Attachment 1.

REVISED ERC 23-6-94

Date 5-4-94

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Principal Investigator Leanne Unicomb

Application No. 94-006 (Revised)

Title of Study A survey of neonatal rotavirus infection in Bangladesh

Trainee Investigator (if any)

Supporting Agency (if Non-ICDDR,B)

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)
   (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)
   (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
   (b) Use of fetal tissue or abortus — Yes (No)
   (c) Use of organs or body fluids — (Yes) No
4. 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 N/A
   (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 N/A
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)

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

Leanne Unicomb
Principal Investigator

Trainee

A-031957

APPLICATION FOR PROJECT GRANT

1.	PRINCIPAL INVESTIGATORS :	Leanne Unicomb ICDDR,B Roger Glass, M.D., Ph.D. Viral Gastroenteritis Unit Centers for Disease Control and Prevention Atlanta, GA, USA
2.	COINVESTIGATORS :	R. Bradley Sack, M.D. Sc.D., ICDDR,B Jon Gentsch, Ph.D. VGU, CDC, Atlanta, GA, USA Medical Officer ICDDR,B Medical Officer, Viral Gastroenteritis Unit CDC, Atlanta, GA, USA
3.	CONSULTANT :	Dr. Nigar S. Shahid ICDDR,B
4.	TITLE OF PROJECT :	A survey of neonatal rotavirus infections in Bangladesh
5.	STARTING DATE :	As soon as possible
6.	DATE OF COMPLETION :	Three months from starting date
7.	TOTAL BUDGET REQUIRED :	US$ 2,845
8.	FUNDING SOURCE :	
9.	HEAD OF PROGRAMME :	

R. Bradley Sack

Dr. R. Bradley Sack
Associate Director
Laboratory Sciences Division

10. ABSTRACT SUMMARY

Rotaviruses are a major cause of morbidity and mortality among infants and young children worldwide, and are responsible for more than an estimated 870,000 deaths each year among children less than 5 years-of-age mostly in developing countries. Several studies suggest that infections in the neonatal period may protect children against severe rotavirus diarrhea upon subsequent infection. While neonatal rotavirus infections acquired in hospital have been documented periodically for many years, a recent survey from New Delhi and preliminary studies from Dhaka suggest that these infections may be much more common than previously expected. Furthermore, rotavirus strains identified and characterized from neonates represent strains and serotypes distinct from those circulating in the normal population.

We propose to conduct a survey of neonatal rotavirus infection among newborns at 6-12 nurseries in Bangladesh to determine the prevalence of newborn rotavirus infection. We will go on to characterize the rotavirus strains obtained from these neonates and compare them with strains normally found in the community. Finally, by using a rapid screening test for the survey, we will attempt to identify risk factors for neonatal nosocomial infection that might help us understand modes of transmission and the potential impact of these infections on subsequent exposures to rotavirus. An improved understanding of the importance of neonatal rotavirus infections could further our understanding of the epidemiology of fatal rotavirus diarrhea in developing countries, could help clarify why these neonatal infections are asymptomatic, and might help identify strains suitable for consideration as future vaccines.

11. INTRODUCTION

a) Objective

The main objective of this survey is to determine the prevalence of rotavirus infection among newborns in various hospitals of Bangladesh. We will also identify risk factors for neonatal infection and in the laboratory, compare rotavirus strains obtained from newborns with those strains circulating in the community at the same time for similarity. This study could provide information on the prevalence of neonatal infections with particular relevance to vaccine strategies.

b) Background

Epidemiology of Neonatal Rotavirus Infections

Rotaviruses were discovered in 1973 and since then, have been recognized as the most common cause of severe diarrhea in infants and children worldwide (1). Nearly a million deaths occur from rotavirus each year and all children are infected with this virus by three years-of-age. The peak incidence of severe rotavirus diarrhea occurs between three- and eighteen months-of-age, and infections in the first three months of life appear to be less common and are often asymptomatic or associated with mild diarrhea.

