Attachment 1.

Date [illegible]

FACE SHEET) ETHICAL REVIEW COMMITTEE, ICDDR,B.

Principal Investigator TASNIM AZIM Trainee Investigator (if any) _____

Application No. 78-003 Supporting Agency (if Non-ICDDR,B) _____

Title of Study CELLULAR + HUMORAL IMMUNITY TO ROTAVIRUS INFECTION IN BANGLADESHI INFANTS AND RELEVANCE TO RV VACCINE STUDIES

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 NA
   (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.

Tasnim Azim
Principal Investigator

Trainee

# APPLICATION FOR A PROJECT GRANT

**TITLE:** Cellular and humoral immune responses to rotavirus infection in Bangladeshi infants and relevance to rotavirus vaccine studies.

**INVESTIGATORS FROM ICDDR,B:**

| | |
|---|---|
| Principal Investigator: | Tasnim Azim, Laboratory Sciences Division |
| Coinvestigators: | M. Abdus Salam, Clinical Sciences Division<br>Goutam Podder, Laboratory Sciences Division<br>M. A. Wahed, Laboratory Sciences Division<br>S. M. Faruque, Laboratory Sciences Division<br>John Albert, Laboratory Sciences Division |
| Consultant: | Leanne Unicomb, Australia |
| **STARTING DATE:** | As soon as possible |
| **ENDING DATE:** | Two years and six months from start |
| **TOTAL BUDGET:** | US$ 201,012 (direct cost) |
| **FUNDING SOURCE:** | USAID |
| **HEAD OF PROGRAMME:** | Director, LSD |

**ABSTRACT:**

Group A rotavirus (RV) accounted for 35% of all diarrhoeal cases in children below 2 years attending the Clinical Research and Service Centre (CRSC) of ICDDR,B in 1996. Control of RV infection will, therefore, have an enormous impact in morbidity and mortality associated with diarrhoeal diseases in Bangladesh and vaccination is now considered the best method for control. The vaccine candidates tested so far have been based on studies of antibody responses but correlation between humoral responses and protection has not always been observed. The question of the best correlates of infection and protection, therefore, remains unanswered. Studies on cellular immune responses in animal models have shown that RV-specific lymphocyte proliferative responses and antibody secreting cells in the peripheral blood reflect events in the intestine. A small study in humans show that the proliferative responses parallel IgA responses and the two together may be a better marker of immunity to rotavirus infection. In this study we aim to study the humoral and cellular immune responses in children with RV infection and to correlate these responses with illness from RV infection. Also, reports from vaccine trials show reduced vaccination efficacy in children from developing countries compared with those from developed countries. The cause(s) (e.g. reduced vaccine take and/or reduced immunity) have not been investigated. As protein energy malnutrition and

micronutrient deficiency are major problems in children from developing countries, it may be that nutritional status is a determining factor in reduced vaccine efficacy observed in children from developing countries. Therefore, this study will also determine whether malnutrition is associated with prolonged RV excretion and lower RV-specific immune responses in RV infected children. For this purpose, the study groups will include children, 7-24 months of age, admitted to the CRSC of ICDDR,B with RV infection only in the extreme categories of nutritional status (weight-for-length $\leq$75% and >90% of the National Centre for Health Statistics median). Thus, the study will provide baseline information regarding the nature and kinetics of RV-specific immune responses and determine the best immune correlate of RV infection as well as the association of nutrition in viral clearance and RV-specific immune responses. Such information is pertinent for future vaccine studies.

## HYPOTHESES:

1. RV-specific antibody responses along with RV-specific cellular responses are a more reliable reflection of the immune response to RV infection than antibody responses alone.

2. Malnutrition is associated with prolonged viral excretion in rotavirus infection as well as lower RV-specific immune responses.

## OBJECTIVES:

1. To determine which immune response is the best correlate of RV infection; the cellular immune response (lymphocyte proliferation), the humoral immune response or the two together. This will be done by comparing the number of children with a positive antibody response with the numbers of children with either a positive cellular response or both positive cellular and humoral responses.

2. To describe the kinetics and nature of the cellular and humoral immune responses in Bangladeshi children with natural RV infection as baseline information for vaccine studies.

3. To determine whether malnutrition is associated with prolonged viral excretion in RV infection and lower RV-specific immune responses .

## BACKGROUND:

RV is the commonest cause of diarrhoea in Bangladeshi children less than 5 years of age and it has been estimated that approximately 20,000 children die from RV infections in Bangladesh each year (Unicomb et al, in press). Vaccination is thought to be the most effective method for reducing infection and mortality and the potential benefit, especially

in countries such as Bangladesh, is enormous. The World Health Organization has, therefore, assigned a high priority to testing and implementation of a RV vaccine in developing countries.

