ICDDR,B LIBRARY
DHAKA 1212

Library

tachment 1.

Date 5-11-95.
7/11/95
29

ETHICAL REVIEW COMMITTEE, ICDDR,B.

rincipal Investigator Leanne Unicomb   Trainee Investigator (if any) ______

pplication No. 95-028   Supporting Agency (if Non-ICDDR,B) ______

itle of Study Investigation of the importance of new viral agents of diarrhea

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

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

. Source of Population:
  (a) Ill subjects — (Yes) No
  (b) Non-ill subjects — (Yes) No
  (c) Minors or persons under guardianship — (Yes) No
. Does the study involve:
  (a) Physical risks to the subjects — Yes (No)
  (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)
. Does the study involve:
  (a) Use of records, (hospital, medical, death, birth or other) — (Yes) No
  (b) Use of fetal tissue or abortus — Yes (No)
  (c) Use of organs or body fluids — (Yes) No
  Are subjects clearly informed about:
  (a) Nature and purposes of study — (Yes) No
  (b) Procedures to be followed including alternatives used — (Yes) No
  (c) Physical risks — Yes No NA
  (d) Sensitive questions — Yes No NA
  (e) Benefits to be derived — Yes No NA
  (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 NA

5. Will signed consent form be required:
  (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
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.

(PTO)

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

Leanne Unicomb
Principal Investigator

A-031976

______
Trainee

CHECK-LIST FOR SUBMISSION OF PROPOSALS TO THE RESEARCH REVIEW COMMITTEE (RRC)

[Please tick (✓) the appropriate box]

1. Has the proposal been reviewed, discussed and cleared at the Division level ?

   Yes [✓]

   No [ ]

   If the answer is 'NO', please clarify the reasons: ______________________

   ____

   ____

2. Has the proposal been peer-reviewed externally ?

   Yes [✓]

   No [ ]

   If the answer is 'NO', please explain the reasons: ______________________

   ____

   ____

3. Does the proposal address gender-issues ?

   Yes [ ]

   No [✓]

   If the answer is 'NO', Please give the reasons.

   ____

   ____

   ____

4. Has a funding source been identified ?

   Yes [ ]

   No [ ] NOT 100%

   If the answer is 'YES', please indicate the name of the donor: ______________

   ____

   ____

Contd... /

5. Whether the proposal is a collaborative one ?

Yes [✓]

No [ ]

If the answer is 'YES', the type of collaboration, name and address of the institution and name of the collaborating investigator be indicated:

(1) VIRAL GASTRO UNIT, CDC, ATLANTA – REAGENTS & TECHNICAL ADVICE WILL BE GIVEN (2) COMMUNITY HEALTH DIVISION : SAMPLES AND CLINICAL INFORMATION WILL BE PROVIDED WHEN NECESSARY. (3) CLINICAL SCIENCES DIVISION – PATIENT SELECTION AND SAMPLE COLLECTI[ON]

6. Has the budget been cleared by Finance Division ?

Yes [✓]

No [ ]

If the answer is 'NO', reasons thereof be indicated: ______________________

7. Does the study involve any procedure employing hazardous materials, or equipments ?

Yes [✓]

No [ ]

If the answer is 'YES', fill the necessary form.

6·11·95
Date

[signature]
Signature of the
Principal Investigator

ASSURANCE ON *HAZARDOUS PROCEDURE* FORM

DECLARATION BY THE PRINCIPAL INVESTIGATORS

Name of the Principal Investigator : Leanne Unicomb.

Title of the Project : Investigation of the importance of new viral agents of diarrhea.

I declare that (check) :

[ ] The above mentioned protocol does not involve any procedure relevent to "Safety/Environmental" hazard.
(P.I. don't fill up the form, if this is the response)

[✓] The above mentioned protocol involves procedure(s) with potentials to cause "Safety/Environmental" hazard, and relevant informations are provided below :

(The following portion to be filled in for only those protocols which need to handle the hazards mentioned and defined in this form)

The nature of hazards (check as many entries as appropriate) :

[✓] Biologicals
[✓] Chemicals
[ ] Non-ionizing radiation
[ ] Radioactive materials
[ ] Ionizing radiation machine

[ ] Other (specify) : ______________________

1.0 Important information :

1.1 Brief description of the objective of the study, relevance and procedure of using hazardous materials, methods of personnel protection, and budget code to cover for the procurement of safety supplies:

① Handling of biologicals will be done as per ICDDR,B Safety committee rules i.e. universal precautions will be taken.
② Hazardous chemicals such as guanidium isothiocyanate, ethidium bromide, phenol and chloroform will be used a) in a fume hood and b) whilst wearing gloves

(The above hazards are defined in the reverse page)

1.2 Summary description of specific training and experience of the Principal Investigator in using hazardous material(s) under consideration.

The P.I has had extensive experience in the use of biological and chemical hazards in the lab.

1.3 Expected average quantity of waste generated (in kg./month)

Radioactive ☐ Chemicals ☑ 1kg Biochemicals ☐ 2kg.