In the late 1970s, a number of investigators began identifying rotavirus infections among neonates that were delivered in-hospital (2,3). These infections were of considerable interest since they usually produced no symptoms, were acquired in hospital, and continued year round, independent of the normal rotavirus season. Ruth Bishop determined that neonates infected with rotavirus were protected against severe diarrhea associated with rotavirus reinfection (4), an observation that led her to suggest that

neonatal immunization with these strains might provide a novel way to protect children against the severe morbidity associated with rotavirus diarrhea.

The rotavirus strains identified from neonates were first characterized by Hoshino for the main neutralization protein, VP7 (5), and subsequently characterized by Flores and Gorziglia for the second outer capsid protein VP4 (6). While neonatal strains contained the full repertoire of main VP7 serotypes, 1 to 4, the VP4 gene was unique to newborn strains (7), which led Flores to consider that these neonatal strains were naturally attenuated. Several groups began preparing neonatal strains as potential vaccine candidates and even testing these in Phase 1 clinical trials. Such Phase 1 testing is currently ongoing with the Australian strain of Ruth Bishop. The development of improved VP4 typing methods led to the occasional identification of neonatal VP4 types in older children with diarrhea, suggesting that it was not necessarily attenuated on the basis of its genotype.

In 1986, a group of novel neonatal rotavirus strains were identified in newborns at the All India Institute of Medical Science in New Delhi (8). This strain has subsequently been characterized as a G9-P11 type, a strain not previously isolated from humans. Follow-up of a cohort of children with this neonatal infection determined that this strain was highly immunogenic, nonreactogenic, and induced good protection against subsequent severe rotavirus diarrhea on reinfection (9). To examine the epidemiology of neonatal rotavirus infections in New Delhi, a survey of six hospitals in New Delhi was conducted for a one-month period of time. Infants in five of the six hospitals were found to have neonatal infections and three unusual types of strains were identified in these children, none of which were common among

strains in the community. This study led to the conclusion that neonatal rotavirus infections in developing countries may be much more common that previously believed. Similar studies conducted in Dhaka through ICDDR,B have identified neonatal rotavirus infections at two hospitals, as well (10).

The consequences of such neonatal infections among children delivered in hospitals are of some importance to vaccine development. An early finding from field trials was that vaccines were less protective among children in developing countries than among children in developed countries (11). A high prevalence of neonatal infections and the year-round incidence of rotavirus in tropical areas would mean that children receiving vaccines in developing countries were being boosted rather than experiencing their primary exposure to rotavirus. Since children can get subsequent rotavirus diarrhea, the protection of this booster vaccine against subsequent rotavirus diarrhea would be less effective than a primary vaccine, a prospect leading to the rejection of a good vaccine on improper grounds.

12. SPECIFIC AIMS

The study described in this proposal will be used to establish the prevalence of neonatal rotavirus infections among newborns at 6-12 hospitals around Bangladesh. This survey will serve to answer the following questions:

a) To measure the prevalence of nosocomial rotavirus infections among newborns in selected hospitals of Bangladesh.

b) To characterize neonatal rotavirus strains collected from newborns and representative children with rotavirus diarrhea.

c) To identify risk factors for nosocomial neonatal rotavirus infection.

13. METHODS AND PROCEDURES

a) *Overview - Survey of neonatal rotavirus infections*

Rotavirus infections occur year-round among children in Bangladesh with a slight seasonal peak in the cooler, winter months (12). Where nosocomial outbreaks have been documented, rotaviruses infect a majority of infants in the newborn units, particularly those who remain in the unit for three days or longer (10). For this study, a field team will visit 6-12 newborn units around Bangladesh over a four-eight week period. Stools of hospitalized newborns present in the unit for 48 hours or more will be sampled and tested with a rapid detection test for the presence of rotavirus antigens. The parents of all newborns positive for rotavirus and a selection of rotavirus-negative controls will be included in a case-control study to examine risk factors for nosocomial infection. All stool specimens identified to be positive for rotavirus in the field will be brought to the laboratory for characterization of the VP4 and VP7 proteins.

b) *Survey Methods*

The sample size of the survey has been estimated from our previous experience with a similar survey in New Delhi as well as experience noted in the literature from other nosocomial neonatal infections. In general, 30-70% of children two or more days-of-age in ongoing outbreaks become infected during the newborn period. Consequently, a sample-size of between 25 and 50 stool specimens in each unit would be adequate to determine whether nosocomial rotavirus infection was present or absent.

