Attachment 1.
(FACE SHEET)

Date March 04, 1991

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Principal Investigator Dr.Nigar S.Shahid
Application No. 90-018(Revised)
Title of Study The impact of infection at birth with rotavirus strains on subsequent rotavirus infection.

Trainee Investigator (if any) X
Supporting Agency (if Non-ICDDR,B) Y
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 minor
   (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
   (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.

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

Nigar S. Shahid 4.3.91
Principal Investigator

Trainee

APPLICATION FOR PROJECT GRANT

1. PRINCIPAL INVESTIGATOR : Dr. Nigar S. Shahid

2. COINVESTIGATORS : Dr. M. John Albert
   Ms. N. Nahar Banu
   Dr. S. M. Faruque
   Ms. Leanne Unicomb
   Prof. Khaleda Banu - DSH[1]
   Dr. B. Elahi - HFH[2]

   CONSULTANTS : a. Dr. R. Bairagi
   b. Dr. R. Glass
   c. Prof. S. Urasawa

3. TITLE OF PROJECT : The impact of infection at birth with rotavirus strains on subsequent rotavirus infection

4. STARTING DATE : When funds are available

5. DATE OF COMPLETION : 2 years from starting date

6. TOTAL BUDGET REQUESTED : $ 99,442.00

7. FUNDING SOURCE :

8. HEAD OF PROGRAMME : Dr. R. B. Sack R. Bradley Sack 4/3/91
   Associate Director
   Community Health Division

9. AIMS OF PROJECT

a) General aim

To determine whether infection with rotavirus in the neonatal period, with "neonatal rotavirus strains" or "community strains" offer clinical protection against subsequent rotavirus infections in the first year of life.

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(1) DSH = Dhaka Shishu Hospital (2) HFH = Holy Family Hospital

b) Specific aims

1) To isolate and characterize group A rotavirus (RV) strains from neonates housed in neonatal wards/baby rooms.

2) To determine whether exposure to any RV strain, in the neonatal period, has an impact on subsequent infections with rotavirus.

3) To determine whether the extent of this impact is related to the nature of the strain (unique nursery or community).

4) To determine whether the serotype of the RV strain influences the outcome of subsequent homotypic or heterotypic RV reinfections.

5) To compare the "nursery strains" isolated from neonates in Bangladesh with similar strains found in other countries.

6) To determine whether the neutralizing antibody levels of colostrum fed to neonates prevents RV infection with circulating nursery strains

c) Significance

Infection of neonates with unique "nursery strains" have been shown to confer protection against severe diarrhoea upon subsequent infection. Our pilot study has shown that a high percentage of babies housed in nurseries/baby rooms in two hospitals in Dhaka shed RV. Should we find that infection with RV in the neonatal period (whether with unique nursery strains or those commonly found in the community) confers protection against clinically significant RV diarrhoea, such a result could have an impact on future RV vaccination programmes.

10. ETHICAL IMPLICATIONS

Subjects will be enrolled from Holy Family Hospital (HFH) and Dhaka Shishu Hospital (DSH). At HFH mothers will be contacted during hospitalization prior to delivery to give information regarding the reason and the rationale for the study, the specimens to be collected and the frequency of collection. At DSH parents or guardians will be informed on hospital admission of the neonate. Consent will be sought from both parents, and signature on the consent forms will be required prior to enrollment. Colostrum will be obtained within two days of birth.

Stool samples will be collected for 7 consecutive days from birth at HFH and first 7 days of admission at DSH. Subjects will be followed up weekly for history of diarrhoea over the first year of life. Weekly stool samples will also be obtained for isolation and characterization of RV. Stools will also be collected during episodes of diarrhoea. Diarrhoeal stool sample will be subjected to identification of diarrhoeagenic *E. coli Shigella* spp, *C. jejuni*, vibrios and parasites Two hundred µl of fingerprick blood collected by HAs using aseptic techniques for IgG estimation will be required from the infant at 3 month interval (total 5 times) to detect mild or asymptomatic infection through measurement of specific antibody. Serum antibodies (IgA and IgG) have been used to indicate recent RV infection. Serum will be collected shortly after birth and at 3, 6, 9 and 12 months thereafter. In case of illness, subjects will be brought to the ICDDR,B Clinical Research Centre for examination by physician. Appropriate treatment will be provided free of cost.

11. BACKGROUND, RESEARCH PLAN AND BIBLIOGRAPHY

a) BACKGROUND

Rotavirus has been shown to be endemic in newborn nurseries of hospitals in different countries, and strains identified have represented each of the 4 major serotypes of group A rotavirus although in each location only a single serotype has been found (1,2,3). When neonates are infected, "nursery" strains are shed as early as the first day of life and often excreted up to 7 days of age (1,4). Despite the serotype diversity it has been shown that these "nursery strains" from different countries show a large degree of genetic homology among their respective gene 4 segments which is not shared by strains found circulating in the community at large (5), suggesting that nursery strains comprise a unique group of rotaviruses (6).

In a prospective, longitudinal study conducted in Melbourne, Australia, it was found that children who were infected in the neonatal period with "nursery strains" of rotavirus developed milder disease during subsequent encounters with rotavirus in the post-natal period (7). Their counterparts who did not have neonatal rotavirus infection, were more likely to develop severe rotavirus diarrhoea in the post-natal period. This study suggested the likely benefit of immunizing babies in the neonatal period and the relative protection acquired through exposure to nursery strains during the neonatal period.

Recently we investigated the presence of neonatal rotavirus strains in Bangladesh. Ninety-eight newborns were enrolled in 2 hospitals in Dhaka, viz Holy Family Hospital (HFH) and Dhaka Shishu Hospital (DSH), and followed consecutively for 7 days. Patient population at HFH is mainly from the well-

to-do section of society whilst at DSH the patients are mostly from the lower socio-economic strata.