2.0 Declaration

I agree to provide the Office of the *Occupational Safety and Environment Programme* (OSEPP) of ICDDR,B with appropriate information related to the study, and to comply with all applicable regulations of the OSEPP (ICDDR,B), other appropriate agencies, scholarly organizations or recognized professional groups. I also agree my participation and participation of all persons involved in my study in safety related training.

Leanne Unicomb 16.10-95

Signature of the Principal Investigator Date

[signature] 6.11.95

Signature of the ~~Division~~/Department Head Date

APPLICATION FOR PROJECT GRANT

1. Title of project : Investigation of the importance of new viral agents of diarrhea

2. Principal Investigator: Leanne Unicomb, ICDDR,B

   Co-Investigators : Roger Glass, CDC, Atlanta
   Nurun Nahar Banu, ICDDR,B
   K. Zahid Hasan, ICDDR,B
   M.J. Albert, ICDDR,B
   Physician, ICDDR,B

4. Potential donor: AUSAid

5. Funding required : US$ 24,759

6. Duration of project : Two years

7. Head of Programme : Dr. M.J. Albert
   Acting Divisional Director
   Laboratory Sciences Division

8. **Abstract summary**

Rotavirus is undoubtedly the most important viral cause of diarrhea in children and so far, found in a far greater proportion of children with diarrhea than other viruses. Other viral agents have been implicated as causative agents of diarrhea yet investigation of their prevalence has been hampered by the lack of availability of diagnostic reagents since many of these viruses cannot be grown in cell culture.

We have investigated the role of enteric adenovirus and astrovirus but have found their contributions to acute diarrheal diseases to be small (~2% each). Recent investigations from our group have suggested a possible association of astrovirus with persistent diarrhea using a small number of samples. Many

studies of etiological agents of persistent diarrhea have been conducted, yet strong associations of particular diarrhea agents are few. We plan to substantiate our findings of the possible association of astrovirus with persistent diarrhea. This may lead to changes in the treatment of patients suffering from persistent diarrhea.

Using limited quantities of virus, molecular techniques have enabled the production of viral proteins using genomic sequence data. An example of this type of work is that with the Norwalk group of viruses. These viruses have been shown to be associated with epidemics of gastroenteritis but due to lack of reagents, their role in endemic disease has not been fully investigated. Serosurvey data using synthetic proteins have suggested that such viruses may contribute significantly to endemic diarrhea. Using samples collected as part of a cohort study, we plan to (a) conduct a serosurvey of antibodies to at least 2 members of the Norwalk group of viruses and once seroconversion data are available (b) attempt to detect virus in stool specimens from within the denoted time period using a PCR and Southern hybridization technique. Using this set of samples, we will be able to ascertain whether infections with these agents are frequently associated with diarrhea, whether there is a high rate of asymptomatic carriage and whether re-infections are common. Should we find that viruses from the Norwalk group are commonly associated with diarrhea, an extensive case-control study would be justified.

9. Background and aims

Even though oral rehydration solution has made significant inroads towards lowering mortality due to diarrhea in children in developing countries, the morbidity continues to be staggering. It has become imperative that ways of preventing diarrhea be pursued. Improved domestic and personal hygiene has been stressed, however it can go only so far in poor countries where the hard economic reality is that massive outlays needed for infrastructural improvements can be ill-afforded. It has also become obvious from the experience of developed countries that viral agents of diarrhea such as rotavirus cannot be contained even with a high standard of hygiene. As a result development of appropriate vaccines against major enteric pathogens has become a priority for both developed and developing countries. Unfortunately, we cannot fully attain the goal of substantial reduction of diarrheal morbidity and mortality unless we know the relative contributions of different etiological agents to the overall diarrhea burden. Two viral agents of diarrhea that need to be singled out are astroviruses and Norwalk viruses.

Lack of sensitive techniques for detection of both of these agents has dogged studies for estimation of their true prevalence. Inability to cultivate Norwalk viruses had hampered the preparation of reagents for more sensitive detection methods. Now this has been largely overcome by utilization of the baculovirus expression system for production of Norwalk virus

antigen in abundance. New studies using recombinant antigen in sensitive immunoassays have discredited the previously held notion that Norwalk viruses are the causative agents of epidemic gastroenteritis only (1), and have demonstrated that they are also significant causative agents of endemic diarrhea (2). In a previous serological survey conducted on specimens from Bangladesh using less sensitive tests, it has been found that up to 80% of children under 5 years of age had evidence of exposure to Norwalk viruses (3). With the advent of new assays, it will now be possible to estimate the true significance of Norwalk viruses in Bangladesh. Therefore, as for rotaviruses, it might be necessary to develop effective vaccination strategies against Norwalk viruses and efforts are underway to develop an effective vaccine.

Previous studies using ELISA as the detection method (4) have probably underestimated the true prevalence of astrovirus infection. A newly developed PCR assay has revealed that up to 10% of hospital admissions for diarrhea in children in the U.S. are due to astroviruses (R.I. Glass, personal communication). Thus astroviruses are emerging as prominent diarrheal agents. Using the conventional ELISA, we have found that approximately 2% of children with diarrhea are infected with astroviruses. But what was striking was that even with the relatively insensitive ELISA up to 10% of a small group of children with persistent diarrhea had evidence of astrovirus infection (unpublished data). Persistent diarrhea is an intractable public health problem in children in developing countries with a staggering 35% mortality,

and up to 10% of children with acute diarrhea develop persistent diarrhea. Persistent diarrhea is multifactional and it is likely that astroviruses may also play an etiological role.