If no rotavirus infections are documented, another hospital will be chosen to continue the survey. However, if any neonates are positive for rotavirus, the study would be continued until 20-25 rotavirus-positive cases and an equal number of age-matched controls for each site, were recruited to the case-control study. This should provide more than 100 cases and 100 controls for analysis. Therefore, a case is defined as an RV infected neonate and a control is uninfected.

Previous studies have documented that duration of hospitalization is the most important risk factor for nosocomial rotavirus infection. Not fully examined have been issues of host factors (breast-feeding, birth weight, vaginal versus C-section delivery), or hospital exposure factors (number of newborns in a ward, lying-in of newborns with mothers, contact of individual nurses with susceptible patients, and delivery suites). By using a rapid antigen screening method, cases with confirmed rotavirus infection can be documented early so that controls can be chosen and case and control interviews (Appendix-I) administered on the same day.

c) *Assessment of Community Strains*

One hundred strains of rotavirus collected from children with diarrhea attending the Dhaka or Matlab hospitals of the ICDDR,B will be characterized using the same techniques for VP4 and VP7 types as part of the protocol (93/023) "Study of the distribution of group A rotavirus P types in Bangladesh" (see Appendix-II). Strains with unusual or different serotypes will be further characterized in a fashion similar to that used for the newborn strains. The distribution of VP4 and VP7

types of these strains will be compared with those of strains found in the community.

d) *Laboratory Methods*

Detection of rotavirus in stool specimens in the hospital: A latex fixation test will be used in the field to detect the presence of rotavirus antigen in stools of neonates. This test is nearly as sensitive as the ELISA, and can yield a positive result in 5-10 minutes. The ability to screen newborns rapidly provides the opportunity to go back and administer questionnaires and choose controls. All latex-positive specimens can be further confirmed using an ELISA assay.

Characterization of rotavirus strains from stool specimens: All strains that are positive by latex fixation will be confirmed by ELISA at the ICDDR,B. These positive strains will be examined by gel electrophoresis and representative strains cultivated in MA-104 cells. Positive specimens will be identified in the culture fluid by ELISA. RNA extracted from these cells will be characterized for VP7 and VP4 genes using the methods of Gouvea and Gentsch (13,14). Strains that can not clearly be characterized will be subjected to further molecular analysis, including sequencing, genogrouping, or sequetyping at CDC.

Analysis of the overall prevalence of neonatal rotavirus infection in each hospital will be compared. Risk factors will be analyzed using the Epi Info Program (5.0) with odds ratios characterized for the risk factors involved.

14. SIGNIFICANCE

Rotavirus vaccines have been less effective among children in developing countries.

One possible explanation for this is the presence of neonatal or early childhood infections with rotavirus that antedates the administration of the vaccine. The effect of this early infection would be to decrease differences observed from the vaccine between placebo and vaccinated groups. This would have the effect of lowering the measured vaccine efficacy observed.

From the laboratory perspective, the study would identify the characteristics of strains circulating among neonates to assess whether these strains were also present in the community. The absence of these strains in the community would suggest that specific factors in the newborn, such as neutralizing antibodies in the mothers that did not protect against this particular serotype, issues of maturation of receptors, mucins or enterocytes could explain the unusual distribution of strains among neonates. Understanding this relationship would clear the way for considering that future vaccines against rotavirus diarrhea be administered to newborns in the neonatal period.

15. FACILITIES REQUIRED

This collaborative study would be conducted based at ICDDR,B in Dhaka. A medical epidemiologist from CDC along with a medical epidemiologist or field workers from ICDDR,B would visit the hospitals selected for the study. All laboratory specimens would be processed in the virology laboratory at ICDDR,B. Preliminary cultivation of strains and characterization of VP4 and VP7 types by PCR would be conducted in Dhaka. Strains that do not conform to known

serotypes would be sent to CDC for further examination and characterization. These arrangements would allow for collaboration in both field studies and laboratory, and could lead to improvements in ability in the Dhaka lab.

