ıcument 1. Library

ETHICAL REVIEW COMMITTEE, ICDDR,B

Date ~~24-6-93~~ 29/6/93

ıcipal Investigator Leanne Unicomb

Trainee Investigator (if any) _____

lication No. 93-023

Supporting Agency (if Non-ICDDR,B) _____

le of Study Study of the distribution of A rotavirus P types in Bangladesh

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

le 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
(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:
   (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)

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

ine Unicomb
Principal Investigator

Trainee

ENTERED 2 1 JUN 1993

93-023
29/6/93

APPLICATION FOR PROJECT GRANT

1.	PRINCIPAL INVESTIGATOR	:	Leanne Unicomb
2.	COINVESTIGATORS	:	Jon Gentsch, CDC, Atlanta Roger Glass, CDC, Atlanta Physician, CSD
3.	TITLE OF PROJECT	:	Study of the distribution of group A rotavirus P types in Bangladesh
4.	STARTING DATE	:	July 1, 1993
5.	DATE OF COMPLETION	:	June 30, 1995
6.	TOTAL BUDGET REQUESTED	:	$ 102,076 ($ 133,720 with 31% overhead)
7.	FUNDING SOURCE	:	
8.	HEAD OF PROGRAMME	:	Dr. R. B. Sack Associate Director Laboratory Sciences Division R. Bradley Sack

9. AIMS OF PROJECT

a) General aim

To determine the frequency and distribution of different P types of group A rotavirus and compare them to the G types (serotypes)

b) Specific aims

i) To set up a polymerase chain reaction (PCR) technique for determination of P type of group A rotaviruses

ii) To conduct P typing using PCR on rotaviruses from various stool collections

iii) To compare P types with G types (serotype), as determined by oligonucleotide probe hybridization and monoclonal antibody-based enzyme immunoassay (MEIA) techniques previously established in the laboratory

iv) Examine the range of symptoms of diarrhoea with respect to P type and P-G type pairs

c) Significance

Rotavirus vaccine trials conducted to date using a single strain or reassortant strains have shown a range of efficacies. The development of heterotypic immune responses have also varied from vaccine to vaccine and heterotypic responses to both neutralization antigens, VP7 (G type) and VP4 seem to be required for protection against rotavirus diarrhoea. P typing classifies RVs according to the protein VP4 which is involved in neutralization and is present in lower numbers on the virion than VP7, the protein that confers serotype or G type. The VP4 is thought to be involved in heterotypic immune response and, therefore, knowledge of the distribution of different VP4 types (P types) will have relevance to vaccine formulation.

10. ETHICAL IMPLICATIONS

Only stool specimens will be used in this study, which have been collected as part of previous studies (e.g. cholera vaccine trial, # 84-001, ICDDR,B surveillance programme at the Clinical Research Centre (CRC)) or will be prospectively collected from subjects attending the CRC as in-patients or as part of the surveillance programme and also from a study of a neonatal birth

cohort (# 90-018). These protocols have already been approved by ERC and are in progress.

11. BACKGROUND, RESEARCH PLAN AND BIBLIOGRAPHY

BACKGROUND

The development of a rotavirus vaccine has been assigned a high priority (1) as it is estimated that an efficacious vaccine may reduce diarrhoeal deaths by 30% in children 6-24 months of age (1). Several candidate vaccines have been tested in infants in both developed (2,3) and developing countries (4,5) and varying degrees of efficacy have been found. Vaccine candidates of human (4) and non-human serotypes (3,5) have been tested. Trials of rhesus rotavirus vaccines have generally resulted in homotypic protection (6) or rather lack of heterotypic protection (18,19). Two bovine candidate strains have been tested (RIT4237, bovine G6 and WC3, bovine G6). Initial trials of RIT4237 suggested that protection against serotype 1 (type G1) rotavirus diarrhoea was afforded (20) but subsequent trials, particularly in developed countries have shown a lack of protection (5). One trial with WC3 has shown protection against rotavirus diarrhoea of type G1 (21) although another study showed no protection during a predominantly type G1 rotavirus season (3). It has been found that lack of protection correlates with lack of production of neutralizing antibodies against the infecting serotypes (3,4).

Early trials of the human neonatal strain, M37, which is a type G1 RV have shown that specific neutralizing antibodies to the homologous vaccine strain were found among more vaccinees than antibodies to a serotype 1 strain that infects infants (4). One trial showed a lack of protection against rotavirus

diarrhoea at a dose of 10^4 PFU/ml and in some cases the diarrhoea was due to type G1 strains (4). Such information suggests that heterotypic immunity needs to be induced by vaccination.