The advent of more sensitive assays for these viral agents of diarrhea presents us with an unique opportunity to pursue the aim of total eradication of diarrhea. This aim will be advanced significantly by defining the relative contributions of these viral agents to the total diarrhea burden.

The aims of this study are:

a) to confirm our findings of the association of astrovirus with persistent diarrhea using a larger collection of samples with corresponding contemporaneously collected acute diarrhea, and nosocomial controls sets.

b) to determine whether Norwalk viruses are important etiological agents of diarrhea in Bangladesh.

10. Research Plan

a) Astrovirus studies

The sample size has been calculated assuming a 2% detection rate in acute diarrhea, non-diarrheal patients and nosocomial controls.

We plan to collect stool samples from:

i) 100 "acute" (admission) stools from patients less than 5 years of age who develop persistent diarrhea. Specimens

will be tested for astrovirus, enteroaggregative *Escherichia coli*, *Aeromonas*, *Cryptosporidium* and *Cyclospora* (because these agents have been reported to be associated with persistent diarrhea). We will also test for group A rotavirus. The tests for group A rotavirus (Appendix-II) and astrovirus (Appendix-III) are (at present) ELISA tests as previously described. Ultimately a subset of samples will be retested using the PCR method (12). Aeromonas will be cultured using standard techniques (7), enteroaggregative *E. coli* will be detected using probe hybridization (Appendix-IV), *Cryptosporidium* will be detected using the Ziehl-Neelsen stain and *Cyclospora* identified by wet mounts.

ii) 100 stools from patients less than 5 years of age with acute diarrhea that do not develop persistent diarrhea, tested as above.

iii) 100 "nosocomial control" stools i.e. from infants less than 5 years of age who are admitted to the hospital with diarrhea, stay for a minimum of 2 days and do not develop persistent diarrhea. These specimens will be tested as above.

iv) 100 stools from non-diarrheal control i.e. infants matched for age in the absence of diarrhea. The samples will be tested as above.

NB: All samples collected will be stored at -20°C for further testing for other viruses as warranted.

Information on clinical signs, nutritional status and duration of diarrhea will be collected from infants with diarrhea (questionnaire - Appendix-I).

b) Norwalk virus studies

Serosurveys

We plan to test sera from:

i) a serum bank representing a cross-sectional survey of single samples from approximately 200 subjects of all ages.

ii) a longitudinal study. A complete set of sera (collected at 6 monthly intervals over a 3-year period) from 100 children recruited at birth will be tested (i.e. total = 700 sera). This will represent a group from which we can determine the timing of infection and re-infections.

The sera will be tested for antibodies to Norwalk virus (NV) and the related Toronto virus (TV, formerly known as minireovirus) (9) using an ELISA test (Appendix-V). Reagents will be provided by CDC, Atlanta. Baculovirus expressed recombination NV and TV antigen will be used to coat ELISA plates as described elsewhere (11) to measure serum IgG, IgA and IgM antibodies.

Detection of NV

Using a PCR technique (Appendix-VI), routine stool samples (collected at monthly intervals) and diarrheal stools from infants in the longitudinal study mentioned above can be tested

ICDDR,B LIBRARY
DHAKA 1212

at the appropriate time intervals to see whether virus detection coincides with seroconversions. Since the test is expensive, identification of the time interval at which a child becomes infected will allow us to target appropriate specimens rather than attempt to screen large numbers. Primers will be provided by CDC, Atlanta.

From this we will be able to deduce:

a) frequency of NV/TV infections (symptomatic and asymptomatic).

b) re-infections with NV/TV

c) symptoms associated with NV/TV diarrhea

In this study we plan to utilize specimens that have mostly come from other studies in order to minimize costs and maximize output from existing studies.

11. Ethical implications

Longitudinally collected blood samples are an approved study (Epidemiology of diarrhea and ARI in a cohort of newborns in Bangladesh #92-024). Serum bank samples will be collected as 'anonymous sera' (age and sex will be known) from samples about to be discarded from the Clinical Biochemistry Laboratory. No further information is required. The remaining samples required for this study are stools, many of which have already been collected. Anonymity of all subjects will be maintained.

12. Policy implications

The study will establish the true diarrhea burden of these two viral agents. If astroviruses are found to be associated with persistent diarrhea, a new treatment modality can be developed to address this serious problem. If Norwalk viruses are also found to be a significant cause of endemic diarrhea, we will be able to add to global efforts for eradication of this infection by joining developed countries in vaccination efforts.

13. References

1. Kaplan JE, Gary GW, Baron RC, Singh N, Schonberger LB, Feldman R, Greenberg HB. Epidemiology of Norwalk gastroenteritis and the role of Norwalk virus in outbreaks of acute nonbacterial gastroenteritis. Ann Intern Med 1987; 96:756-761

2. Lew JF, Valdesuso J, Vesikari T, Kapikian AZ, Jiang X, Estes MK, Green KY. Detection of Norwalk virus and Norwalk-like virus infections in Finnish infants and young children. J Infect Dis 1994; 169:1364-7.