16. ETHICAL REVIEW

Ethical review would be secured for the study at ICDDR,B. Informed consent would be secured from all parents. The newborns would only submit a soiled diaper and no invasive procedures would be performed.

17. REFERENCES

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ABSTRACT SUMMARY for ERC

Project title : A survey of neonatal rotavirus infections in Bangladesh

Principal investigators: Leanne Unicomb, LSD, ICDDR,B
Roger Glass
Viral Gastroenteritis Unit, CDC, Atlanta

This study concerns rotavirus (RV) infection of neonates, an infection which appears to be beneficial by resulting in protection against subsequent symptomatic RV infection. We have looked for the presence of such infections in neonates in 3 hospitals in Dhaka only and found the majority of neonates born or housed in hospitals in Dhaka to be infected.

This study is designed to look at how common neonatal RV infection is in babies born in various hospitals around Bangladesh.

We have two main interests in performing this study. Firstly, we would like to gain some insight into the mode of transmission of this type of infection, and secondly, to look at how different the RV strains are from different locations.

Study of the mode of transmission will involve assessment of risk factors associated with infection using a questionnaire. This will be done at various hospitals by an EIS officer from CDC. A rapid test will be employed at the bedside for the selection of RV positive and negative individuals. All samples will be retested in the Dhaka lab.

The variability of the RV strains from hospital-to-hospital will be examined by testing for various antigenic characteristics as outlined in the protocol.

We have sent out a questionnaire on participation to a total of 8 hospitals representing 7 different cities. Four positive responses have been received.

Specific information required by ERC (points 1 to 8):

1. The subjects to be enrolled in this study are neonates. A stool sample is required, and following the outcome of a bedside test of stool samples for rotavirus, the subjects may be enrolled. If enrolled, a questionnaire will be administered to the mother if consent is given and the consent form is signed.

2. There are no potential risks to the neonate or mother.

3. N/A

4. Confidentiality will be maintained by keeping all results and questionnaires in a locked filing cabinet.

5. Neonate-mother pairs will only be enrolled after obtaining the mother's signature/thumb impression on the consent form in the protocol.

6. An interview of the enrolled mothers will take place at the bedside in hospital. The interview will take no longer than 10 minutes.

7. There are no foreseen direct benefits to the subjects although there are no anticipated risks. The information gained from completion of this study could have an impact on formulation of rotavirus vaccine strategies.

8. The activities will require information from the nurses, i.e. infant:nurse ratio and rooming type, from the hospital charts - any symptoms of diarrhoea; from the mother - information on diet and from the neonate - a stool sample.

LU:mh/L11:ERCABS

18. BUDGET

Lab costs for ICDDR,B

a) Rotavirus detection for 200 specimens

Latex (provided by CDC)	...	US$	0
ELISA	...		400
Gel	...		200
b) Culture costs for 10 samples	...		100
ELISA	...		30
Gel	...		15
c) PCR for VP4 and VP7 types for 30 strains			600
d) Specimen containers			300
			---- US$ 1,645

Othr costs

Field worker for 3 months	...		400
Local transport	...		800
			----- 1,200
		Total	US$ 2,845

RG:LU:mh/L11:RV-BANGL.PRT

APPENDIX-I

NATIONAL ROTAVIRUS INFECTION
FACTORS ASSOCIATED WITH ACQUISITION

Hospital code _____ Child's ID number _____

Ward _____ Bed _____

Child's name _____ Mother's name _____

Date of birth _____ Date of discharge _____

Time of birth _____ Birth weight _____(gm)

Gestation _____ (weeks)

Method of delivery _____ (1=Vaginal; 2=C.section)