Since primary RV infections are symptomatic and subsequent infections are generally milder (Bishop et al, 1983), it is thought that primary infections elicit an immune response sufficient to protect against subsequent symptomatic reinfection. Such responses should ideally be mounted by a vaccine and have therefore, been the basis of the vaccine candidates so far tested. However, of the vaccine trials conducted, correlation between humoral responses after vaccination and efficacy has not always been observed (Bernstein et al, 1995). This has raised questions regarding the best correlates of infection and protection (immune response) among infected children. Moreover, the failure of vaccine candidates to provide the desired level of protection highlights the need for a better understanding of both humoral and cellular immune responses to human RV infection and the identification of host factors associated with these responses. This study will investigate these factors which are discussed at length below.

*Immune correlates of RV infection.*

It is thought that humoral immune mechanisms play the primary role in immunity to viruses at mucosal surfaces. Studies on mice have shown that both B cells and CD8+ T cells are required for viral clearance but B cells may mediate clearance even in the absence of CD8+ T cells (Franco et al, 1995). Furthermore, protection from reinfection appears to be primarily a B cell function (Franco et al, 1995). It is generally thought that antibodies capable of neutralising viral infectivity are protective (Matson et al, 1993; Chiba et al, 1986) yet non-neutralising antibodies have been shown to confer protection in animal models (Burns et al, 1996). Various studies on natural RV infection or volunteers challenged with RV have shown that antibodies may be associated with protection but antibodies did not consistently confer protection from infection or illness (Black et al, 1982; Kapikian et al, 1983; Chiba et al, 1986; Ward et al, 1989; Bernstein et al, 1991; Clemens et al, 1992; Matson et al, 1993). The variation in correlation between antibody responses and protection may reflect variation in the sensitivity of the tests used but it is also possible that other responses, along with antibodies, may better reflect the immune response to RV infection.

Studies on cellular immune responses to RV infection are limited and have been conducted almost entirely in animal models. Using a pig model, correlation was shown between protection against subsequent infection and RV-specific lymphocyte proliferative responses (Ward et al, 1996) and antibody secreting cells (ASC) (Yuan et al, 1996). Although the systemic ASC and lymphocyte proliferative responses were lower than the intestinal responses, the peripheral blood responses mirrored the intestinal response. Similar studies in mice showed that intestinal RV-specific IgA ASCs were higher than RV-specific IgG ASCs and were detectable within four days of the infection (Merchant et al, 1991). In humans, the RV-specific lymphocyte proliferative response was compared to the RV-specific antibody response in eight children with RV infection (Offit et al, 1993) where RV-specific proliferative responses were demonstrated in seven

and RV-specific antibodies were detected in six children. These findings suggest that both RV-specific IgA and lymphocyte proliferative responses reflect the intestinal immune response to RV infection.

*Kinetics of RV-specific cellular and humoral immune responses.*
There is limited published information of antibody response of Bangladeshi children infected with RV. In a longitudinal study, Black et al (1982) have shown high levels of RV-specific antibodies in the serum of children and antibody levels correlated negatively with the risk of subsequent symptomatic RV infection. Similar findings were obtained from a case-control study conducted in rural Bangladesh (Clemens et al, 1992). In a separate and more recent study, we examined RV-specific antibody responses as part of a birth cohort study of Bangladeshi children where serum neutralising antibody responses were detected following 56% of RV infection (Unicomb, unpublished data). Yet, when second samples were collected from those children within 8 weeks of the first, a four-fold rise in RV-specific neutralising antibodies occurred in 70% of children (Unicomb et al, unpublished data). This suggests that neutralising antibodies peak before 8 weeks of the onset of infection. In a study of North American children, it was shown that all children (3/3) with symptomatic RV infection had at least a four-fold rise in RV-specific IgA titers in their plasma (Losonsky et al, 1989) when samples were collected 4 weeks after the first sample during the acute illness. The kinetics of antibody responses of naturally infected Bangladeshi children (using carefully collected acute and convalescent samples) have not been described. It is possible that the responses among RV infected children from developing countries may differ from more developed countries. A single study on the kinetics of lymphocyte proliferative responses in children showed that these responses could only be demonstrated during convalescence (2-8 weeks after onset of infection) which paralleled the IgA response (Offit et al, 1993). For a vaccine to be efficacious it must mimic the natural infection as much as possible. For this reason it is essential that the kinetics of the immune response of Bangladeshi children to natural RV infection be described.

*Malnutrition as a host factor in determining outcome of RV infection and vaccine.*
Vaccine efficacy has been shown to be lower in developing countries and the reasons suggested include interference with the vaccine (from maternal antibodies, oral polio vaccine and enteric viruses), lack of boosting, waning immunity and challenge with large doses or unusual RV types (Vesikari, 1997). However, these have not been investigated. We initially observed that RV infection occurred more commonly in children who were better nourished. But, further analysis showed that the malnourished children with symptomatic RV infection were older than their better nourished counterparts. This suggested that secondary infections were symptomatic only in poorly nourished children, as older children were likely to have had a primary infection at a younger age, thus indicating that nutritional status may affect outcome of RV infection (Unicomb et al, unpublished data). We have also observed that Bangladeshi children tend to have RV infection of longer duration than well nourished children (Unicomb, unpublished data). In animal studies, prolonged diarrhoea was observed in malnourished pigs compared to well nourished pigs who were challenged with RV (Zijlstra et al, 1996). Also, in a study