3. Black RE, Greenberg HB, Kapikian AZ, Brown KH, Becker S. Acquisition of serum antibody to Norwalk virus and rotavirus in relation to diarrhea in a longitudinal study of young children in rural Bangladesh. J Infect Dis 1982; 145:483-489.

4. Cruz JR, Bartlett AV, Herrmann JE, Caceres P, Blacklow NR, Cano F.Astrovirus-associated diarrhea among Guatemalan ambulatory rural children. J Clin Microbiol 1992; 30:1140-1144.

5. Unicomb LE, Bingnan F, Rahim Z, Banu NN, Gomes JG, Podder G, Munshi MH, Tzipori SR. A one-yearsurvey of rotavirus strains from three locations in Bangladesh. Arch Virol 1993; 132:201-208.

6. Moe CL, Allen JR, Monroe SS, Gary HEJ, Humphrey, CD, Herrmann JC, Blacklow NR, Carcamo C, Koch M. Kim KH, Glass RI. Detection of astrovirus in pediatric stool samples by immunoassay and RNA probe. J. Clin Microbiol 1991; 29:2390-2395.

7. George WL, Jones MJ, Nakata MM. Phenotype characteristics of *Aeromonas* species isolated from adulthumana. J Clin Microbiol 1986; 23:1026-1029.

8. Lew JF, Petric M, Kapikian AZ, Jiang X, Estes MK, Gree KY. Identification of minireovirus as a Norwalk-like virus in pediatric patients with gastroenteritis. J Virol 1994; 68:3391-3396.

9. Moe CL, Gentsch J, Ando T, Grohmann G, Monroe SS, Jiang X, Wang J, Estes MK, Seto Y, Humphreys C, Stire S, Glass RI. Application of PCR to detect Norwalk virus in fecal specimens from outbreaks of gastroenteritis. J Clin Microbiol 1994; 32:642-648.

10. Graham DY, Jiang X, Tanaka T, Opekun AR, Madore HP, Estes MK. Norwalk virus infection of volunteers: new insights based on improved assays. J Infect Dis 1994; 170:34-43.

11. Grohmann GS, Glass RI, Pereira HG, Monroe SS, Hightower AW, Weber R, Bryam RT for the Enteric Opportunistic Infection Working Group. Enteric viruses and diarrhea in HIV-infected patients. N Engl J Med 1993; 329:14-20.

12. Birnboim HC, Doly J. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl Acid Res 1979; 7:1513.

13. Maniatis T, Fritsch EF, Sambrook J. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory, New York, 1982.

14. Feinberg A, Volgelstein B. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 1983; 132:6-13.

15. Feinberg A, Volgelstein B. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 1984; 137:266-267.

16. WHO 1987. Programme for control of diarrhoeal diseases (CDD/83.3, Rev.1) In: Manual for laboratory investigations of acute enteric infections. World Health Organization, Geneva.

14. Budget

	First year	Second year	Total
1. Personnel			
N. Nahar Banu (GS5-9) 75%*	3000	6500	9,500
	----	----	-----
Total	3000	6500	9,500
2. Reagents, plasticware and office supplies	3900	4100	8,000
3. Interdepartmental costs	700	700	1,400

Total			18,900
Overhead 31%			5,900

Grand Total			24,759
			======

*First year 50%, second year 100%

APPENDIX-I

Clinical information required from persistent diarrhea patients

ID No. .. ____________________

1. Age (months) .. /__/__/
2. Sex (M/F) .. /_____/
3. Duration of diarrhea prior to admission (days) /__/__/
4. Duration of fever prior to admission (days) /__/__/
5. Weight (kg) /_/_/. /__/
6. Height (Cm) /_/_/_/
7. Family members with diarrhea in last month (Y/N) /_____/

During observation period

8. Type of stool (liquid/watery/mucoid/bloody) /_____/
9. Vomiting (Y/N) /_____/
9. Fever (Y/N) /_____/
10. Specimen day (after hospitalization) /_____/

APPENDIX-II

ELISA FOR DETECTION OF GROUP A ROTAVIRUS IN STOOL SAMPLES (5)

1) Wells will be coated with:

   a) Rota-positive sera (this is an equal mixture of rabbit anti-SA11, RV4, RV5, RV3, ST3)

   b) Rota-negative sera

   Both diluted 1:5000 in 0.06 M carbonate buffer pH 9.6 and plates will be incubated for 1 hr at room temperature.

2) Plates will be washed 3 times with PBS 0.05% Tween-20 (PBST) and 100 ul of 10% (w/v) stool extract in PBS will be added to rota-positive well and 100 ul to rota-negative well and plates incubated for 1 hr at room temperature.

3) Plates will be washed 3 times with PBST and 100 µl of anti-human rotavirus-HRP conjugate (Dakopatts) diluted 1:500 in SMP diluent will be added to all wells.

   SMP diluent is 2% (w/v) skim-milk powder dissolved in PBST.