HFH is an obstetric hospital where newborns are housed in a separate nursery. We enrolled 58 newborns either at birth or day 1, of whom 45 became infected with rotavirus by the sixth day, none of which displayed any evidence of diarrhoea. Rotavirus strains from this hospital had a single distinct electrophoretic pattern and were found to be serotype 1.

DSH has a neonatal unit where babies born in other hospitals in Dhaka are admitted with clinical illnesses such as neonatal jaundice, respiratory distress, etc. For the purpose of our study, we enrolled babies who were less than 21 days of age so that the 7-day-follow-up would be completed before the end of the neonatal period. Of 30 neonates enrolled, 15 (50%) shed rotavirus by 6th day after enrolment. Rotavirus strains from this hospital had a distinct RNA electropherotype and belonged to serotype 4. Rotavirus from diarrhoeal stools of children seen at the Clinical Research Centre of ICDDR,B during January to March, 1990, were found to belong to all 4 major serotypes of human rotavirus. Co-electrophoresis was performed with a sub-sample of strains from HFH and DSH. Eight strains from each hospital were tested which showed that RV strains from each hospital had identical PAGE patterns and between hospitals were different.

It is important that this study should now be extended to find out whether the strains infecting newborn babies are unique "nursery strains" or "community" strains and whether protection is afforded to subsequent rotavirus disease. It is not known whether the serotype of rotavirus infection during the neonatal period can influence the outcome of subsequent homotypic or

heterotypic rotavirus reinfection. The serotype of nursery RVs from the Melbourne study have rarely been detected in the community. However, our pilot study has demonstrated that RVs infecting neonates and circulating in the community are found to be the same serotype.

Maternal immunity is transferred to the baby across the placenta in the anti-natal period and via colostrum and breast-milk in the post-natal period. It has been shown that cord blood, colostrum and breast-milk contain group and serotype specific neutralizing antibodies to rotaviruses (8). We wish to determine possible correlation between neutralizing antibody levels in colostrum against specific serotype and infection in neonates with the prevailing nursery strains, i.e. were infants infected with serotype 4 RV fed colostrum containing lower levels of anti-serotype 4 neutralizing antibodies than infants that were not infected with the same serotype..

We wish to further investigate the presence and nature of these neonatal rotavirus strains causing asymptomatic infection in Bangladesh and compare them with similar strains found in other countries.

Representative strains will be adapted to cell culture for further antigenic and genetic analysis, some of which will be carried out by a collaborator(s) which we hope to identify once we have the strains cultivated.

To rule out the probability of mixed infections with rotavirus in the diarrhoeal specimens and to determine episodes of diarrhoea caused only by RV, we propose to look for bacterial organisms viz diarrhoeagenic *E. coli*, *Shigella*, *Vibrios*, *C. jejuni* and parasites (9,10). Recent studies at ICDDR,B have shown that diarrhoeagenic *E. coli* may contribute to a high proportion of incidence of diarrhoea in this age group (unpublished observation). We hope

to utilize current techniques set up at ICDDR,B and propose to characterize the diarrhoeagenic *E. coli* strains by using DNA probes.

Enteric adenovirus (EAd) has been shown to be a causative agent of gastroenteritis in infants (11). We intend to test stool samples collected at the time of a diarrhoeal episode for the presence of EAd using an ELISA test established in the Virology Laboratory.

B) RESEARCH PLAN

Materials and Methods

Recruitment of infants

Babies born in Holy Family Hospital and babies admitted to the neonatal unit of Dhaka Shishu Hospital, whose parents reside in Dhaka City within a radius of 6 km from ICDDR,B will be approached to join the study. Parents willing to enroll their children will be made aware of the number and sequencing of clinical samples required for the study and made familiar with the background and rationale of the study. Access to a telephone will be considered an advantage as the parents will be required to contact study personnel if and when the child develops diarrhoea. Sample size estimated is 30 index infants (cases) and 30 controls (see sample-size calculations below). We intened to recruit 100 infants to cover for the dropout 40% rates expected since blood will be required.

Duration

This study will be of 2 years duration with recruitment being conducted during the first 6 months and follow-up for the next year. Laboratory tests, data

entry and final analysis will be conducted, simultaneously and should be completed during the last 6 months.

Inclusion criteria

Babies who have asymptomatic rotavirus infection within the first 7 days of life will be considered as INDEX CASES and those who are not shedding rotavirus as CONTROLS initially. A true control will be redefined as having no IgA antibody at 3 months after delivery. Babies with congenital abnormalities, low-birth weight, or are a twin, etc. will not be included.

Collection and testing of specimens obtained at follow-up

The following specimens will be collected from index cases and controls and their mothers:

a) Mothers:

Maternal colostrum - approximately 1.5 ml of breast milk will be collected between 0-2 days post-partum for determination of RV neutralizing antibodies to serotypes 1, 2, 3 and 4.

b) Babies

Weight and height reading will be taken at birth, then at 3 month intervals. Weight and height also will be measured at the time of onset of diarrhoea and on recovery. Weight and height data will be transferred to anthropometric measures of nutritional status--weight-for-age and height-for-age using NCHS standard (12). This will enable use to determine whether anthropometric status differs between RV infected and unifected neonates.

Estimation of IgA and IgG antibodies will also be performed on serum samples (0.5 ml of blood drawn on each occasion) collected by health workers using aseptic techniques at 3 monthly intervals for the first year of life. Estimating serum IgA and IgG levels at regular intervals will help detect RV infections that are asymptomatic or unreported episodes with very mild clinical symptoms (13).

Stool samples will be collected for the detection of RV for 7 consecutive days from birth (0-7 days) and then weekly from the 2nd week of life for 1 year. Parents will be asked to contact study personnel in the event that the child develops diarrhoea. Stool samples will be collected from infants with diarrhoea by the health worker or the mother who will be supplied with the containers and will be asked to collect the specimen at the time of onset of diarrhoea.