investigating the role of vitamin A in RV specific antibody responses following challenge with RV, the antibody response was lower in vitamin A deficient mice than non-deficient mice (Ahmed et al, 1991). Malnutrition is known to prolong illness in children with diarrhoeal diseases (Black et al, 1984) and one of the possible mechanisms is lowered cell mediated immune response observed in these children (Baqui et al, 1993). It is possible that malnutrition prolongs illness from RV infection perhaps by impairing the mechanisms for clearance of virus, although we have no evidence to support this hypothesis. Clearance of RV infection involves both humoral and cellular immune mechanisms (Franco et al, 1995) and both humoral and cellular responses may be affected by nutritional status. Also, RV-specific antibody responses have been found to be lower in stunted children than in control children (Brussow et al, 1995) and, furthermore, we have observed lower numbers of total T cells (CD3+ cells) in the peripheral blood of poorly nourished RV infected children compared to well nourished RV infected children (Azim, unpublished data). These data suggest that nutrition may indeed play a role in the extent and nature of immune responses to RV infection which may then influence outcome. In addition to protein energy malnutrition, micronutrients, such as zinc and vitamin A, can play a major role in outcome from diarrhoeal diseases (Sazawal et al, 1995). Although no differences in RV-specific antibody levels were observed in children with zinc deficiency, anaemic children were found to have lower levels of RV-specific antibodies than normal children (Brussow et al, 1995). It is therefore possible that poorly nourished children with RV infection have a more prolonged illness and/or are more severely ill, as has been observed for other diarrhoeal diseases (Azim et al, 1996) and that this is secondary to the effect of nutrition on RV-specific immune responses.

This study will attempt to investigate the best immunological correlate, levels of RV-specific IgA and neutralising antibodies and RV-specific lymphocyte proliferation, in children with natural RV infection and describe the kinetics of these responses in children during the acute illness and convalescence. In addition, as both malnutrition and rotavirus are major problems in Bangladesh, we aim to investigate whether malnutrition is associated with prolonged viral excretion and altered RV-specific immune responses. Such information will be relevant for future vaccine studies.

## METHODS:

### a) Study population:

Children with acute watery diarrhoea due to RV infection attending the CRSC of ICDDR,B and having the following inclusion criteria will be enrolled.

*Inclusion criteria:*

- age between 7 and 24 months
- nutritional status $\leq$75% and >90% weight-for-length of the National Center for Health Statistic (NCHS) median *
- duration of diarrhoea 0-3 days, where diarrhoea is defined as $\geq$3 loose stools in 24 hrs
- detection of rotavirus antigen in stool

*Children belonging to these two groups of nutritional status have been chosen as differences in responses are more likely to be observed when comparisons are made between the extremes of nutritional status.

*Exclusion criteria:*

- presence of other enteric pathogens
- history of measles in the last six months

From all children enrolled in the study, a medical history will be obtained and a thorough physical examination will be performed. Children with RV infection will be evaluated at least once a day during their hospitalisation. Disease severity will be assessed based on previously described criteria (Riepenhoff-Talty et al, 1981) for which information regarding diarrhoea duration, stool frequency, frequency of vomiting, extent of dehydration and temperature will be collected daily. On admission, approximately 3 ml of venous blood will be collected for routine blood tests which include a complete blood picture and serum electrolytes. For the purpose of the study, samples of peripheral blood (7 ml) and stool will be collected. Samples will be collected on enrolment, at 6-8 days and 19-21 days (convalescence) after the onset of diarrhoea for immunological assays. Additionally, we will collect daily stool samples for 5 days after enrolment for determination of viral clearance. Children will be hospitalised till diarrhoea resolves and health assistants will visit their homes to ensure their return for follow-up sampling.

Nutritional status will be determined based on anthropometry as well as biochemical measurements. Weights will be obtained to the nearest 10g using a balance scale (Gebrüder Soehnle, Murrhardt, Germany) and the mean of three consecutive measurements will be recorded. Recumbent length will be measured using a slideboard infantometer (Harpenden, St. Albans, UK). The mean of two consecutive length measurements to the nearest 0.1cm will be recorded as the observed value. Children will be divided into two groups of $\leq$75% and >90% weight-for-length of the NCHS median. The biochemical measurements include albumin, retinol binding protein (RBP), retinol and zinc in serum. Nutritional measurements will be taken on enrolment and at convalescence.