   Plates will be incubated for 1 hr at room temperature.

4) Plates will be washed 4 times with PBST and substrate containing TMB will be added to all wells.

   The reaction will be stopped after incubation at room temperature for 10 mins and plates read in a spectrophotometer.

5) A positive is defined as a samples giving an OD reading of 0.1 OD units higher in rota-positive well than rota-negative well.

APPENDIX-III

ELISA for the detection of astroviruses in stool samples (6)

1. Immunolon II plates will be coated with anti-astro rabbit antisera and preimmune antisera diluted in carbonate-bicarbonate buffer and incubated for 1 h at 37°C.

2. Phosphate buffered saline (PBS) containing 1% (w/v) BSA and 0.1% (v/v) Tween-20 (PBS-BSA-T) will be added to all wells and plates will be incubated for 1 h at 37°C.

3. Plates will be washed 6 times with PBST and stool samples, positive and negative controls, will be added to anti-astro and preimmune sera coated wells. Plates will be incubated for 1 h at 37°C.

4. Plates will be washed, as above, and monoclonal antibody 8E7 will be diluted in PBS-BSA-T and added to all wells. Plates will be incubated for 1 h at 37°C.

5. Plates will be washed and HPR-conjugated anti-mouse IgG will be diluted in PBS-BSA-T and added to all wells. Plates will be incubated for 1 h at 37°C.

6. Plates will be washed, substrate containing 3,3',5,5'-tetramethylbenzidine-(TMB) will be added and plates will be incubated for 10 mins at room temperature. The reaction will be stopped with 2 N H_2SO_4 and the OD will be read at 450 nm in a microplate reader.

7. A sample will be considered positive if: P/N >2 and P-N $\geq$0.07, where P is the average OD of wells coated with positive capture antisera and N is the average OD of wells coated with negative capture.

APPENDIX-IV

Detection of diarrheagenic *Escherichia coli* using probe hybridization

1. Preparation of probe DNA fragments

Recombinant clones carrying the probes of interest (enteroaggregative) will be grown in nutrient broth containing the appropriate antibiotic. Plasmids will be isolated by modifications of the alkaline lysis method of Birnboim and Doly (12) and will be purified by using a commercially available column (NACS-52 PREPAC, BRL). The recombinant plasmids will be digested with the appropriate restriction enzymes to excise the inserts and the digests will be electrophoresed in agarose gels. The probe DNA fragments (inserts of recombinant plasmids) will be extracted from agarose gels by electroelution into dialysis bags as described by Maniatis *et al.* (13).

2. Preparation of specimens

Colony blots, dot blots, or stool blots will be prepared by standard techniques (12) using nylon membranes (HYBOND-N, AMERSHAM). For each set of specimens, a number of replica filters will be prepared for hybridization with different probes. The colonies will be lysed and the DNA denatured by placing the filters on pieces of Whatman 3MM sheets soaked in denaturing solution (0.5 M NaOH, 1.5 M NaCl). The filters will then be neutralized by placing on Whatman 3MM sheets soaked in neutralizing solution (1 M Tris-Cl pH 8.0, 1.5 M NaCl). The DNA will be fixed to the nylon membranes by exposing the membranes to UV light for 2-5 minutes on a UV transilluminator.

For each set of specimens, a set of master filters carrying live bacterial colonies will be properly stored for possible future studies on the hybridization-positive colonies.

3. Radioactive labelling of DNA probes

The probe DNA will be radioactively labelled by the method of Feinberg and Volgelstein (14,15) with a-^{32}P-dATP (10 uCi ul, 3000 Ci/mM, AMERSHAM) and oligonucleotide primers [P(dN)$_6$, PHARMACIA] using the Large Fragment of *E. coli* DNA polymerase I. In case of small DNA probes (less than 200 base pairs) the method of choice will be 5'-end labelling with -^{32}P-dATP using a 5' end-labelling kit

(BRL). Radio-labelled probes will be denatured by boiling followed by quick chilling on ice, before using these for hybridization experiments.

4. Hybridization of DNA blots

Hybridization with labelled probe will be carried out as described by Maniatis *et al.* (13). The filters will be prehybridized in the presence of denatured salmon sperm DNA to block unspecific binding sites on the filters, and will then be hybridized with the denatured probe DNA for 12-16 hours at the appropriate temperature. After hybridization, the filters will be washed under conditions of increasing stringency.

5. Preparation of autoradiographs

The hybridized filters will be exposed to X-ray films in metal cassettes at -70°C for the appropriate time. In case of weak signals, intensifying screens will be used. The exposed X-ray films will be developed and fixed by standard procedure.

APPENDIX-V

ELISA for the detection of anti-Norwalk virus antibodies (10)

1. Polysorb microtiter plates will be coated with recombinant Norwalk virus (NV) or Toronto virus (TV) antigen diluted in phosphate buffered saline (PBS) and incubated for 4 h at 37°C.

2. The contents of wells will be removed and PBS containing 0.05% Tween-20 (PBST) containing 2% (w/v) skim milk powder (PBST-SMP) will be added to all wells and plates will be incubated overnight at 4°C.