Although testing faecal samples once per week alone is inadequate for monitoring a likely asymptomatic infection with RV, the reason for doing so are as follows:

Since a weekly visit to the household is necessary to collect data in relation to an occurrence of diarrhoea during the past week, the collection of stools will be simple enough. Mothers will be requested to collect faecal specimens on the day of the proposed visit in sample containers left in the previous visit by the health workers, or by the mothers if diarrhoea had occurred during that week. This will also ensure that if and when diarrhoea occur, a stool specimen will be available. Mothers will be advised to send stool samples to the laboratory in case of diarrhoea. Transportation cost will be reinbursed.

Study personnel will be imparted training on assessment of dehydration and use of ORS in accordance with WHO recommendations prior to the commencement of this project (14). Stool frequencies, duration, vomiting and fever will be recorded and a grading system of severity of clinical disease based on that of Riepenhoff-Talty will be used (15) (Appendix A). Timely immunization of infants of the 5 major childhood diseases will be ensured. We hope to be able to give feedback to mothers on their infants' status of nutrition and advise them on appropriate feeding during weaning and thereafter. We will ensure the free treatment in case of diarrhoea are brought to the CRC at ICDDR,B and ARI referred to Dhaka Shishu Hospital.

Definition of diarrhoea in babies will be defined as the passage of 3 or more loose or liquid motions in any 24-hour period. A diarrhoeal episode will be defined as a period of 3 diarrhoea free days during each episode. The nature of diarrhoea will be characterized as follows:

Watery: never exhibiting blood

Never watery, but loose: never exhibiting blood

Diarrhoea with blood regardless of consistency

Laboratory Methods

a) Collection of samples for analysis

1) Colostrum

1.5 ml sample of colostrum will be collected on day 0-2 post-partum from all mothers where possible and stored immediately at $-20^{o}C$. Prior to testing, breast milk will be centrifuged at

2500 X *g* at 4°C for 20 min. The upper lipid layer will be discarded and the aqueous layer retained for antibody tests. Neutralizing antibodies in these samples will be tested against the 4 major group A rotavirus serotypes (see Appendix B).

2) Serum

0.5 ml blood will be collected in glass tubes and transferred immediately to Eppendorf tubes for serum separation, and sera will be stored at -20°C. Serum will be tested for anti-rotavirus IgA and IgG estimation (see Appendix C).

3) Stool

1-10 g of stool will be collected and stored at -20°C. Prior to testing, 10% (W/V) PBS extract will be made and ELISA for RV will be performed using an ELISA test, performed in the Virology Laboratory. RV positive specimens will then be tested for RNA electropherotype and serotype (see Appendices D and E). Neonatal rotavirus strains will be adapted to cell culture (see Appendix F) for further characterization of their gene 4 sequence. Collaborative efforts will be established with appropriate laboratories performing RV sequence analysis. Faecal IgA estimation will be performed for asymptomatic infection when raised serum IgA and IgG levels are observed. This might help estimate the approximate time of infection/exposure in asymptomatic cases.

1 g of fresh stool will be carried in transport media to be plated for S/S and MacConkey plates for isolation of *E. coli* and *Shigella* and further characterization by DNA probes, tissue

culture assay and animal studies. Three colonies will be stored on agar slopes and fixed on nitrocellulose filters and refrigerated (Appendices G and H).

b) Sample size estimation

Assuming that a child, who does not have rotavirus infection at the neonatal period, will have 2 episodes of rotavirus diarrhoea during infancy (< one year); and who developes rotavirus infection in the neonatal period will have 1 episode of rotavirus diarrhoea during infancy, (i.e. 50% reduction in the episodes of rotavirus diarrhoea due to immunogenicity produced during the neonatal period from the nursery strain), the required sample size of each group for 5% level of significance and 90% power is

$$\frac{1}{1 \sqrt{2/n}} = Z_{\alpha} + Z_{\beta} = 30$$

Assuming 50% of the new born will have rotavirus during neonatal period and standard deviation of the number of episode is 1.

Therefore, from the above equation

Controls = 30

Index cases = 30

c) Statistical analysis

Statistical comparisons will be made by t test, X^2 test and regression analysis (22). Relative risk will be estimated as the rates of asymptomatic infection and degree of severity in the observed neonatal rotavirus positive group against the corresponding rates in the control group. The actual antibody titers or the logarithmic antibody titers

will be tested by McNemar test and Wilcoxon-type test respectively.

Specimens summary

Numbers and type

			Sample size	To be recruited (assuming 25% dropout)
Subjects	=	60 (30 index cases, 30 control)	= 60	100
Mothers	=	50 breast milk for colostrum	= 50	50
Blood				
Cord			= 50	50
First year of life (60 x 4)			= 240	400
			290	450
Stool				
First week of life (60 x 7)			= 420	700
Second week to 52nd week (60 x 52)			= 3120	5200
Diarrhoea specimens @ 4 episodes/year for 1 year (60 x 4)			= 240	400
			3780	6300

c) REFERENCES

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2. Madeley CR, Cosgrove BP and Bell EJ. Stool viruses in babies in Glasgow. II. Investigation of normal newborns in hospital. J Hyg, 1978; 81:285-294.

3. Murphy AM, Albrey MB and Crewe EB. Rotavirus infections of neonates. Lancet, 1975; ii:1149-1150.

4. Perez-Schael I, Georgette D, White L, *et al.* Rotavirus shedding by newborn children. J Med Virol, 1984; 14:127-136.

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8. Rengerbergs M, Albert J. Davidson GP *et al.* Serotype-specific antibody to rotaviruses in human colostrum and breastmilk and in maternal cord blood. J Infect Dis. 1988; 158(2):477-479.

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22. Draper N and Smith H. Applied Regression analysis. 1981, John Wiley & Sons Ltd., USA.