The outer capsid layer of RV patients comprises 2 proteins, VP4 and VP7, both of which elicit antibodies capable of neutralizing rotavirus infection. It has been shown that VP7 binds to a cell surface receptor and VP4 is required for selective internalization of RV particles (22). RV serotypes (or G tpes) were initially assigned by performing cross-neutralization experiments with hyperimmune antisera (23) and serotypes (G types) correlated with antibody binding to VP7. Epitopes on VP7 that are shared by serotypes have been found (7) although it is thought that heterotypic serological responses are mostly elicited by the VP4 protein (8,9,24), and vaccine studies have suggested that heterotypic immunity involving neutralizing antibodies to the VP7 and VP4 proteins is required for protection against rotavirus diarrhoea (4).

The VP4 protein has a molecular weight of around 84 kDa (for human strains) and comprises 1.5% (by weight) of the RV particle compared with VP7 which comprises 30% (17). VP4 is coded by segment 4, is the haemagglutinin, is responsible for growth restriction and antibodies directed against VP4 have been shown to passively protect mice against heterologous challange (25).

The VP4 gene of human rotaviruses is approximately 2359 b.p. long and codes for a protein of 775 amino acids. It contains one long open reading frame. The deduced amino acid sequences of various strains show that there are two conserved regions flanking the trypsin cleavage site (presumably serving to hold the trypsin cleavage site in proper confirmation (8)) and a conserved hydrophobic region (amino acids 385 to 401) which is thought to be the region involved in cell fusion (8).

The VP4 protein is cleaved by trypsin (and other proteolytic enzymes (31) into VP8* (MW = 28 kDa, N-terminal) and VP5* (MW = 60 kDa). The putative fusion region is found on VP5, whereas the area of high sequence variation between P types is found on the N-terminal VP8 and antibodies directed to VP8 are responsible for P type-specific neutralization (26).

Studies of the nucleotide sequence of gene 4 have found 5 genetically distinct gene 4 types (10). A study using antisera produced against baculovirus expressed VP4s representing the 4 major types has shown that the division of VP4 groups based on sequence correlates well with reactivity of antisera with RVs from predicted VP4 groups (P types) (14). Recently a hybridization technique (11) and a PCR technique for P typing of stool human RVs (12), as well as an enzyme immunoassay technique incorporating monoclonal antibodies (27) have been described.

Limited information is available on circulation of various P types (12,27). Based on amino acid sequences of established RVs, human strains that infect infants and children with long RNA patterns are type P8, with short RNA patterns are type P4, those that infect neonates are type P6 (regardless of whether they have a long or short RNA pattern), strain K8 (G type G1) is type P9 (17) (see Appendix-I).

Recent studies have shown that unusual P types of RV strains have been found in India and Thailand (28,29), and these results suggest that transmission from animals to human is likely. Such findings are important additions to the current pool of knowledge of RV strain variation and have relevance to vaccine formulation.

A study of P types of 40 RV specimens from Bangladesh was conducted (13) using a polymerase chain reaction technique (12). Of the 40 specimens tested, 39 were P typed and 36 of these were expected P and G type combinations. Three were found to have unexpected P and G type combinations. It is therefore likely that the current scheme of expected P and G type combinations is somewhat preliminary and further P typing will allow examination of the diversity of VP4 and ultimately the role of VP4 in eliciting cross-protective antibodies.

We, therefore, plan to characterize RVs from different patient groups with respect to their P type for correlation with G type and with clinical signs of diarrhoea. We plan to examine P types of RVs that infect children that have been followed as part of a neonatal cohort (both with and without RV infections with P6 strains as neonates) and to examine (in detail) RV strains that cannot be P typed or have interesting P and G type combinations (methodology given later). Since little is known about the frequency of the different P types (and P and G type combinations), this study plans to investigate around 1000 specimens from different patient groups.

RESEARCH PLAN

Specimens obtained from the following groups of subjects will be P typed by PCR.

a) Specimens from neonates followed for 18 months from birth (expected total = approximately 400 RV positive specimens)

b) Asymptomatic infants (expected total = 40 specimens collected/year when it is anticipated that approximately 2000 control stools will be

collected. From data of Bhan *et al.* (15), 2% are expected to be positive, viz 40 specimens per year.)

c) Symptomatic adults ≥12 years of age (from expected total = 40 specimens collected over a one-year period from the surveillance system of the Clinical Research Centre of ICDDR,B - based on the figures from Unicomb *et al.* (16)).

d) Symptomatic infants

i) Collected so far, G typed by probe hybridization (Appendix-V) and categorized according to severity of dehydration (<2 years of age with no other pathogens)

	G typed	Not typeable
Mild	250	136
Moderate	168	80
Severe	67	17

ii) Collected and/or will be collected from patients attending the Clinical Research Centre (CRC) of ICDDR,B either as in-patients or surveillance patients and producing a stool sample from May, 1992 to December, 1994): expected number of specimens = 3750 (1½ years), expected RV positive (15%) = 562, expected number of infants RV+ (97%) = 545.