3. Plates will be washed 6 times with PBST and test sera diluted in PBST-SMP will be added in duplicate. Plates will be incubated for 2 h at 37°C.

4. Plates will be washed, as above, and HRP conjugate anti-human IgG or IgA or IgM diluted in PBST-SMP will be added. Plates will be incubated for 2 h at 37°C.

5. Plates will be washed and substrate containing TMB will be added, as given in Appendix-III.

6. On each plate, a serially diluted standard and a negative control will be included.

APPENDIX-VI

Reverse transcriptase polymerase chain reaction for the detection of Norwalk-like virus (9) and southern blot analysis

A. RNA extraction

1) Stool extracts will be mixed with a buffer containing 4.6 M guanidium thiocyanate, 20 mM EDTA and 2% (v/v) Triton X-100, then extracted with phenol-chloroform (1:1).

2) The mixture will be added to silicon dioxide particles, mixed for 10 mins at room temperature, then centrifuged at 850 *g* for 1 min.

3) The supernatant will be removed and the SiO_2 particles will be washed 3 times with a solution containing 70% (v/v) ethanol, then with 100% acetone.

4) SiO_2 particles will be dried under vacuum for 20 mins, DEPC treated water will be added and particles will be incubated at 65°C for 10 mins.

5) Particles will be centrifuged at 10,000 *g* for 10 mins and supernatant will be harvested. The particles will be re-extracted by addition of DEPC water and incubation at 65°C and supernatants pooled.

6) 0.11 volumes of 3M sodium acetate and 2 volumes of ethanol will be added and RNA will be precipitated overnight at -20°C.

7) RNA will be pelleted at 14,000 rpm for 30 mins, washed with 70% ethanol, centrifuged at 14,000 pm for 10 mins then dried, resuspended in 20µl of DEPC-water, incubated at 56°C for 5 mins then stored at -70°C.

B. RT-PCR

8) 1 ul of RNA (from step 5) will be denaturated at 95°C for 3 min in 30 ul denaturation buffer (30 mM Tris-HCl, pH 9.0, 100 mM KCl, 4.5 mM $MgCl_2$, 0.2% [v/v] Triton X-100, 2 mM 2-mercaptoethanol, 1.67 mM [each] dNTPs, 1 uM each primers SR33, SR48, SR50, SR52 [G-1 primer set] or SR33 and SR46 [G-2 primer set]).

9) RT and PCR will be carried out sequentially in one reaction tube by adding RT-PCR buffer (15 mM Tris-HCl, pH 9.0, 50 mM KCl, 2.25 mM $MgCl_2$, 0.1% Triton X-100, 1 mM 2-mercaptoethanol, 0.5 mM [each] dNTPs, 0.3 uM [each] G-1 and G-2 primers, 1 mM dithiothreitol, 40 U human placental RNAase inhibitor, 12 U avian

myeloblastosis virus super reverse transcriptase and 5U of amplitaq DNA polymerase) to a final volume of 100 ul.

10) RT-PCR was performed as one-cycle of RT at 42°C for 1 h followed by denaturation at 94°C for 3 mins. Then 40 amplification cycles with denaturation for 1 min at 94°C and annealing for 1.5 min at 5°C and extension for 2 min at 60°C. A final cycle of incubation at 72°C for 7 min will be given.

11) Amplification product (123 bp long) will be analysed by electrophoresis in 3% agarose gels and visualised under UV illumination after staining with 0.5 µg/ml ethidium bromide for 1 h.

C. Southern Hybridisation:

12) Gels will be denatured, neutralised and then blotted overnight in 20x SSC onto nylon membranes.

13) Blots will be hybridised with the appropriately matched digoxigen labelled probe to genotypes P1A, P1B, P2A, P2B at 58°C for 6h then washed 5 times.

14) Blots will be incubated with anti-digoxigen-phosphate for 30 mins at RT.

15) Blots will be washed 3 times then incubated with substrate (Lumi-phos 530) and exposed to X-ray film.

Description of oligonucleotide primers

Primer set[a]	Identification	Sequence							Polarity[b]	Location (nucleotides)[c]
Primer(s)										
G-1, G-2	SR33	tgt	cac	gat	ctc	atc	atc	acc	-	4856-4876
G-2	SR46	tgg	aat	tcc	atc	gcc	cac	tgg	+	4754-4773
G-1	SR48	gtg	aac	agc	ata	aat	cac	tgg	+	4754-4773
G-1	SR50	gtg	aac	agt	ata	aac	cac	tgg	+	4754-4773
G-1	SR52	gtg	aac	agt	ata	aac	cat	tgg	+	4754-4773

[a]Primer set including individual primer components

[b]-, negative; +, positive

[c]Equivalent location within the NV genomic sequence (M87661)

APPENDIX-VII

CONSENT FORM: PERSISTENT DIARRHEA STUDY

We are conducting a study on persistent diarrhea and we are trying to determine whether a virus known as astrovirus may be responsible for some cases of persistent diarrhea.

In order to conduct our study, we will need a small amount of stool from your child (about 5 ml) and we will need to look at your child's medical charts.