TASKS OF EACH INVESTIGATOR

Dr. Nigar S. Shahid

- Collaborate with HFH and DSH staff for enrollment of subjects, collection of clinical information and biological samples
- Ensure that careful processing, storage and cataloging of study samples is maintained
- Collaborate with field staff to ensure proper collection of samples
- Maintain an accurate record of all clinical, epidemiological and laboratory information
- Adaptation of neonatal RV strains to cell culture
- Collate and write-up virology results

Dr. M. John Albert

- Ensure proper processing of samples for bacteriology.

Prof. K. Banu and Dr. B. Elahi

- Collaborators at DSH and HFH
- Enrollment of neonates at the above hospitals

Ms. N. Nahar Banu

- Performance of RNA gel electrophoresis

Dr. S. M. Faruque

- DNA analysis of *E. coli* colonies (approx. 1,500) with 8 probes

Ms. L. Unicomb

- Collaborate with Dr. Shahid in matters relating to the performance and setting-up of assays and analysis of results provide technical assistance and interpretation of results, advice when needed

Dr. R. Bairagi

- Assist with data analysis, interpretation and documentation

Dr. Roger Glass

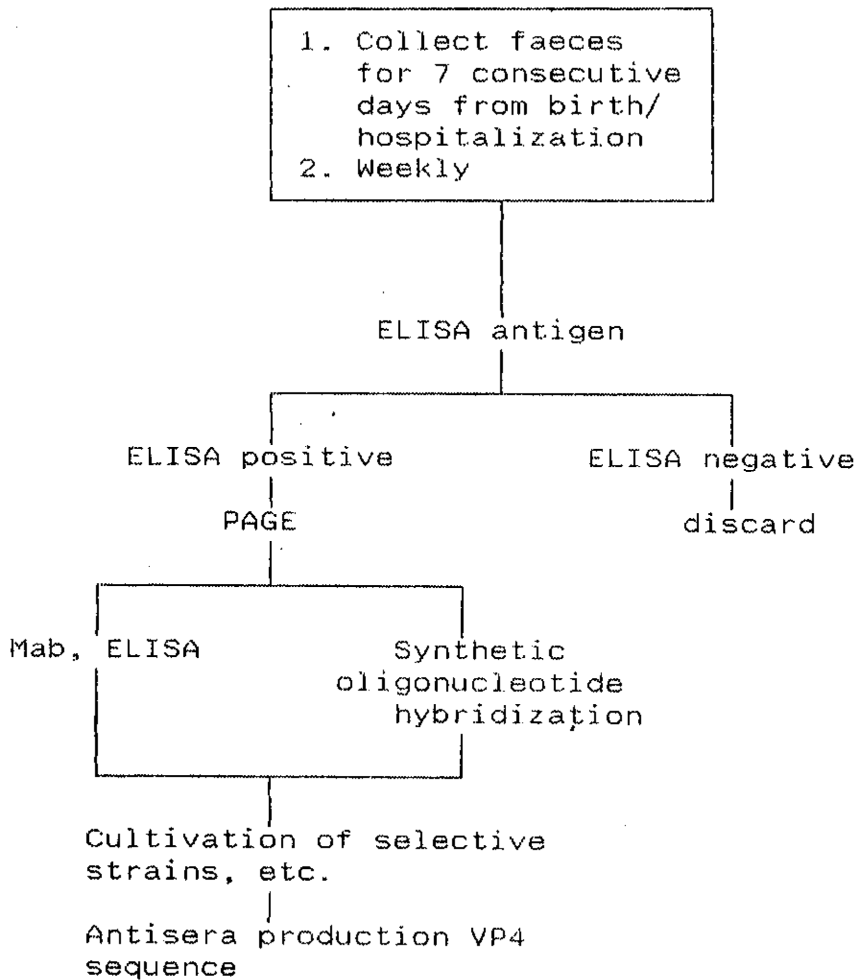
- Advise on epidemiologic methods and strategic feedback from existing studies elsewhere.

Prof. S. Urasawa

- Collaboration in gene sequence analysis of our "nursery" strains.