RV status _____

If RV positive: 1. On what day of life? _____

2. No. of days RV excreted? _____

3. RV serotype: G type _____

P type _____

4. PAGE pattern _____ (1=Long; 2=Short)

Child's ID number _____

SPECIMEN CODE				
DATE OF STOOL COLLECTION (DD/MM/YY)				
TIME OF STOOL COLLECTION (AM,PM)				
SYMPTOMS				
Vomiting				
Fever				
STOOL CHARACTERISTICS				
Formed				
Loose				
Watery				
Loose and watery				
No. OF STOOLS IN PREVIOUS 24 HOURS				
DIET				
Breastmilk				
Other milk				
Water				
Other substances				
INFANT:NURSE RATIO				
<8:1				
≥8:1				
ROOMING				
Special care <5 infants/room				
Special care ≥5 infnats/room				
Normal with mother <5 infants/room				
Normal with mother ≥5 infants/room				
In mother's bed				
In own cot				

Appendix 2 - Research plan, data analysis plan and methodology from protocol 93-023 "Study of the distribution of group A rotavirus P types in Bangladesh"

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). These specimens comprise the RV positive samples from an ongoing neonatal cohort study (#90-018) in which specimen collection will be completed by December, 1993.

b) Asymptomatic infants (expected total = 40 specimens collected/year when it is anticipated that approximately 2000 control stools will be collected. These specimens will come from the ongoing "Surveillance case-control study" for which Dr. A.S.G. Faruque is a co-investigator. 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 as mentioned under 'b' above - 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, as described above under 'b' and 'c': 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 validated 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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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

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

Neonatal rotavirus study Consent Form

Rotavirus infection causes diarrhoea and many people are working towards a vaccine against rotavirus infection. It has been found that neonates can have this infection but they don't get diarrhoea. We would like to find out whether babies in this hospital have these infections.

To help us to find out whether these infections are found in hospitals in Bangladesh, we would like to test some of your baby's stool. If you agree to be part of the study, we will take some sample - a procedure which will not be harmful to you or your baby.

Depending on the outcome of the stool test, we may like to ask you some questions such as the way your baby was delivered, whether your baby is being breastfed and whether your baby is staying in the nursery. All information will be kept confidentially.

If you decide to participate can you please sign this form or give a thumb impression.

Date:---------- Signature/thumb----------

Impression of Guardian

Date:---------- Signature of Investigator----------

Date:---------- Signature of Witness----------

72/Consent.Frm/LU:kn.

আন্তর্জাতিক উদরাময় গবেষণা কেন্দ্র
বাংলাদেশ
সম্মতি পত্র

রোটাভাইরাস সংক্রমণ দ্বারা ডায়রিয়া হয় জেনে আমরা রোটাভাইরাস সংক্রমণের বিরুদ্ধে ভ্যাকসিন তৈরির জন্যে কাজ করছেন। শিশুদের দুই সংক্রমণ হতে পারে কিন্তু তাদের ডায়রিয়া হয়না। আমরা জানতে চাই এই হাসপাতালের শিশুদের রোটাভাইরাস সংক্রমিত হয়েছে কিনা?

এই সংক্রমণ বাংলাদেশের হাসপাতালে হয়েছে কিনা সেটা জানার জন্য আমরা আপনার শিশুর পায়খানা পরীক্ষা করব। এই গবেষণায় যদি আপনি সম্মত নাহন, তবে আমরা কিছু পায়খানা সংগ্রহ করব, যার আপনার কিংবা আপনার শিশুর জন্য কোন প্রকার ক্ষতি করবেনা। পায়খানা পরীক্ষা করার পড় আমরা আপনাকে কিছু প্রশ্ন জিজ্ঞাসা করব যেমন - কিভাবে আপনার শিশু প্রসব করানো হয়েছে, আপনার শিশুকে বুকের দুধ খাওয়ান কিনা এবং আপনার শিশু নামরীতে থাকে কিনা?

এই গবেষণা তথ্য সংগ্রহের পড়ে সেগুলোর গোপনীয়তা রক্ষা করা হবে। যদি আপনি এই গবেষণায় অংশগ্রহন করতে চান তবে নিম্নকরে এই অঙ্গীকার পত্রে স্বাক্ষর করবেন বুড়াঙ্গুলের ছাপ দিবেন।

তারিখ _____ সম্মতি / বুড়াঙ্গুলের ছাপ _____
অভিভাবকের / কিতাগনের

তারিখ _____ গবেষকের স্বাক্ষর _____

তারিখ _____ সাক্ষীর স্বাক্ষর _____