If you choose not to participate in the study your child will still continue to get standard treatment. If you choose to participate, please sign or give your thumbprint on this form.

All of the information that we collect will be kept confidential and no one other that the investigator will have access to the information.

------------------	------------------------------
Date	Signature/Thumb impression of guardian

------------------	------------------------------
Date	Signature of investigator

------------------	------------------------------
Date	Signature of witness

LU1/lu.newvir1.prt

Appendix VIII

সম্মতি পত্র

রোটা ভাইরাস সংক্রমনে ডায়রিয়া হয়। বেশ কিছু বিজ্ঞানী রোটাভাইরাস সংক্রমন প্রতিরোধে টিকা নিয়ে কাজ করেছেন। আমরাও জানতে চেষ্টা করছি, যে টিকা শিশুদের মুখে খাওয়া যাবে, সেটি বাংলাদেশে ব্যবহার করার উপযোগী কি-না এই কাজের জন্যে আমরা রোটা ভাইরাসে আক্রান্ত শিশুদের "এন্টিবডিজ" (antibodies) নির্নয় করব।

আপনার শিশুর মল পরীক্ষার পর আমরা জেনেছি যে, আপনার শিশু রোটা ভাইরাসে আক্রান্ত। আপনার শিশুর 'এন্টিবডিজ' নির্নয় করার জন্যে, আমাদের আপনার শিশুর ৫ মি.লি. শিরার রক্ত এবং লালা দরকার। ৩ সপ্তাহ পর আমরা আবার শিশুর রক্তের এবং লালার নমুনা নেব। আমরা সুই দিয়ে রক্ত নেব, এ জন্যে সামান্য ব্যথা ছাড়া আর কোন বিপদ হবে না। আপনার শিশুর লালার নমুনা নেওয়া হবে মুখে বিশুদ্ধ তুলোর কাঠি দিয়ে।

আপনি গবেষনায় অংশগ্রহনে অসম্মতি বা পরবর্তিতে সম্মতি প্রত্যাহার করলেও, আপনার শিশু স্বাভাবিক চিকিৎসা পাবে। গবেষনায় অংশগ্রহনে রাজী থাকলে, দয়া করে আপনার দস্তখত বা বুড়ো আঙুলের ছাপ এই ফর্মে দিবেন। যে কোন সময় আপনার গবেষনা থেকে সম্মতি প্রত্যাহারের স্বাধীনতা থাকবে। ৩ সপ্তাহ পরে হাসপাতালে আসার (যাতায়াত) খরচ আপনাকে দেয়া হবে। গবেষনালব্ধ তথ্যাদি গোপন রাখা হবে এবং এ গবেষনায় সম্পৃক্ত গবেষক ছাড়া অন্য কেউ এ তথ্য পাবেন না।

তারিখ ..... অভিভাবকের দস্তখত ....

/বুড়ো আঙুলের ছাপ

গবেষকের দস্তখত ....

Response to reviewers' comments :

Reviewer #1 :

Point 1: The reviewer has suggested that other agents apart from ASV and NV should be sought. We have investigated the role of adenoviruses in diarrhoeal disease and have produced 3 publications. At present we either do not have access to reagents (for viruses) or existing reagents and/or methods do not appear to be satisfactory (HCV and PBV) to examine these agents. However, samples will be kept for future analysis including B & C rotavirus.

Point 2: Regarding non-group A rotaviruses, we have previously screened stool samples from patients with acute diarrhoea by gel electrophoresis and found less than 1% of patients to be infected. A much larger sample size would be required to study these agents more thoroughly and therefore such an investigation cannot be justified within the guidelines of this study.

Point 3: Parasites : We will look for cyclospora, in wet mounts and Cryptosporadium using Ziehl-Neelson staining.

Point 4 Lower nos of sera will be tested (see page 7).

Point 5: ELISA is not the best screening technique for ASV, PCR has been shown to be more sensitive. As given in the protocol we have already observed a difference in ASV detection rates among acute diarrhoea (AD) and persistent diarrhoea (PD) patients using this technique. Since our aim is to compare the rates of detection in each group, ELISA offers a cheap and convenient method to perform this. If true prevalence rates in AD cases were to be sought, PCR would be preferable.

Point 6: NV-PCR : The extraction method has been revised from that given in the original protocol. The new method is that developed in the Viral Gastroenteritis Unit of CDC (June 95) and also incorporates a) primers for genotypes 1 and 2 and b) southern hybridization with probes to genotype P1A, P2A, P2B and P1B.

Point 7: Sample collections : I assume this is in relation to the NV study. We have proposed to select stool samples from children from a cohort study who show a sero-conversion to NV antigen. These samples have been stored adequately.

Point 8: Our laboratory has adequate facilities for the handling of biohazardous material and employs safe disposal methods.

aa:lu4(revcomnt.920)

#1

Title: Investigation of the importance of new viral agents of diarrhea

Summary of Referee's Opinions: Please see the following table to evaluate the various aspects of the proposal by checking the appropriate boxes. Your detailed comments are sought on a separate, attached page.

