.

(i) Read fluorescing cells using a filter suitable for fluorescein at low power.

APPENDIX L

Detection of Faecal neutralizing antibodies to rotavirus (Flourescent focus reduction neutralization (FFN) assay in faeces)

This method was adapted from that described previously, for estimation of rotavirus-neutralizing antibody levels in hybridoma supernatant fluids (Coulson et al. 1985) and its development will be described in detail elsewhere (Coulson et al., manuscript in preparation). Rotavirus stocks were activated with trypsin prior to incubation with faecal extracts. Stocks were diluted to contain 2.5×10^4 fluorescing cell-forming units (FFU)/ml containing 5 ug/ml porcine trypsin in Dulbecco modified Eagle medium DMM (Sigma). After incubation at 37°C for 30 min., the activated virus was further diluted to 2.0×10^3 fofu/ml in DMM containing 1 ug/ml trypsin and 1% (vol/vol) foetal calf serum (DMM-T-S) found to be free of rotavirus antibodies by EIA and FFN assay. The diluted virus was mixed with an equal volume of faecal extract diluted 1:20 and 1:200 in DMM-T-S in duplicate giving final dilution of stool of 1:200 and 1:2000, respectively. The mixture was incubated at 37°C for 1h, and 50 ul per well was inoculated in duplicate onto confluent monolayers of MA104 cells in microtitre plates. After centrifugation at 1,200 x g for 30 min., the plates were incubated at 37°C in an atmosphere of 5% CO^2 and 95% relative humidity overnight. The cell supernatants were removed, and the monolayers fixed in 70% (vol/vol) acetone for 5 min. and air dried. Rabbit hyperimmune antiserum to SA11

(50 ul) at optimal dilution (1:500) in PBS was added to each well, and the plates incubated at 37°C for 30 min. The plates were washed in PBS before addition of 25 ul/well of fluorescein isothiocyanate-labelled sheep anti-rabbit IgG F $(Ab)^2$ (Silenus, Australia) diluted 1:100 in PBS. After 30 min. at 37°C, the plates were washed, air dried and wells examined for specific flourescence as described previously (Coulson et al., 1985). The neutralization titre of each faecal sample was expressed as the reciprocal of the faecal dilution giving 50% reduction in 40) containing no antirotaviral IgA, IgM, IgG by EIA all gave titres <200 by FFN assay. Samples giving titres below 200 were therefore considered to be negatives for neutralizing antibody to the rotavirus under test.

APPENDIX M.

Indirect Enzyme Immunoassay for Titration of Rotavirus-specific Antibodies in Human Sera and Secretions

1. Coat 96F NUNC tray with 100 ul/well of rotavirus EIA antigen or cell control antigen prepared as described in Appendix G and diluted in 0.06:1 sodium carbonate-bicarbonate buffer, pH 9.6. Generally, virus and cell control are diluted 1:100-1:300. The exact dilution is determined by checkerboard titration.

2. Incubate trays at 37°C for 2 hours in a moist environment, or overnight at 4°C.

3. Wash x 3 with phosphate buffered saline pH 7.2 + 0.05% (v/v) Tween 20 (PBS-T-.05).

4. Add 100 ul of samples to virus-coted wells as follows :

   Serum : Serial 2-fold dilutions in SMP solution stating at 1 in 100.

   Saliva : Serial 2-fold dilutions in SMP solution starting at 1 in 12.5.

   Faeces : Prepare 10% homogenates in PBS or 0.01M Tris HCL buffer pH 7.2 containing 0.15M NaCl and 10 MM $CaCl_2$. Centrifuge at 2,000 x g fcr 10 min. to remove solid debris. The supernatant is assayed for antibody by serial 2-fold dilution in SMP solution starting at a 1 in 10 dilution (1 in 100 dilution of faeces), or by reference to a standard positive with defined unit value. The highest

concentration of each sample is also reacted with wells coated with cell control antigen.

5. Incubate overnight at $4^{o}C$.
6. Wash as in 3.
7. Add 100 ul of anti-human IgA, IgM, IgG or secretory piece (Sc) conjugated to HRPO, to each well as appropriate. The dilution to be used, usually 1:50 - 1:1,000, is determined by checkerboard titration with a known positive sample.
8. Incubate for 1.5 hours at $37^{o}C$ in a moist environment.
9. Wash as in 3.
10. Add 100 ul TMB substrate to each well.
11. Incubate at room temperature for 10 minutes.
12. Stop reaction with 50 ul of 2M H_2SO_4 per well.
13. Either read plates visually or with the aid of a spectrophotometer at 405 nm.