Of the specimens listed above:

Number requiring RV detection = 3,750

Number requiring G-typing = 562

Summary of specimens for P typing:

	Nature of specimen	No.	Expected/known G type	No. available/ expected to be P typed
1.	Symptomatic infants from Matlab (severity information available)	485	485	400
2.	Symptomatic infants from CRC (May 1992 - December 1994)	562	340	200
3.	Neonates	400	240	240
4.	Asymptomatic infants	60	40	40
5.	Symptomatic adults	60	40	40
6.	Non-typeable RVs	100	0	100
	TOTAL			1020

(See Appendix-II for PCR method for P typing)

The RT-PCR technique (as described in Appendix-III) will initially be valiadated in our laboratories using culture adapted RVs of known P and G type (Wa, RV4, RV5, RV3, SA11, ST3, F45, B37, AT176). Stool samples will be tested using the con 3 and con 2 primers and primers 1T-1, 2T-1, 3T-1, 4T-1, 5T-1 for P types 8, 4, 6, 9 and 69M-like respectively. The resulting PCR product (expected sizes for each P type given in Appendix-II) will be identified by ethidium bromide staining of agarose gels following electrophoresis of the products (12). A selected subset of samples will be tested using confirmation primers (see Appendix-III). Controls will be tested in each PCR run.

Number of RV positive specimens expected to yield unusual G and P type combinations (from 40 specimens tested at CDC, 3 were found with unusual combinations (i.e. 7.5%)), therefore, approximately 80 specimens are expected.

These specimens will be:

- retested for P type at CDC
- retested for G type by oligoprobe hybridization and MAb-EIA (see Appendix-V)
- tested for G type by PCR
- tested for subgroup by monoclonal enzyme immunoassay (see Appendix-VI)
- tested for electropherotype by PAGE
- sequenced (at CDC initially and subsequently at ICDDR,B)

Data analysis

- G and P type comparisons will be made by tabulation of results
- Clinical signs of diarrhoeal disease will be correlated with P type and P and G type using pairwise analyses. The type of clinical information available for different patient groups is given in Appendix-V.

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12. PUBLICATIONS OF PRINCIPAL INVESTIGATOR

Leanne Unicomb

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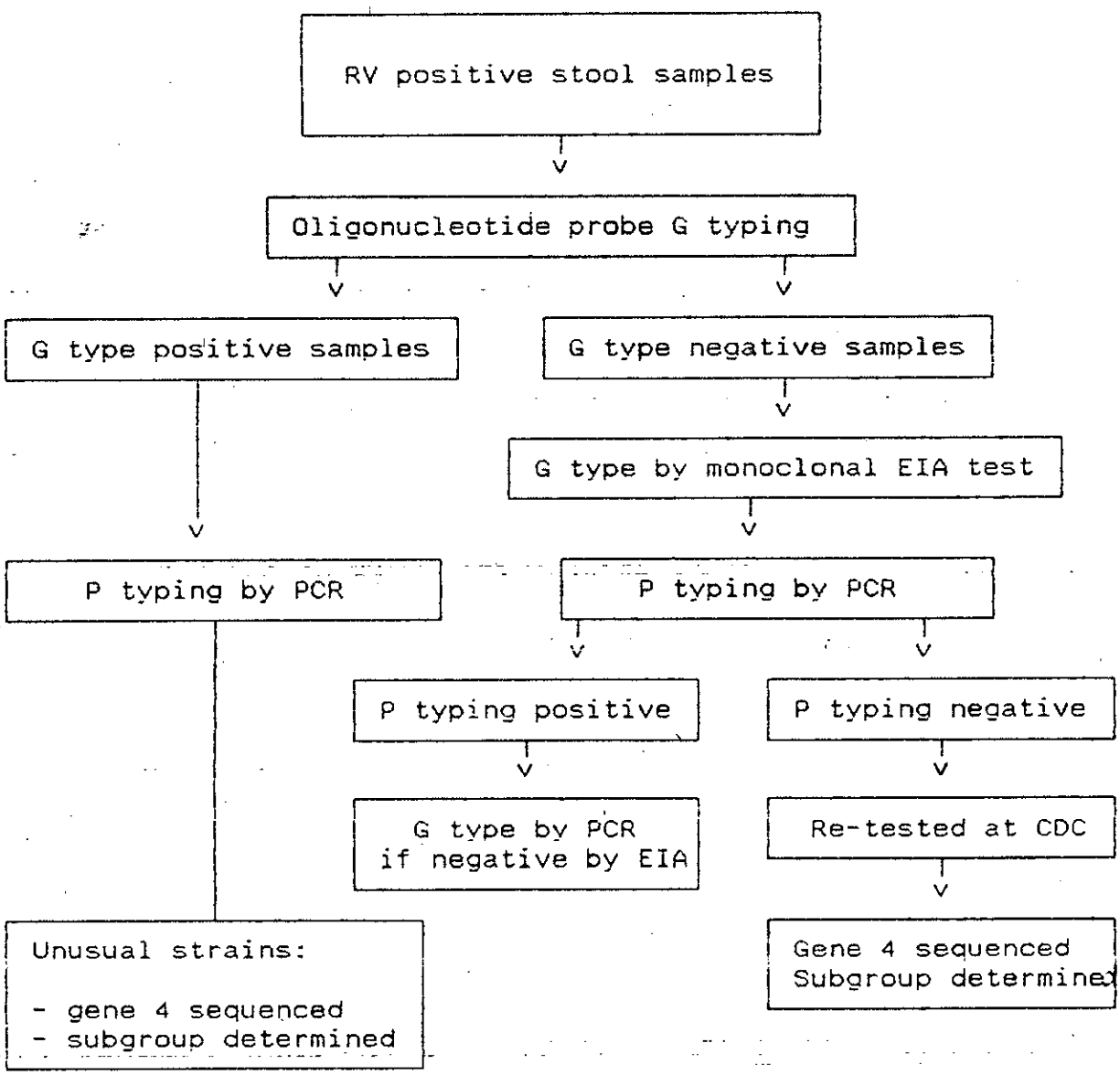
13. FLOWCHART