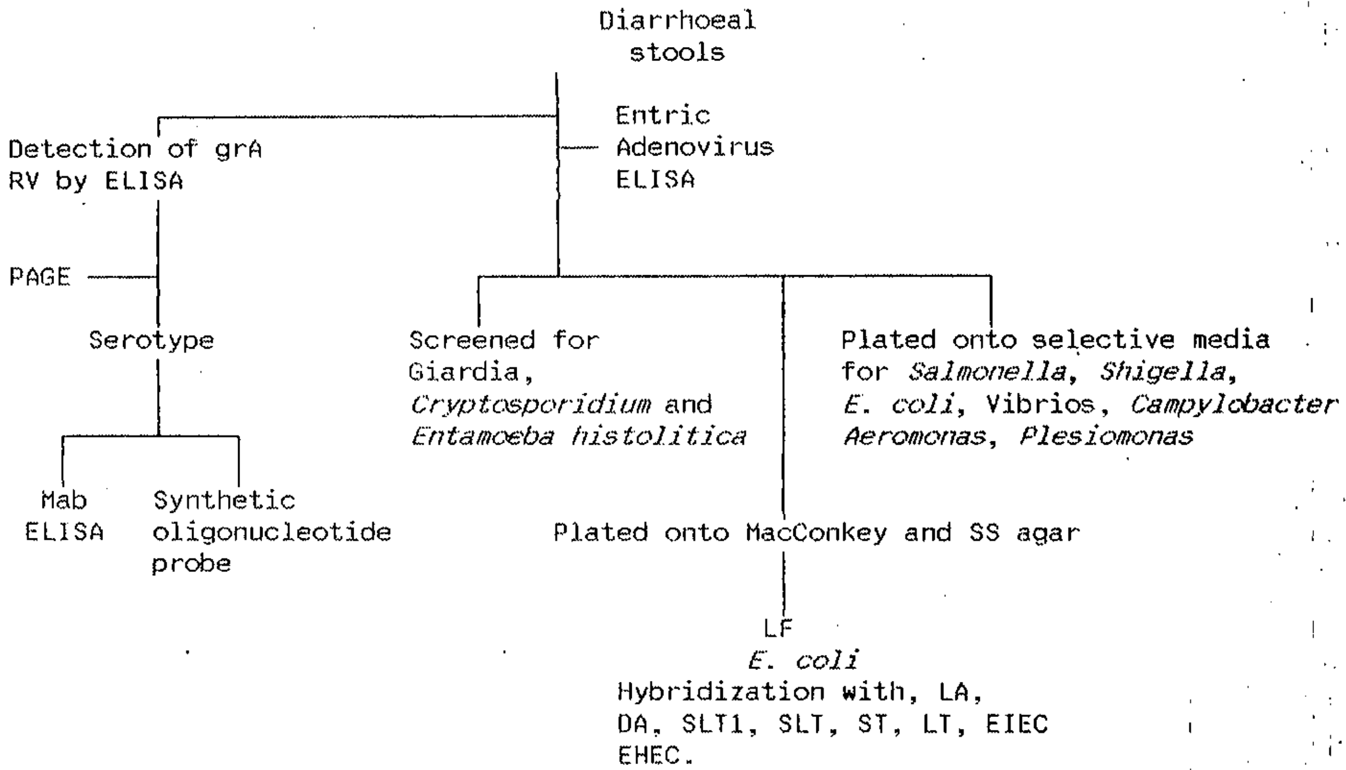
FLOW CHART 1

Processing of surveillance (non-diarrhoeal) stool specimen



FLOW CHART 2

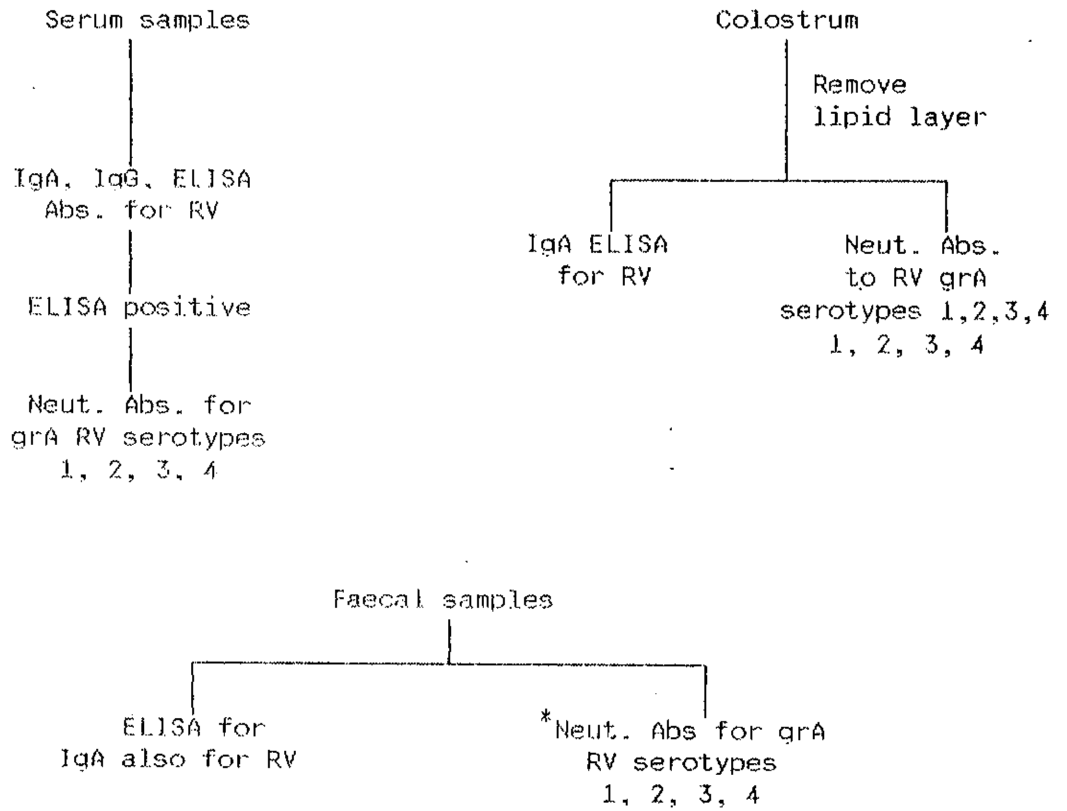
Processing of diarrhoeal stool samples



*   Approx 110 x 4 = 440 spc/year
                   = 9 spec/week

FLOW CHART 3

Rotavirus (RV) antibody studies



*RV neutralization abs. will be performed on serum positive ELISA and on the corresponding stool specimen for IgA specific antibody.

ABSTRACT SUMMARY

The objective of this protocol is to determine whether exposure after birth to rotavirus strains influences the outcome of a subsequent infection with community strains. We propose to enroll neonates from two hospitals, viz. Holy Family Hospital and Dhaka Shishu Hospital with asymptomatic rotavirus infection and without, who are admitted in the neonatal wards of these hospitals. Asymptomatic RV strains have recently been isolated from the neonatal strains of these hospitals.

Subjects will be followed-up daily for the first 7 days after enrollment (neonatal period) and weekly for the first year of life. Stool samples will also be collected on each visit and at the time of diarrhoea episode. Diarrhoeal stools will be subjected to analysis for rotavirus, parasites, diarrhoeagenic *E. coli*, Vibrios, *Campylobacter* and *Shigella*. Non-diarrhoeal stool samples will be subjected to rotavirus analysis only.

Colostrum will be obtained from mothers for serotype specific RV antibodies. Blood samples (0.5 ml) obtained quarterly, i.e. 4 times during the course of the study will be subjected to αRV IgG estimation. Anthropometry (Ht, Wt., MAC, TFT) will be carried out quaterly, at diarrhoeal onset and recovery.

This protocol will provide a unique opportunity to study the dynamics of infection with rotavirus and other aetiologies of diarrhoea in developing countries in the first year of life.