*Sample size calculation:*

The sample size was calculated for the following outcome variables:

a) determination of the best immune correlate of infection - RV-specific antibody responses or/and lymphocyte responses
b) kinetics of immune response in children with RV infection
c) comparison of viral excretion between well nourished and malnourished children
d) comparison of RV-specific humoral and cellular responses between well nourished and malnourished children

a) Determination of the best immune correlate of infection:
Offit et al (1993) found that 6/8 (75%) children had a positive antibody response while 7/8 children had a positive cellular response (88%). Using this data and the following formula:

$$\frac{P1(100-P1) + P2(100-P2)}{(P1-P2)^2} \times f(\alpha\beta)$$

where, P1 = proportion of children with a positive cellular response, P2= proportion of children with a positive antibody response, and $\alpha$ (type I error)=0.05 and $\beta$ (type II error)=0.2, the sample size in each group was estimated to be 137.

For determination of the kinetics of the immune response we used data from Matlab (a field station of ICDDR,B) where a four-fold rise in antibody response was observed in approximately 39% of children with RV infection (Unicomb et al, unpublished data). A sample size of 143 was obtained using the following formula $n=z^2pq/d^2$ where,

z = 1.96
d = allowable error
p = proportion of children who are expected to have a four fold rise in response (39%)
q = 1 - p

b) For comparison of viral excretion between well nourished and malnourished children, sample size calculation has been based on unpublished information from hospitalised patients <2 years of age in Matlab. The number of days in hospital was used as a proxy indicator of viral excretion. The percentage of RV infected children who spent >3 days in hospital were: children <75% weight-for-length = 18% and children >90% weight-for-length = 8%. Using this data and the following formula:

$$\frac{P1(100-P1) + P2(100-P2)}{(P1-P2)^2} \times f(\alpha\beta)$$

where, P1 = proportion of well nourished children hospitalised for >3 days, P2= proportion of malnourished children hospitalised for >3 days, and $\alpha$ (type I error)=0.05 and $\beta$ (type II error)=0.2, the sample size in each group was estimated to be 175.

d) For comparing antibody and cellular responses between nutritional groups in RV infected children data on RV antibody responses and cell mediated immune responses in vitamin A deficient mice versus mice without vitamin A deficiency (Ahmed et al, 1991) was used for sample size calculation.

In vitamin A deficient and non-deficient mice the mean$\pm$SD of antibody levels were 594$\pm$485.2, and 1100$\pm$169.9, respectively. Using the formula:

$$n = \frac{2 \times (SD)^2 \times f(\alpha\beta)}{d^2}$$

where, d=difference between means of the two groups,
$\alpha$ (type I error)=0.05 and $\beta$ (type II error)=0.2, the sample size in each group is 2.

Similarly, for cell mediated immune responses where, for the vitamin A deficient mice mean=53 and SD=13.4 and for pair fed mice, mean=67 and SD=6.4, using the above formula, the sample size in each group was estimated to be 4.

The number of children that we will enrol will be the maximum sample size, 175, in each nutritional subgroup.

The CRSC surveillance data of 1995 (where every 25th patient was studied) showed that 6,000 children 7-24 months of age were admitted to the CRSC with a 0-3 days history of diarrhoea from RV infection. Of these, 300 (5 %) were $\leq$75% weight-for-length while 2,575 (42.9 %) were >90% weight-for-length. In order to collect daily stool samples for 5 days as well as blood and stool samples at 6-8 days and 19-21 days after the onset of diarrhoea in 350 children (175 x 2), home visits by health assistants will be essential to ensure complete sampling. Under these circumstances, a drop-out rate for follow-up at ~30%, may be expected. Because of patient loss during follow-up, competition with other ongoing studies on rotavirus and our stringent inclusion and exclusion criteria, we estimate that patient sampling will be complete within two years of the initiation of the study.

**b) Laboratory tests:**
The following laboratory investigations will be performed on the study children:

- Stool microscopy for semiquantitation of leukocytes and erythrocytes.
- Stool culture for enteropathogens (WHO, 1987).
- Stool for identification of *Escherichia coli* by probe hybridisation (Faruque et al, 1992).
- Stool for RV antigen by ELISA (Unicomb et al, 1993). This ELISA is currently under an external quality control programme including exchange of specimens between Northfield Laboratories Pty Ltd, Adelaide, Australia and ICDDR,B.
- RV G types by ELISA (Appendix I) and G and P types by RT-PCR (Appendix I).
- Total and differential white blood cell (WBC) counts and haematocrit.

**c) Tests for cellular immune responses:**
Lymphocyte proliferation responses will be measured by stimulating peripheral blood mononuclear cells (PBMs) with Concanavalin A (as a positive control) and RV strains of appropriate G and P serotypes (Offit et al, 1993). PBMs will be frozen in foetal bovine serum containing 10% DMSO and stored in liquid nitrogen. Assays will be done once patient enrolment is complete and only with samples from children with complete follow-up. Frozen PBMs will be used as has been done before in studies on shigellosis (Islam et al, 1995) and in our hands, recovery of cells is approximately 80%.