14. TASK OF EACH INVESTIGATOR

Leanne Unicomb

- perform PCR for P typing and G typing
- collate lab results and clinical data
- analyse data
- write manuscripts/reports

Jon Gentsch

- oversee performance of PCR for P typing
- provide technical back-up
- give advice regarding PCR technique and results
- provide assistance in testing and analysing results pertaining to "unusual strains"

Roger Glass

- advise on matters relating to results, manuscript preparation

Physician

- advise on analysis of clinical information

Shahjahan Miah

- performance of ELISA tests

Masudur Rahman

- maintenance and washing of laboratory glassware and equipments
- preparation of buffers, solutions and stool extracts

15. BUDGET

Detailed Budget

			1993-94	1994-95	Total
a) Personnel costs					
Leanne Unicomb	P2-12	50%	27,686	27,686	55,372
Shahjahan Miah	GSIII-20	100%	4,380	4,650	9,030
Masudur Rahman	GSI-5	100%	$ 1,833	$ 1,893	$ 3,726
Total			$ 33,899	$ 34,229	$ 68,128
b) Operating costs					
RV detection (n=3750) $2/test			$ 2,500	$ 5,000	$ 7,500
Oligoserotyping (n=562) $4/test			1,200	1,048	2,248
P typing - PCR (n=1020) $10/test			6,000	4,200	10,200
Further characterization of unusual RVs (n=100) $20/strain			-	2,000	2,000
Interdepartmental			2,500	2,500	5,000
Equipment*			3,000	-	-
International travel			4,000	-	4,000
Total			$ 19,200	$ 14,748	$ 33,948
GRAND TOTAL			$ 53,099	48,977	$ 102,076

*Equipment = DNA gel electrophoresis apparatus - $ 500
Shaking water bath - 2,500

LU:mh/L9:GAROT.PRT

16. JUSTIFICATION FOR BUDGET

International travel

We plan to investigate the gene 4 segment of selected RV strains (non-P-typeable, unusual P and G type combinations) by examination of the nucleic acid (and predicted amino acid) sequence. Some non-P-typeable strains will also be retested. It is planned that this will take place in Dr. Roger Glass's laboratory at CDC, Atlanta. The PI will spend around one month at CDC to learn (and perform as much as possible of) gene 4 sequencing and also to retst non-typeable strains. The gene sequencing technique will subsequently be carried out at ICDDR,B (when necessary).

Appendix-I

P typing and corresponding G type of human group A rotaviruses

Serotype (G type)	P type (following scheme of Estes and Cohen[a])				
	P8	P4	P6	P9	P?
G1	G1		NG1[b]	G1-K[c]	
2		G2	NG2		
3	G3		NG3		
4	G4		NG4		
5					
6					
7					
8					G8
9	G9				

[a]Estes MK, Cohen J. Microbiol Rev 1989; 53:410-449 (17)

[b]NGI = neonatal G type 1

[c]GIK = G type 1 - K8 like strains

Appendix-II

P typing of group A rotavirus by polymerase chain reaction (PCR)
(Gentsch *et al.* J Clin Microbiol 1992; 30:1365-1372 (12))

Two amplification steps will be used; the first uses primer pairs that contain highly conserved sequences of gene 4 of P types 8, 4, 6, 9, 69M-like and the second uses a cocktail of P type specific primers yielding a PCR product of each P type of different sizes.