DETAILED BUDGET

a) Personnel cost				1991	1992
Dr. Nigar S. Shahid	-	80%	NOC-7	$ 12,200.00	13,420.00
Co-Investigator	-	20%	NOB-3	2,070.00	2,377.00
*Mr. N. Rahman	-	10%	GS-VI	1,253.00	626.00
Sr. Research Officer	-	20%	GS-VI	810.00	891.00
Sr. Lab Technician (1)	-	50%	GS-IV	2,240.00	2,460.00
*Health Assistant (6)	-	100%	GS-III	14,285.00	8,000.00
Data Entry Technician		59%	GS-III	1,022.00	1,124.00
Laboratory Attendant	-	100%	GS-I	1,640.00	1,750.00
				35,520.00	30,648.00

b) Operating cost	1991	1992
1) Supplies	$ 800.00	400.00
2) Miscellaneous charges	600.00	350.00
3) Cost of tests for rotavirus	6,000.00	2,124.00
4) Cost of neutralization tests	2,000.00	2,000.00
5) Cost of tests for microbiologic culture & Ecoli probes	8,000.00	5,000.00
6) Travel	3,000.00	3,000.00
	20,400.00	12,874.00
Total	55,920.00	43,522.00

GRAND TOTAL : $ 55,920.00 + 43,522.00 = 99,442.00

APPENDIX A

FORM 1 Enrollment Form

Child No. |__|__|__| Sex |__| 1=Male, 2=Female DOB |__|__|__|__|__|__| dd mm yy

Hosp. No. |__|__|__|

Where delivered |__| 1=Holy Fam. Hosp.; 2= DMCH; 3=Other Hosp.; 4= Maternity; 5=Private Clinic; 6= Home

Father's name: ______________________________

Years of schooling |__|__|

Father's Occupation: |__| 1=Service; 2=Bussines; 3=Profesional; 4=Agriculture; 5=Rickshowpuller; 6=Daylabour 9=Other (specify)

Mother's name: ______________________________

Years of schooling |__|__|

Occupation: |__| 1=House wife; 2=Service; 3=Profesional; 4=Day labour; 5=Maid servant

Residence address: ______________________________

____

Date of enrollment |__|__|__|__|__|__| (dd/mm/yy)

Why is child in hospital: |__| 1=Delivery; 2=Respiratory; 3=Jaundice

Birth wt. |__|__|__| (to nearest 0.1 kg)

Type of delivery: |__| 1=Normal; 2=Forceps; 3=Caeserian section

Full-term baby |__| 1= Yes; 0=No

Was Colostrum given |__| 1=Yes; 0=No

Is your child breastfed |__| 1=Yes; 0=No; 9=Uncertain

No. of stool in last 24 hours |__|__|

Type of stool |__| 1=Normal; 2=Watery; 3=Loose; 4=Bloody; 9=Uncertain

Diarrhoea |__| 1=Yes; 0=No

Day 1- 7 |__| Day

Diarrhoea |__| 1=Yes; 0=No

No. of stools/day |__|__|

Vomiting |__| 1=Yes; 0=No

No./day |__|__|

Day 1

Specimens collected

Cord blood |__| 1=Yes; 0=No

Colostrum |__| 1=Yes; 0=No

Stool |__| 1=Yes; 0=No

Urine flow |__| 0=Empty bladder; 1=Normal
2=Reduced amount and dark
3=None passed for several hours

Filled out by: |__|

Day 2-7

Stool collected |__| 1=Yes; 0=No

Form 2 Follow-up (weekly)

Date	|__|__|__|__|__|__|	dd/mm/yy
Child No.	|__|__|__|	
Week No.	|__|__|	
Is the child breastfed	|__|	1=Yes; 0=No
Diarrhoea during past week	|__|	1=Yes; 0=No
Food intake	|__|	1=Breast-milk only; 2=With supplementary food; 4=Supplementary food only
Supplementary food contains	|__|__|	01=Cows milk; 02=Formula milk; 03=Suji; 04=Rice; 05=Muri; 06=Bread; 07=Biscuit; 08=Khichuri; 10=Suji+milk; 11=Rice+Dal; 12=Rice+Milk; 13=Bread+Milk 14=Family food;
Source of water	|__|	1=Tap-inside house; 2=Tap outside house; 3=Tubewell water; 4=Pond water
Diarrhoea during past week	|__|	1=Yes; 0=No
If yes, Duration of diarrhoea in days	|__|	
Any other family member has diarrhoea	|__|	1=Yes; 0=No
No. of days diarrhoea continued	|__|__|	
Thirst	|__|	1=With; 0=Without
Eyes	|__|	1=Normal; 2=Sunken; 3=Very sunken
Skin elasticity	|__|	1=Retracts immediately; 2=Slowly; 3=Very Slowly
Pulse	|__|	1=Normal/rate & vol; 2=Rapid; 3=Rapid/feeble,/impalpable
Axillary temperature	|__|__|__|	(^{o}C)

Vomiting during past week	|__|	1=Yes; 0=No
If yes, Number per day	|__|__|	
Duration in days	|__|	
Was ORS given	|__|	1=Yes, 0=No
Antibiotic prescribed	|__|	1=Yes, 0=No
Name of Antibiotic	|__|	1=Ampicillin; 2=Cloxacilline; 3=Penicillin; 4=Tetracycline; 5=Gentamycin; 6=Nalidixic Acid; 7=Amoxicillin; 8=Mecillinam; 9= Other
Specimens collected : Stool	|__|	1=Yes, 0=No
Blood	|__|	1=Cord; 2=13th; 3=26th; 4=39th; 5=52nd week.
Weight	|__|__|__|	kg 1 dec.
Height	|__|__|__|	cm
Filled-out by	|__|	

APPENDIX B

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

1. Preparation of Cell Monolayers :

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

2. Neutralising Antibody Assay :

a) Dilute serum or breask milk in DMM + NP with 1 μg/ml Porcine trypsin type IX.