The end-point of the titration is the highest dilution of the sample with a colour greater than that of the cell control well (or 2 x the OD405 of the cell control well).

Appendix N

Methods for detection of "other" enteric viruses in stool specimens

A. Enteric adenoviruses

i. ELISA test

Monoclonal antibodies to enteric AdV are being sent by Dr. JE Herrmann from the Division of Molecular Genetics and Microbiology, University of Massachussetts Medical School, Worcester, Massachusetts and will be used according to the method of JE Herrmann et al (1987) Antigen detection with monoclonal antibodies for the diagnosis of adenovirus gastroenteritis. J.Infect.Diseaes. ISS: 1167-1171.

ii. DNA probes

We have written to Dr. HE Takiff of Medical Virology Section, Natyional Institute of Allkergy and Infectious Diseaes, Bethesda, Maryland. for the BgL II D clone of AdV 41 which has been shown to differentiate between enteric and non-enteric adenoviruses, according to the method Takiff et al (1985). Detection of enteric adenoviruses by dot-blot hybridization using a molecularly cloned viral DNA probe. J.Med.Virol. 16: 107-118.

B. Group B rotavirus.

ELISA test : An ELISA test utilizing gpBRV specific monoclonal antibodies will be used for the detection of gPBRV according to the method of Yolken et al. (J.Clin.Microbiol (1988) 26: 1853-1856.

C. Group C rotaviruss. We have written to Dr. L. Saif of Ohio Agricultural Research and Development Centre, the Ohio State University, Wooster, Ohio to obtain some cultivable porcine group C rotavirus and antisera to group C rotavirus in order to cultivate group C rotavirus and produce hyperimmune reagents in order to develop an ELISA test in due course.

We have also written to Dr. R. Yolken of Department of Paediatrics, Johns Hopkins School of Medicine, Baltimore, Maryland to obtain monoclonal antibodies directed to double stranded RNA to use in the ELISA test as quated in the reference :

JS Kinney et al (1989) Monoclonal antibody assay for detection of double stranded RNA and application for detection of group A and non-group A rotaviruses. J.Clin.Microbiol. 27:6-12.

D. Astrovirus, Calicivirus, norwalk virus.

ELISA tests are currently being developed at the Viral Gastroenteritis Unit, Centre for Diseases Control, Atlanta, Georgia and will be forwarded to ICDDR,B by Dr. Roger Glass.

Budget

a. Personnel :

Mr. J. Gomes	100 % salary	GS 4,Stp.15	5,160	
Ms. N. Nahar	100 % salary	GS 5,Stp.03	4,452	
Mr. B. Paul	100 % salary	GS.4,Stp.10	4,848	
Dr. G. Podder	100 % salary	GS.6,Stp.06	7,440	
Dr. N. Shahid	50 % salary	NO.C,Stp.05	7,035	
To be named	200 % salary	GS.4,Stp.15	10,320	
			39,255	
			US$	39,255

b. Operating costs.

Per year	US$	31,000

c. Capital equipment

1. Microcarrier cell culture system for large scale cultivation of viruses	2,000	
2. Bio Rad Electrophoresis powerpak	3,000	
3. Beckman 45 Ti ultracentrifuge rotor	20,000	
	25,000	
	US$	25,000

d. Travel

Return travel from USA for consultant working on methods for detection of enteric viruses	US$	4,000

First year

Capital equipment	25,000		
Personnel	39,000		
Operating cost	31,000		
Travel	4,000		
	99,000		
		US$	99,000

Second year

Personnel	43,000		
Operating cost	35,000		
	78,000		
		US$	78,000

Third year

Personnel	47,000		
Operating cost	38,500		
	85,000		
		US$	85,000
	TOTAL COST	US$	262,000

16. Justification of Budget

One of the principle aims of the protocol is the establishment of a virology laboratory. The amount of money required for capital expenses is quite low, as virology techniques do not require a high degree of technology and much of the equipment for cell culture and ELISA tests is already available at ICDDR,B.

The other aims are to study the epidemiology and importance of a variety of enteric viruses and importantly to carry out a diagnostic function. The diagnosis hopefully performed by a virology laboratory at ICDDR,B may allow a pathogen to be implicated in a greater number of cases of diarrhoea, thereby improving patient management and care.