A. FIRST (GENERAL) AMPLIFICATION

1) Double stranded RNA (dsRNA) will be extracted from stool samples using a glass powder preparation (RNAid)

2) 1.5 µl of dsRNA will be added to 3.5 µl of dimethylsulphoxide and will be denatured by heating to 97°C for 5 mins

3) After cooling for 5 mins on ice, the sample will be centrifuged at 10,000 X *g* for 10 sec

4) The following reverse transcriptase-PCR mixture will be made:

12.0-13.5 µl distilled water

16.0 µl of deoxynucleosidetriphosphate (dNTP) mixture
(1.25 mM each of dATP, dGTP, dCTP, dTTP)

5.0 µl of [10X] buffer II (100 mM Tris-HCl, pH 8.3, 500 mM KCl)

3.5-5.0 µl of 25 mM $MgCl_2$

2.0 µl of primer (25 mM each of "conserved" primers
con 3 + con 2)

1.5 µl reverse transcriptase (RT) - Amplitaq/RT-Taq mixture
(9 U RT and 1.9 U Taq)

41.5 µl total
===

5) Denatured dsRNA will be added to the above mixture (final volume approximately 50 µl)

6) 100 µl of mineral oil will be added and mixed and centrifuged at 10,000 X *g* for 10 sec

7) Sample will be subjected to one cycle of RT and 30 cycles of PCR

Each PCR cycle = 1 min 94°C, 2 min 50°C, 2 min 72°C, then finally cooled at 17°C at completion of 30 cycles.

B. SECOND (TYPING) AMPLIFICATION

1) 0.5 to 5.0 µl (5.0 µl if no product is visible) of first amplification product will be added to the following reaction mixture:

19.5 µl of distilled water

16.0 µl of dNTP mixture

5.0 µl [10X] buffer II

3.0 µl of 25 mM $MgCl_2$

45.0 µl total

=======

2) To the above mixture, a further 1 µl of typing primer cocktail (20 mM each of primers con 3, 1T-1, 2T-1, 3T-1, 4T-1 and 5T-1) will be added along with 0.5 µl of (2.5 U) Amplitaq and overlayed with mineral oil

3) After mixing and centrifugation (10 sec at 10,000 X *g*), the specimens will be subjected to PCR as above

4) The resulting products will undergo agarose gel electrophoresis for determination of P type. The expected size of PCR products for P types 8, 4, 6, 9 and 69M-like are 345, 483, 267, 391 and 583 base pairs respectively.

C. CONFIRMATION OF P TYPING

In selected case, confirmation of P type may be required and this will be done using a one-step reverse transcriptase and PCR amplification procedure using pairs of primers (e.g. 1C-1 and 1C-2 for type 8), i.e. one plus sense and one minus sense primer for each pair. dsRNA will be extracted using RNAID (as previously described) and the expected sizes of PCR products for P types 8, 4, 6 and 9 are 180, 504, 206 and 261 base pairs respectively.

Sequence of primers (5'->3') for P typing (con 3 to 5T-1) and confirmation (1C-1 to 4C-2)

		Nucleotide position
con 3	TGGCTTGCCATTTTATAGACA	11 to 32
con 2	ATTTCGACCATTTTATAACC	868 to 887
1T-1	TCTACTTGGATAACGTGC	339 to 356
2T-1	CTATTGTTAGAGGTTAGAGTC	474 to 494
3T-1	TGTTGATTAGTTGGATTCAA	259 to 278
4T-1	TGAGACATGCAATTGGAC	385 to 402
5T-1	ATCATAGTTAGTAGTCGG	575 to 592
1C-1 (+)	GGACTGCAGTAGTTGCTA	314 to 331
IC-2 (-)	TTAGTATCAGAAGTTAGTGTA	474 to 494
2C-1 (+)	ATACGAACACGTACAATAAAC	1324 to 1344
2C-1 (-)	CATCATTTACTGAGTCAGTT	1809 to 1828
3C-1 (+)	GAATCCAACTAATCAACA	261 to 278
3C-2 (-)	TGTTGAAATTCGGCACTAACA	446 to 467
4C-1 (+)	ACCTCACTCAACTTAGT	223 to 239
4C-2 (-)	ATAATGTTGAATATTGAGTGT	404 to 484

Source of primers: Viral Gastroenteritis Unit, Centers for Disease Control, Atlanta, GA, USA

030785

Appendix-III

Clinical and other information collected for different patient groups to be examined

The following parameters have been recorded for these patient groups:

1) Symptomatic infants from Matlab 1987-1989

Age, gender, type of diarrhoea, duration of diarrhoea, number of loose motions in 24 h period prior to presentation, history of vomiting, presence of abdominal pain, presence of fever, weight (admission and discharge), height, ORS treatment, IV fluid treatment, various parameters of dehydration, breastfeeding practices, presence of shigellae, salmonellae, vibrios and adenoviruses in stool.