Rank Score

	High	Medium	Low
Quality of Project		✓	
Adequacy of Project Design		✓	
Suitability of Methodology		✓	
Feasibility within time period	✓		
Appropriateness of budget	✓		
Potential value of field of knowledge	✓		

CONCLUSIONS

I support the application:

a) without qualification ✓

b) with qualification ☐

- on technical grounds ☐
- on level of financial support ☐

I do not support the application ☐

Name of Refere

Signature:....

Position:

Institution:

Detailed Comments

Please briefly provide your opinions of this proposal, giving special attention to the originality and feasibility of the project, its potential for providing new knowledge and the justification of financial support sought; include suggestions for modifications (scientific or financial) where you feel thay are justified.

(Use additional pages if necessary)

Title:

PI:

Reviewer:

nvestigation of the importance of new viral agents of diarrhoea

tific Merit: The objectives and plan for this project are sound. It is hoped to apply existing technology to mine the importance of astroviruses (ASV) and Norwalk virus (NV) as causes of diarrhoea. Both these viruses probably turn out to be important causes of diarrhoea and their investigation is warranted and should be a ity for diarrhoeal disease research in children.

questions or points to consider:

le ASV and NV are a clear priority, other agents such as Adenoviruses (ADV), Caliciviruses (HCV), viruses (TV) and picobirnaviruses (PBV) may be highly relevant. Why not expand the study to determine the emiology of these agents in children. Adenoviruses in particular should be sought and all of these agents may ssociated with persistent diarrhoea. Even the investigation of a sub-set of collected stool material would be of rest. Alternatively, samples can be stored (4oC)

proposal will also seek a number of bacteria and rotavirus A in stools from patients with persistent diarrhoea. what about Rotaviruses B & C ? will RNA gels be run ? These emerging viruses could be missed.

o, there is no mention of screening for parasites - how will these organisms be taken into account ??

proposal to screen 500 blood bank sera seems excessive - 100 - 200 samples should give a reasonable answer.

ELISA the best screening technique for ASV ? There are some doubts about the tests referred to and in later ers PCR is clearly a more sensitive test.

NV PCR, the proposed extraction method may not be suitable. Estes has described a method involving PEG & AB which appears to be better for NV nucleic acid extraction. This question should be addressed. Also, hing is stated on the control of inhibition, the use of positive and negative controls or the confirmation of so led positives for NV PCR. Which primer sets will be used and why ? These points should be seriously nsidered.

e use of samples from previous collections may also pose some problems - have they been adequately stored? Do y represent a reasonable cross-section of samples ?

ally, the question of laboratory safety should be considered - are the facilities adequate for handling blood nples which may harbour agents such as HIV, Hepatitis viruses etc.

ick record: Excellent.

dget: Adequate, - low. 31% overhead cost seems excessive

#2 Ms. Leanne Unicomb

Title: Investigation of the importance of new viral agents of diarrhea

Summary of Referee's Opinions: Please see the following table to evaluate the various aspects of the proposal by checking the appropriate boxes. Your detailed comments are sought on a separate, attached page.

Rank Score

	High	Medium	Low
Quality of Project	✓		
Adequacy of Project Design	✓		
Suitability of Methodology	✓		
Feasibility within time period	✓		
Appropriateness of budget	✓		
Potential value of field of knowledge	✓		

CONCLUSIONS

I support the application:

a) without qualification ☑

b) with qualification ☐

- on technical grounds ☐
- on level of financial support ☐

I do not support the application ☐

Name of Refer[redacted]

Signature:.. [redacted] /95

Position: Head

Institution:

School of Microbiology
La Trobe University
Bundoora Vic 3083
Australia

Detailed Comments

Please briefly provide your opinions of this proposal, giving special attention to the originality and feasibility of the project, its potential for providing new knowledge and the justification of financial support sought; include suggestions for modifications (scientific or financial) where you feel thay are justified.

(Use additional pages if necessary)

Title:

Investigation of the importance of new viral agents of diarrh ea.

PI:

Reviewer:

It is very logical that the possible true importance of astrovirus and Norwalk virus in diarrhoea be investigated in Bangladesh, a country where diarrhoea is a major national concern. This is although similar studies to aspects of the ones proposed, have been and are being carried out in other countries. With the introduction of more widespread testing these viruses appear to be emerging as being far more important in diarrhoea generally than was previously suspected and any further information on this at this stage would be both important and helpful.

Given the new reagents and primers available and proposed to be used, and the availability of faecal specimens and sera, there is no doubt that the project is feasible. The new knowledge gained will provide invaluable baseline data which would be required in the consideration or future implementation of any vaccine strategies with respect to these two viruses. As well, it will add to the international body of knowledge on the general significance of these viruses, and as mentioned above this is important at this stage.

I would, however, suggest that in the case of the detection of astrovirus the applicants consider the use of the primers used in the recently published PCR test (Jonassen *et al.*, J. Virol. Meth. 52 (1995), p. 327), which appears to be more useful than previous such tests, rather than or in conjunction with the probably less sensitive ELISA test proposed. As a consequence the results are likely to be even more meaningful, as was the case with testing for Norwalk virus when PCR tests started to be used.

The outlined budged expenditure appears to be reasonable, so in conclusion I strongly recommend the support of this project.