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

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

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

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

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

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

iii) aspirate PBS;

iv) repeat (ii) and (iii)

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

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

f) Add 50 µl of DMM with 1 µg/ml trypsin to each well.

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

3. Fluorescent Stain :

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

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

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

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

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

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

g) Incubate at 37°C for 45 mins.

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

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

APPENDIX C

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

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

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

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

4. Add 100 µl of samples to virus-coted wells as follows :
   Serum : Serial 2-fold dilutions in SMP solution stating at 1 in 100.
   Saliva : Serial 2-fold dilutions in SMP solution starting at 1 in 12.5.

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

5. Incubate overnight at 4°C.

6. Wash as in 3.

7. Add 100 µl of anti-human IgA, IgM, IgG or secretory piece (Sc) conjugated to HRPO, to each well as appropriate. The dilution to be used, usually 1:50 - 1:1,000, is determined by checkerboard titration with a known positive sample.

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

9. Wash as in 3.

10. Add 100 µl TMB substrate to each well.

11. Incubate at room temperature for 10 minutes.

12. Stop reaction with 50 µl of 2M H_2SO_4 per well.

13. Either read plates visually or with the aid of a spectrophotometer at 405 nm.

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

APPENDIX D

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

1. Extraction of ds RNA

a) Adjust pH to 5.6

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

OR

Make up faecal homogenate in 0.1M sodium acetate pH 5.6

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

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

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

For 10 ml

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

recipe for phenol mixture:500g crystalline phenol + 70g m-cresol + 0.5g 8.OH quinoline + 200g H_2O.

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

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

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

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

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

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

a) Cut stacking gel from the top of the gel and soak gel for a minimum of 30 mins. in fixer (10% ethanol, 0.5% acetic acid) - gels can be kept overnight in fixer if required.

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

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

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

0.75M NaOH, 0.1M formaldehyde and

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

N.B.: use 200 ml volumes.

APPENDIX E

DNA Hybridization for detection of serotypes of group A rotaviruses

Rotavirus RNA Extraction

1. Suspend 50-100 mg faecal sample in 0.5 ml of 0.1M sodium acetate, 1% SDS pH5.

2. Extract with phenol-chloroform mixture and keep the supernatant (dsRNA).

Blotting of RNA onto Nitrocellulose

1. Mix the RNA solution with one volume of 6.15M formaldehyde, 10X SSC.

2. Incubate at 65°C for 15 mins.

3. Spot on the nitrocellulose using Bio-dot apparatus.

4. Rinse with 10X SSC.

5. Air dried and bake at 80°C for 2 hrs.

Oligonucleotide probes labeling

1. 5-end labelled 8 pmol of probe with r-32p-ATP (3000 Ci/mmol).

2. Separate the labelled probe from unincorporated ATP by passing through Sephadex G-25 column.

Probe Hybridization

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

Probe concentration : 10^6 cpm/mL hybridization buffer

Hybridization Temperature :

38°C for HuG2Ac and HuG3Ac
42°C for HuG1Ac, HuG4Ac, BoG6Ac and PoG5Ac
45°C for Hug8Ac

Washing condition :

1. 3 X SSC at Room temperature for 30 mins.

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

3. 2 X SSC at room temperature for 1 hr.

APPENDIX F

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

Selection of Stool Specimens :

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

Preparation of Sample for Inoculation :

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

Preparation of MA-104 Cells :

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

Inoculation of Trypsin Activated Filtrate :

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

Detection of Rotavirus in Inoculated Tubes :

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

Further Passages :

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

APPENDIX G

1. DNA probes available at the ICDDR,B

DNA probes presently available at ICDDR,B for the detection of enteric pathogens are summarized as follows:

Determinant	Recombinant plasmid	Probe DNA (base pairs)
SLT I	pJN37-19	1154
SLT II	pNN110-8	842
EIEC	pMR17	17000
EHEC	pCVD419	3400
DA	pSLM852	450
LA	pJPN16	1000
LT	pCVD403	1300
STh	pCVD402	216

2. Preparation of probe DNA fragments

Recombinant clones carrying the probes of interest 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 (17) 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.* (18).

3. Preparation of specimens

Colony blots, dot blots, or stool blots will be prepared by standard techniques (17) 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.

4. Radioactive labelling of DNA probes

The probe DNAs will be radioactively labelled by the method of Feinberg and Volgelstein (19,20) with a-^{32}P-dATP (10 uCi µl, 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.

5. Hybridization of DNA blots

Hybridization with labelled probes will be carried out as described by Maniatis *et al.* (17). The filters will be prehybridized in the pesence 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.

6. 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.

NSS:mh/N3:ROTNEO4.PRT

APPENDIX H

Scoring for severity of disease* [Riepenhoff-Talty M, Bogger-Goren S. Li P, Yrem Carmody PJ, Barrett HJ and Ogra PL. (1981). Development of serum and antibody response to rotavirus after naturally acquired rotavirus infection in man. Journal of Medical Virology, 8:215-222.]

Symptom	Symptom/score	Symptom/score	Symptom/score
Diarrhoea			
Number of stools per day	<4 = 1	5 = 2	>8 = 3
Duration of diarrhoea in days	1-4 = 1	5-7 = 2	8-14 = 3
Vomiting			
Number per day	1-3 = 1	4-6 = 2	>6 = 3
Duration in days	2 = 1	3-5 = 2	>5 = 3
Dehydration			
In degree	<5% = 1	≥5% = 2	≥10% = 3
Fever			
Degree in centigrade	<38.5 = 1	≥38.5 = 2	≥39 = 3

*Score were graded as follows:

Severity.	Total score
Mild-moderate	0-12
Severe	13-18

Prolonged = Diarrhoea for ≥ 14 days

ABSTRACT SUMMARY FOR ERC

1. The objective of this protocol is to determine whether exposure after birth to rotavirus (RV) strains influence the outcome of a subsequent infection with rotavirus. Neonatal infections are usually asymptomatic and mild, and these strains have been shown to be endemic in newborn nurseries in some countries. Analysis of the genome segment 4 of the "nursery strains" have revealed differences from community strains indicating that this could account for its attenuated nature. Recently we have also identified such asymptomatic RV strains in 2 hospitals in Dhaka, viz. Holy Family Hospital (HFH) and Dhaka Shishu Hospital (DSH). Hence, children need to be enrolled during the neonatal period and followed during infancy to study the dynamics of infection with RVs in developing countries.