Delayed type hypersensitivity responses (DTH) will be measured by skin tests using the Multitest CMI kit (Pasteur MÉRIEUX, Lyon, France) whereby seven antigens and a glycerine control solution will be injected into the skin of the paravertebral area of the back of each child and the induration measured at the end of 48 h. An induration of 2 mm or more will be considered to be a positive response. The antigens present in the kit include tetanus (550,000 Mérieux units/ml), diphtheria (1,100,000 Mérieux units/ml), *Streptococcus* (group C) (2000 Mérieux units/ml), tuberculin (300,000 IU/ml), *Candida albicans* (2000 Mérieux units/ml), *Trichophyton mentagrophytes* (150 Mérieux units/ml) and *Proteus mirabilis* (150 Mérieux units/ml). This will serve as a measure of the general immune status of children at the time of enrolment.

**d) Tests for the humoral immune responses:**

RV-specific antibodies will be measured in plasma and stool to determine the effect of infection and malnutrition on RV-specific immune responses. The following assays will be done:

- RV-specific IgA and IgG and their subclasses by ELISA (Matson et al, 1993; Friedman et al, 1996).
- RV neutralising antibodies (Appendix I).
- RV-specific IgA1 and IgA2 by ELISA (Friedman et al, 1996).

**e) Tests for nutritional status:**

In addition to anthropometry, the following biochemical estimations of nutritional status will be done on two sampling days, on enrolment and convalescence:

- Serum retinol will be measured by HPLC as described before (Wahed et al, 1995)
- Serum RBP will be by single radial immunodiffusion using commercially available plates containing monospecific antisera against RBP (Binding Sites, UK).
- Zinc will be measured in serum using an atomic absorption photometer
- Serum albumin levels will be determined by electrophoresis.

**ANALYSIS PLAN:**

For objective (1):

The number of children with RV infection who have a positive antibody response will be compared to the number of children with a positive lymphocyte response and also with the total number of children with either positive antibody or positive lymphocyte responses. For these comparisons, the chi square statistic and Fisher's exact test will be used when appropriate.

For objective (2):

In order to determine the kinetics of response in RV infected children within the same nutritional subgroup, the Wilcoxon matched-pairs signed rank test will be used to compare the responses at different time points.

For objective (3):
Viral excretion and immune responses will be compared between malnourished and well nourished children with RV infection. The Mann Whitney U test (for non-parametric data) and the t-test (for parametric data) will be used for continuous variables while the chi square statistic or, where appropriate, Fisher's exact test will be used for categorical variables.

## SIGNIFICANCE AND IMPACT ON POLICY:

This study will provide a better understanding of the cellular and humoral immune response in natural RV infection in Bangladesh which has so far relied on the antibody response alone. In addition, it will describe the kinetics of the immune response in natural infection in Bangladeshi children which has not been described before. Such information is relevant for future vaccine studies as appropriate indicators of response as well as collection of samples at appropriate times is essential for a successful vaccine trial. This study will also investigate whether malnutrition is associated with prolonged excretion of RV and reduced RV-specific immune responses. This will be relevant for vaccine strategies in developing countries where vaccines have been found to be less efficacious.

## TIME FRAME:

Initial three months will be required for hiring of personnel, ordering reagents, etc. The next two years will be required for patient enrolment and laboratory assays. The last three months will be used to complete laboratory assays, data analyses and writing up.

## ETHICAL CONSIDERATIONS:

We will approach diarrhoeal patients 7-24 months of age for enrolment into our study and a consent form outlining the nature of the study, the possible pain and risk involved, procedures to be followed, their ability to withdraw at any time without jeopardising treatment and details of maintenance of anonymity will be read to their guardians. If the guardians consent, we will collect a stool sample which will be tested for RV by ELISA and for other enteropathogens. From diarrhoeal patients, stool and blood (7 ml) will be taken by venepuncture at 3 time points (as given above). Such volumes of blood will not be detrimental to the patient.

| BUDGET (in US$) | year 1 | year 2 | year 3 (6 months) |
|---|---|---|---|
| **1. Personnel** | | | |
| Dr. T. Azim (50%) | 6,779 | 7,254 | 3,808 |
| Dr. G. Podder (50%) | 6,074 | 6,378 | 3,348 |
| Dr. M. A. Salam (10%) | 1,911 | 2,045 | 1,074 |
| Research Officer (1) (GS-5) | 4,789 | 5,125 | 2,691 |
| Health Assistants (2) (GS-3) | 6,138 | 6,568 | |
| Research Fellow (1) | 2,040 | 2,040 | |
| **Subtotal** | **27,731** | **29,410** | **10,921** |
| **2. Travel** | | | |
| Local (patient follow-up) | 1,000 | 1,000 | |
| International | 3,500 | 3,500 | |
| **3. Laboratory supplies** (reagents, plastic ware, etc.) | 23,000 | 17,000 | 19,000 |
| **4. Printing and publication** | 500 | 500 | 500 |
| **5. Interdepartmental** | | | |
| Patient hospitalisation | 12,000 | 12,000 | |
| Routine lab tests (TC, DC, hct, stool C/S etc.) | 5,000 | 5,000 | |
| Fax, email, communication | 400 | 400 | 400 |
| Library charges | 100 | 100 | 100 |
| Test for *E. Coli* | | | 3,000 |
| Biochemical tests for nutrition | | | 17,150 |
| **6. Equipment** | | | |
| $CO_2$ incubator | | 7,000 | |
| Liquid nitrogen tank | 800 | | |
| **TOTAL (direct cost)** | **74,031** | **75,910** | **51,071** |