2) Symptomatic infants and adults from the surveillance patient group

Age, gender, chemotherapy, feeding practices, duration of diarrhoea prior to presentation, vomiting in the last 24 h, duration of stay in hospital, source and nature of water supply, socioeconomic status, fever, type of diarrhoea, abdominal pain, clinical assessment of dehydration, respiratory symptoms, presence of shigellae, salmonellae, vibrios, adenoviruses, *Giardia lamblia*, *Entamoeba histolytica*, *Cryptosporidium*, *Campylobacter* spp., *Aeromonas* spp., diarrhoeagenic *E. coli*, *Plesiomonas* spp., weight on discharge, height, arm circumference, weight/height (%).

3) Neonatal cohort (symptomatic infections)

Age, gender, socioeconomic status, birth weight, history of breastfeeding, type of stool, history of vomiting, duration of diarrhoea, source of drinking water, fever, parameters of dehydration, ORS treatment, chemotherapy, weight, height.

Appendix-IV

Enzyme immunoassay (EIA) for serotyping group A human rotavirus using monoclonal antibodies B (according to the method of Coulson *et al.*, 1987)[7]

1. Coat 96F NUNC tray with 100 µl/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-RV3 (serotype 3)	...	1:6,000
	4	Anti-ST3 (serotype 4)	...	1:8,000

2. Incubate tray at 37°C for 2 hours to a moist environment.

3. Wash X3 with phosphate buffered saline, pH 7.2, containing 0.5% Tween-20 (PBST)

4. Add 75 µl of skim milk powder solution (SMP) to each well, followed by 25 µl of 10% (w/v) faecal supernatant or 100 µl 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°C for 16-20 hours

6. Wash as in step 3

7. Add 100 µl of mouse monoclonal antibodies to each well, diluted in SMP as follows:

Row	1	MAb RV4:2	Protein A fraction 1:200
	2	MAb RV5:3	Protein A fraction 1:2,000
	3	MAb RV3:1	Protein A fraction 1:2,000
	4	MAb ST3-1	Protein A fraction 1:1,000

8. Incubate at 37°C for 2.5 hours in a moist environment

9. Wash as in step 3

10. Add 100 µl/well anti-mouse immunoglobulins conjugated to horseradish peroxidase at 1/800 (Dako) diluted in SMP, or Silenus branch (1/2,000)

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

12. Wash as in step 3

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

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

APPENDIX-V

DNA hybridization for detection of serotypes of group A rotavirus

Rotavirus RNA extraction

RNA is extracted as for polyacrylamide gel electrophoresis following the method of Herring *et al.* (30).

Blotting of RNA onto nitrocellulose

1. The RNA solution is mixed with one volume of 6.15 M formaldehyde, 10X SSC and incubated at 65°C for 15 minutes

2. 15 µl is then spotted onto nitrocellulose sheets using a Bio-dot apparatus. The spots are rinsed with 10X SSC (100 µl) and the filters are air-dried and baked at 80°C for 2 hours.

Oligonucleotide probes labelling

1. 8 pmol of each probe will be 5'-end labelled with r-32p-ATP (3000 Ci/mmol) using a commercial kit (BRL)

2. The labelled probes will be separated from unincorporated ATP by passing them through Sephadex G-25 column by centrifugation

Probe hybridization

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

Probe concentration: 10^6 cpm/ml hybridization buffer

Hybridization temperature

41°C for Hug2Ac and Hug3Ac

42°C for HuG8Ac

46°C for HuG1Ac, HuG4Ac, HuG4Ac, BoG6Ac and PgG5Ac

(filters will be hybridized for 16-18 hours)

Washing condition

1. 3X SSC at room temperature for 30 minutes

2. 3X SSC at the hybridization temperature for 10 minutes, twice

3. 2X SSC at room temperature for 1 hour

Following washing, filters will be air-dried then exposed to X-ray film for 16-18 hours at -70°C

APPENDIX-VI

ELISA FOR DETECTION OF GROUP A ROTAVIRUS IN STOOL SAMPLES

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 µl of 10% (w/v) stool extract in PBS will be added to rota-positive well and 100 µl 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-VII

Detection of group a rotavirus subgroups by enzyme immunoassay (following the method described by Svensson *et al.*, 1988 [32])

1. Plates will be coated with rabbit antirotaviral hyperimmune antisera and preimmune rabbit sera at a dilution of 1/5000 in 0.06 M carbonate-bicarbonate buffer, pH 9.6 (100 µl/well) and incubated at 37°C for 1 hour.