2. a) Colostrum will be obtained from mothers for estimation of serotype specific antibodies. There is no potential risk involved. Consent will be obtained before collection of samples.

   b) Cord blood will be obtained at HFH. 0.5 ml of blood will be obtained from the babies at 3, 6, 9 and 12 months of age for estimation of αRV IgA and IgG. This will involve discomfort to the infants but no potential risk as aseptic precautions will be taken.

3. Aseptic precautions will be taken when blood is obtained.

4. All information will be coded. Strict confidentiality will be maintained. All records will be kept under lock-and-key.

5. Both parents will be required to sign the consent form. All enrolled children will be provided treatment for diarrhoea and respiratory infections.

6. Home visits will be made after the child is discharged from hospital. Approximately five minutes will be required to take the history. Mothers will be requested to collect stool samples on specific days in containers provided earlier.

7. All enrolled children will be provided treatment for diarrhoea and respiratory infection. Should we find that infection with RVs during the neonatal period confer protection against significant RV diarrhoea, such a result could have an impact on future RV vaccination programme.

8. Colostrum and cord blood from mother and venous blood from infant will be required.

INTERNATIONAL CENTRE FOR DIARRHOEAL DISEASE RESEARCH, BANGLADESH

CONSENT FORM

Rotavirus (RV) is a common problem in young children in Bangladesh. Mortality due to rotavirus disease is high. It is hypothesized that babies who develop rotavirus infection with special strains during the first month of life have milder symptoms of rotavirus subsequently. Such strains have been found to be present in Holy Family Hospital and Dhaka Shishu Hospsital. Since your child has been admitted in one of the 2 hospitals, it is possible that your baby be colonized with these strains. Such strains may help in the formulation of future rotavirus vaccine.

If you decide to join the study, we will appreciate your allowing your child to participate in our study. We will visit your child daily for the first week and then weekly up to the end of first year. Stool samples will be requested at these visits. You will have to come with the baby and stool of the child in case he/she develops diarrhoea. The treatment of diarrhoea and respiratory infection will be provided by us. Your child will be monitored for his/her nutritional status and advice will be rendered.

Colostrum will be requested from the mother for anti-rota estimation of mother's milk. Simiarly, 0.5 ml of blood will be drawn from the baby's vein at the end of the 3rd, 6th, 9th and 12th month of life for testing.

If you decide to participate, please put your signature/thumb impression in the following place.

Signature of mother : ______________________

Signature of father : ______________________

Signature of investigator : ______________________

সম্মতি পত্র

বাংলাদেশে রোটাভাইরাস অল্প বয়স্ক শিশুদের জন্য একটি সাধারণ সমস্যা এখানে রোটাভাইরাস জনিত ডাইরিয়ার প্রকোপ বেশী। ধারণা করা হয় জীবনের প্রথম মাসে রোটাভাইরাস দ্বারা আক্রান্ত শিশুর পরবর্তীতে রোটাভাইরাসের লক্ষণ সমূহ কম প্রকাশ পায়। এ ধরনের বিশেষ রোটাভাইরাস হলিফ্যামিলি ও শিশু হাসপাতালে পাওয়া গেছে। যেহেতু আপনার শিশু এই দুইহাসপাতালের একটিতে ভর্তি আছে সে জন্য আপনার শিশুর বিশেষ ধরনের রোটাভাইরাস হওয়ার সম্ভাবনা আছে। এ ধরনের রোটাভাইরাসের উপর গবেষণা ভবিষ্যতে এই রোগপ্রতিরোধক টিকা আবিষ্কারের সহায়ক হবে।

আপনি যদি এ গবেষণায় অংশ গ্রহণে ইচ্ছুক থাকেন তবে আমরা আপনার শিশুকে আমাদের গ্রহণ করবো। প্রথম সপ্তাহে প্রতিদিন তারপর এক বছর পর্যন্ত প্রতিসপ্তাহে একবার করে আপনার বাড়ীতে যাবো এবং প্রত্যেকবার আপনার শিশুর মলের নমুনা সংগ্রহ করবো। যদি কখন ও ডাইরিয়া দেখা দেয় তবে মলের নমুনা সহ হাসপাতালে নিয়ে আসতে হবে। ডাইরিয়া এবং শ্বাসনালীর প্রদাহের চিকিৎসা করা হবে।
আপনার শিশুর পুষ্টিমান নিরূপণ করা হবে এবং প্রয়োজনীয় ব্যবস্থাদি দেওয়া হবে। মায়ের দুধের রোটাভাইরাস প্রতিরোধক ক্ষমতা কতটুকু আছে তা পরীক্ষা করা হবে। শিশুর রক্তে এ রোগ প্রতিরোধক ক্ষমতা নিরূপনের জন্যে তৃতীয়, ষষ্ঠ, নবম এবং দ্বাদশ মাসের শেষে 0.৫ মিলিলিটার রক্তের প্রয়োজন হবে। যদি আপনি এ গবেষণায় অংশ গ্রহন করতে চান তবে আপনি দস্তখত/টিপসই দিন।

মাতার দস্তখত - - - - - - - - - - - - -

পিতার দস্তখত - - - - - - - - - - - - -

গবেষকের দস্তখত - - - - - - - - - - - - -

To Chairman ERC

From Dr. Nigar Shahid Nigar S. Shahid

Sub: Acceptance of Manuscript

The manuscript describing the results from the pilot study (90-901) has been accepted for publication in the Archives of Virology .The acceptance letter is attached .

Thanks