S. Moin 10/9/97
Shamima Moin
Controller, Budget & Costing

**JUSTIFICATION FOR BUDGET:**

1. The $CO_2$ incubator in the Virology laboratory has developed leaks and needs replacement. A $CO_2$ incubator is essential for the lymphocyte proliferation assays which will be carried out in this study and for maintenance of the cell lines necessary for viral replication. As PBMs will be frozen initially and lymphocyte proliferation assays will commence in the second year of the study the incubator can also be purchased in the second year of the study.

2. A liquid nitrogen tank for freezing of PBMs will be needed as soon as the study starts. We will now share a -20°C freezer has been removed from our requirements and with another investigator in the Division.

3. Travel: The co-principal investigator will be leaving for Melbourne in mid-1997. It will be necessary for her to return in the first year for the initiation of the study and towards the end of the second year for an interim analyses.

4. Biochemical tests for nutrition including albumin, retinol binding protein (RBP), retinol and zinc in serum have been added. The cost for these assays are RBP = US$ 12/test, albumin = US$ 4/test, retinol = US$ 5/test and zinc = US$ 3.5/test. Therefore, per sample US$ 24.5 will be required and for 350 children in whom testing will be done twice, the cost will be 350 x 24.5 x 2 = US$ 17,150.

5. As the number of children for immunological tests has increased from 60 to 175, the costs for Laboratory Supplies will also increase. However, as transferrin has been excluded the costs for which were US$ 5/test with a total of US$ 2,350 for all samples this amount will now be used for immunological tests. Also, we have reduced US$ 1,000 from Printing and Publication in the third year and added that to Laboratory Supplies. Also, costs for routine laboratory tests have been increased by US$ 1,000 in the first and second years each (total by US$ 2,000).

## REFERENCES:


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# APPENDIX I

## LABORATORY METHODS

### 1) MEASUREMENT OF NEUTRALISING ANTIBODIES

RV neutralising antibodies will be measured using a cell culture assay. Serially diluted plasma samples will be mixed and incubated with prototype RV strains of the major RV G serotype.(G1-4 and G9). The plasma-virus mixture will be added to washed MA104 cell monolayers in 96 well cell culture plates and incubated overnight at 37°C in a 5% $CO_2$ incubator. Controls of virus only, without plasma will be included. The monolayer will be fixed with 70% acetone and hyperimmune anti-RV rabbit sera will be added and incubated at 37°C for 30 min. Anti-rabbit antibodies conjugated to horse radish peroxidase (HRP) will then be added for 30 min. at 37°C following which cells will be stained with 3-amino-9-ethylcarbazole. Readings will be taken using a light microscope. Results will be expressed as titers which are the dilution at which 50% reduction in the number of stained cells are observed when compared to the number of stained cells in the corresponding wells where virus is incubated without plasma.

### 2) RV G TYPING BY ELISA

This ELISA is a monoclonal antibody (mab) based using G type-specific mabs obtained from Silenus Laboratories, Melbourne, Australia and given as gifts by Dr. K. Tanaguchi, Sapporo, Japan. The method is as described by Coulson et al (1987). Briefly, stool samples will be added to microtitre plates coated with mabs (as ascites fluid) to VP7 and G1-4 and incubated overnight at 4°C. Tissue culture adapted RV of different G types will be used as positive controls. After washing, hyperimmune anti-RV rabbit serum will be added and incubated. This will be followed by washing and addition of and incubation with HRP conjugated anti-rabbit antibodies for 60 min. at 37°C. Substrate containing 3,3',5,5' tetramethyl benzidine (TMB) will then be added and the reaction will be stopped using 2 N sulfuric acid after color development. The optical density will be measured at 450 nm in a spectrophotometer.

### 3) RT-PCR FOR G AND P TYPING

RNA will be extracted from stool samples using glass powder as described by Gentsch et al (1992). RNA will be tested for size specific PCR products in an RT-PCR assay using primer pairs specific for RV gene 9 (to determine G type) and gene 4 (to determine P type) as described by Gouvea et al (1990) and Gentsch et al (1992), respectively. These two tests will allow us to determine G types 1, 2, 3, 4 and 9 and P types 4,6, 8, 9, 10 and 11.

## APPENDIX II

### Cellular and humoral immune responses to rotavirus infection in Bangladeshi infants and relevance to rotavirus vaccine studies.