2. After washing 3 times with phosphate buffered saline, pH 7.2 (PBS) containing 0.05% (v/v) Tween-20 (PBST), 75 µl of 2% (w/v) skim milk powder in PBST (SMP) will be added followed by 25 µl of 10% (w/v) stool extract in PBS. All samples will be tested in quadruplicate on anti-RV coated wells and in duplicate on preimmune sera coated wells. Two anti-RV coated wells and one preimmune sera coated well will receive each subgroup monoclonal. Plates will be incubated at 4°C overnight.

3. After washing 3 times with PBST, subgroup I and subgroup II monoclonal antibodies diluted 1/500) in SMP will be added (100 µl/well) to respective wells. Plates will be incubated for 1 hour at 37°C.

4. After washing with PBST, HRP-conjugated anti-mouse immunoglobulins diluted 1/1000 in SMP (100 µl/well) will be added. Plates will be incubated for 1 hour at 37°C.

5. After washing, substrate containing 3,3',5,5'-tetramethylbenzidine will be added (100 µl/well) and incubated for 10 minutes at room temperature. The reaction will then be stopped and OD at 450 nm determined in an ELISA reader.

6. Subgroup will be determined by comparison of OD_{450} ratios with the 2 monoclonal antibodies. A subgroup I RV will, therefore, be one with ratio of ≥ 2.5 of subgroup I wells OD_{450}/subgroup II wells OD (and vice-versa for subgroup II strains).

ABSTRACT SUMMARY

This study is designed to survey group A rotavirus (RV) strains for their "P type". This new typing scheme will identify different P types on the basis of the sequence of the gene for one of the neutralization proteins, VP4. VP4 is an important component of RVs as it stimulates production of antibodies capable of neutralizing virus infectivity and is important for pathogenesis.

There is much interest in the variation of P types since not much is currently known. Recent reports have suggested that some RV infections may be zoonotic and this is possible to study by determining P types since animal RVs possess VP4s that are distinct from human strains.

Furthermore, distribution of P types of RV strains, particularly in developing countries, will be of importance for the formulation of RV vaccines since vaccine candidates tested so far have only provided limited protection against RV illness.

Review #1

Title: Study of the distribution of group A rotavirus P types in Bangladesh.

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 Referee:

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)

SEE ATTACHED SHEET.

Title:

PI:

Reviewer: .....

REVIEW OF ICDDR,B PROTOCOL:

"STUDY OF THE DISTRIBUTION OF GROUP A ROTAVIRUS P TYPES IN BANGLADESH" by L. Unicomb, J. Gentsch and R. Glass

This is a well written proposal dealing with an important subject (the identification and distribution of rotavirus P serotypes) which has important implications for the development of rotavirus vaccines. The methodology is clear and has been proven useful in previous studies. Chances for success are therefore high.

It may be useful to take into consideration the following minor points:

- two new "P" serotypes of human rotaviruses have been identified. Although the corresponding manuscripts have not yet been published, it is likely that they will be appearing in the next few months. The investigators may want to consider addition of primers to detect these additional human P serotypes once the information is available.

- the new "P" serotype corresponding to bovine strain B223 has been identified in human neonates, and therefore its inclusion in the study will be of interest.

- it would be advisable that specimens of "unknown" P serotype be tested with the same procedures by using primers corresponding to the various animal rotavirus P types.

- correlating specific P types with the severity of diarrheal disease may be difficult in the absence of parallel serological studies to question whether the outcome of infection is the product is influenced by previous exposure to rotavirus.

- it is likely that all the specimens from neonates be similar (in view of the observation of previous similar studies); therefore the expense of examining a large number of them may not be justified (unless the infants are subsequently followed to evaluate the protective effect of neonatal infection).

- the flow-chart on page 14 of the protocol should be closed at the bottom (P-type negative specimens from G-type negative samples also would need sequencing of the gene 4).

- with respect to the budget: a) the cost of P-typing ($ 10/test) seems exaggerated; b) a shaking water bath does not appear to be needed for this study; c) what are "interdepartmental" operating costs?

Review #2

STUDY OF THE DISTRIBUTIONS OF GROUP A ROTAVIRUS P-TYPES IN BANGLADESH

PRINCIPAL INVESTIGATOR: DR. L. UNICOMB

REFEREE'S COMMENTS ON PROPOSAL

I have studied the above research proposal with enthusiasm. Knowledge of the epidemiology of rotavirus P-types and their correlation with rotavirus G-type is urgently needed. Most clinical trials of candidate rotavirus vaccines conducted thus far have been performed without any consideration of the epidemiology of rotaviruses with regard to P-types and knowledge of the role of P-types in immunity. Furthermore, the importance of rotavirus protein VP4 (P-type protein) in pathogenicity and as a marker of zoonotic infection is emerging.

The methodology descibed is, on the whole, tried and tested and should not present any dificulties.

The proposed budget is reasonable, provided the majority of the oligonucleotides are obtained early on, thus avoiding inevitable increase of the cost of phosphoramidites.

I am familiar with the published work of Dr. Unicomb who has made a significant contribution to rotavirus research and hopefully will continue to do so with this work. Similarly, I have great respect for the abilities of the propsed co-investigators.

My more specific comments on the proposal are minor:-

1. The original method of rotavirus detection is not given (p14).
2. The G-typing of rotaviruses by EIA does not appear in the flow chart (p14).
3. The subgrouping method (albeit standard) is not included although it is refered to in the text.
4. They propose to test for rotavirus G-types 1,2,3 and 4 only. Is this wise, given that at least 7 G-types have been identified in humans to date?
5. The G-type 2-specific monoclonal antibody 5:3 might not the best choice. I and others have found the G-type 2 monoclonal antibody produced Dr. H. Greenberg is more avid, the proposers should consider obtaining it, (try N.I.H., U.S.A.).

I hope these observations are of some use to you.

Title: Study of the distribution of group A rotavirus P types in Bangladesh.

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 Referee:

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 fc modifications (scientific or financial) where you feel thay are justified.

(Use additional pages if necessary) See attached.

Title: Study of group A rotavirus P type in Bangladesh

PI: Dr. Leanne Unicomb

Reviewer:

Response to reviewers comments

Reviewer #1

1. Regarding a) newly described p types of human rotaviruses;
2. b) bovine B223-like p type in humans;
c) "animal" -p types requiring testing in the case of p type negative strains:

We will be receiving probes for bovine p types from Dr. Linda Saif and primers for B223-like bovine p type from Jon Gentsch and Roger Glass. Drs. Glass and Gentsch have also assured us that primers for newly described p types of human RVs will be prepared and sent to ICDDR,B.

2. Regarding correlations of severity of diarrhoea and p types, we do not plan to perform any serological tests for the majority of diarrhoeal cases, with exception of the group of neonates that have been followed. As part of another study (#90-018) serum and stool antirotaviral antibodies will be measured.

In the cases where antibodies will not be tested, we will employ epidemiological techniques for severity correlations as our group (1) and other investigators have in the past for correlating severity of RV illness with various properties of RV strains (2).

3. Regarding specimens from neonates, the protocol was not entirely clear as to what specimens would be tested. The reviewer has understood that only RV strains detected in the neonatal period will be tested as these strains have been found to be a homogenous group with regard to their p type. The intention is to test RV strains sequentially detected from individuals therefore attempting to look at re-infection with RV strains of various p types. Hence the need to test these strains.

4. Regarding the flow chart - the recommendation has been included (p14).

5. Regarding the budget (a) cost for p typing $10. We received an estimate for reagents only (from CDC) at $6/specimen. Costs for reagents plus air-freight (30%) which is an additional expense that we face at ICDDR,B that is not required in USA brings the cost to $8/specimen and we added $2/specimen for disposable plasticware that was not in the CDC estimate. (b) shaking water : is required for oligonucleotide probe hybridization for determination of G type and for probe hybridization for determination of bovine p types. Our original apparatus is no longer functional.

Reviewer #2

1. Regarding RV detection method - this is now included (appendix VI).

2. Regarding EIA for G typing not in the flow chart - it has now been included (p14).

3. Regarding subgrouping not included - it has now been incorporated (appendix VII).

4. Regarding addition of further reagents for G types other than 1 to 4 - from a survey of over 500 RV strains from Matlab, we did not find any G type 5, 6 or 8 strain. It is likely that some non-typeable strains may be of G types other than 1-4 and selected strains may be taken to CDC for G typing by PCR when justified.

5. Regarding the monoclonal antibody for detecting type G2 RVs, we have approached Dr. Harry Greenberg to obtain the reagent.

References:

1. See Principal Investigators publications : Bern *et al* (1992)

2. Steele, AD, Bos P, Alexander JJ. Clinical Features of acute infantile gastroenteritis associated with human rotavirus subgroups I and II. J Clin Microbiol 1988 26: 2637-2649.

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