### CONSENT FORM

Your child is suffering from watery diarrhoea which is caused by infection due to a germ called rotavirus. There is no treatment available for this disease yet and vaccines are being developed and tried for its prevention. However, in order to assess whether a vaccine is effective we must first understand the immune response that is generated in natural infection. Also, the role of malnutrition in affecting the disease course from rotavirus is not understood. We are conducting a study at this Centre for better understanding the immune response in natural infection and whether that is affected by malnutrition. We hope that the results of the study will help future vaccine trials and the development of other preventive strategies. We request you for participation of your child in this study since s/he is suffering from diarrhoea due to rotavirus. If you agree, the following will be done to your child:

1. Your child will be admitted in a research ward of this hospital until his/her condition permits discharge from the hospital. During this period, s/he will receive the usual good care of this hospital.

2. On the day of admission we will ask you questions related to the illness of your child, perform a thorough physical examination, and record all information. We will ask about medical problems and perform physical examinations on your child each day that s/he remains in this hospital.

3. On the day of admission, some laboratory tests which are almost routinely done at this hospital such as a rectal swab culture, microscopic examination of a stool sample, complete blood picture and electrolytes (a total of 3.0 ml or 1/2 teaspoon of blood) will be done. For the special tests of this study, an additional 7.0 ml (about 1.5 teaspoon) of blood will be drawn from a vein in the arm of your child on the day of admission, and 6-8 days and 19-21 days after the onset of diarrhoea. Other than temporary pain due to the needle stick, drawing of this amount of blood will not cause any harm. On the same days, stool samples will also be collected. For the collection of the 2$^{nd}$ and 3$^{rd}$ samples, your child will have to be brought back to the Centre.

4. We will collect daily stool samples from your child for 5 days after enrolment for special tests.

5. It is you who will decide whether your child participates in this study, and you may withdraw your consent any time during the study. Your child will receive the good

care of this hospital if you do not include you child in this study, and also if you withdraw your consent during the study.

6. Participation in this study may not give additional benefit to your child. However, the society may benefit from the results of this study.

7. Information obtained from the history and the laboratory investigations of your child will be kept strictly confidential and none other than the investigators of this study and the Ethics Committee of this Centre will have access to the information. Analysis of information obtained from your child will be done by assigning a code number to your child, not using his/her name.

8. If you want to know results of any or all investigations we will happily provide those to you, subject to their availability. We'd like to inform you that results of most of the special tests will be available only after completion of this study.

If you agree to our proposal for participation of your child in this study, please put your signature or left thumb impression at the specified space below:

Thank you for your co-operation.

| Signature of the investigator: | Signature/LTI of parent/guardian: | Signature of the witness: |
|---|---|---|
| _____ | _____ | _____ |
| Date: _____ | Date: _____ | Date: _____ |

# আন্তর্জাতিক উদরাময় গবেষণা কেন্দ্র, বাংলাদেশ
## বাংলাদেশী শিশুর রোটাভাইরাস সংক্রমনে "সেলুলার ও হিউমোরাল রেসপন্স" ও এদের সাথে রোটাভাইরাসের টিকা গবেষনার সংগতি।

## সম্মতি পত্র

রোটাভাইরাস নামের এক জীবানুর সংক্রমণে আপনার শিশুর ডায়ারিয়া হয়েছে। এ জীবাণুর বিরুদ্ধে কার্যকর কোন ঔষধ নেই, তবে এর প্রতিরোধক টিকার আবিস্কার করা হয়েছে এবং তার কার্যকারীতা পরীক্ষা করা হচ্ছে। কিন্তু এ টিকা কার্যকর হবে কিনা তা জানতে হলেএ রোগে রুগীর স্বাভাবিক প্রতিরোধ ব্যবস্থা সম্মন্ধে জানা দরকার। অধিকন্ত অপুষ্টির সাথে এ ভাইরাসের কারনে সৃষ্ট ডায়ারিয়ার মেয়াদের কোন সম্পর্ক আছে কিনা তাও জানা নেই। আমরা এ বিষয়ে জানার জন্য এ কেন্দ্রে গবেষণা করছি। আমরা মনে করি এ গবেষণার ফলাফল থেকে এ রোগের বিরুদ্ধে রুগীর প্রতিরোধ ব্যবস্থা সম্মন্ধে আরো ভাল জানতে পারবো, যা ভবিষ্যতে এ টিকা গবেষণায় ও প্রতিরোধমূলক ব্যবস্থা নির্নয়ে সহায়তা করবে। যেহেতু আপনার শিশু এ ভাইরাসের কারণে সৃষ্ট ডায়ারিয়ায় ভুগছে, সেজন্যে আমরা এ গবেষণায় আপনার শিশুর অন্তর্ভুক্তির অনুমতি চাচ্ছি। আপনি আমাদের প্রস্তাবে সম্মত থাকলে ঃ

১. আপনার শিশুকে তার অবস্থার উন্নতি না হওয়া পর্যন্ত এ হাসপাতালের গবেষণা প্রকোষ্টে ভর্তি রাখা হবে, এবং এ সময় সে তার রোগের উপযুক্ত চিকিৎসা পাবে।

২. ভর্তির সময় এবং ভর্তি থাকাকালীন প্রতিদিনই আমরা আপনাকে আপনার শিশুর রোগ সম্মন্ধে বিভিন্ন প্রশ্ন করবো, তার শারীরিক পরীক্ষা করবো, ও প্রাপ্ত ফলাফল লিপিবদ্ধ করবো।

৩. ভর্তির পরপরই যে সব পরীক্ষা সচরাচর ডায়ারিয়া রোগীদের ক্ষেত্রে করা হয়ে থাকে যেমন- মলের পরীক্ষা ও রক্তের পরীক্ষা সে সব আপনার শিশুর জন্যও করতে হবে, এবং এজন্যে ৩.০ মি.লি. (০.৫ চা চামচের সামান্য বেশী) রক্তের প্রয়োজন হবে। গবেষণার বিশেষ পরীক্ষার জন্য প্রতিবারে ৭.০ মি.লি. (প্রায় ১.৫ চা চামচ) করে রক্ত ভর্তির সময়, ভর্তির ৬-৮ দিন পরে এবং ভর্তির ১৯-২১ দিন পরে, মোট তিন বারে ২১.০ মি. লি. (প্রায় ৪ চা চামচ) রক্ত তার হাতের শিরা থেকে সংগ্রহ করা হবে। রক্ত সংগ্রহের সময় সূচের তাৎক্ষনিক সামান্য ব্যাথা ছাড়া এতে করে আপনার শিশুর আর কোন ক্ষতি হবে না। এ সব সময়ে আপনার শিশুর মলের নমূনাও সংগ্রহ করা হবে। দ্বিতীয় ও তৃতীয় বারের পরীক্ষার জন্য আপনার শিশুকে হাসপাতালে আনতে হবে।

৪. ভর্তি থাকাকালীন সময়ে ৫ দিন, বিশেষ পরীক্ষার জন্যে আপনার শিশুর মলের নমূনা সংগ্রহ করা হবে।

৫. এ গবেষণায় অংশ গ্রহনের ব্যাপারে আপনার সিদ্ধান্তই চূড়ান্ত, এবং অংশ গ্রহনের পরেও যে কোন সময়ে আপনি আপনার সম্মতি প্রত্যাহার করতে পারবেন। এ গবেষণায় অংশগ্রহন না করলে, এমনকি অংশগ্রহনের পরে সম্মতি প্রত্যাহার করলেও আপনার শিশু এ হাসপাতালের প্রচলিত সচিকিৎসা পাবে।

৬. এ গবেষণায় অংশ গ্রহনের ফলে আপনার শিশু বাড়তি কোন সুবিধা পাবে না, তবে এর ফলাফলে সমাজ উপকৃত হবে।

৭. আপনার শিশুর সমস্ত তথ্য ও পরীক্ষার ফলাফল গোপন রাখা হবে, এবং শুধুমাত্র এ গবেষনার গবেষক ও এ কেন্দ্রের "এথিক্স কমিটি" ছাড়া অন্য কেউই এ সব তথ্য দেখতে পাবে না। ফলাফল নিয়ে কাজের সময় আপনার শিশুর নামের পরিবর্তে আমরা সাংকেতিক সংখ্যা ব্যবহার করবো।

৮. আপনি চাইলে আপনার শিশুর যে কোন, বা সমস্ত পরীক্ষার ফলাফল আমরা আমরা আপনাকে জানাব। আমরা আপনাকে এও জানাতে চাই যে কিছু কিছু পরীক্ষা এ গবেষণার কার্যক্রমের শেষ পর্যায়ে করা হবে।

আপনি এ গবেষণায় আপনার শিশুর অন্তর্ভুক্তির প্রস্তাবে রাজী থাকলে অনুগ্রহ করে নীচের নির্দিষ্ট স্থানে আপনার স্বাক্ষর / টিপসই দিন। আপনার সহযোগীতার জন্য ধন্যবাদ।

| গবেষকের স্বাক্ষর | সাক্ষীর স্বাক্ষর | পিতা-মাতা/ অভিভাবকের স্বাক্ষর / টিপ সই |
|---|---|---|
| তারিখ ঃ | তারিখ ঃ | তারিখ ঃ |

26
R1-I

EVALUATION OF CHILD HEALTH RESEARCH PROPOSAL Review #1

Reviewer's name:

Name of proposal: Cellular and humoral immune responses to rotavirus infection in Bangladeshi infants and relevance to rotavirus vaccine studies.

Name of proposed investigator: Tasnim Azim

Date of review: June 25, 1997

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This proposal addresses basic immune responses in protection against rotavirus infections to establish a foundation of rotavirus vaccine development. In essence, a principal aim is the search for a reliable correlate of protection. A second aim is to determine if immune responsiveness is influenced by nutritional status. The hypotheses and objectives as stated are confusing. For instance, are the investigators trying to determine which immune response correlates with RV infection (exposure) as stated or which correlates with protection from infection? The Background section is lucid and contains a good review of relevant literature. Of particular importance is the discussion of the association of peripheral RV-specific responsiveness with intestinal responses. This correlation is critical, especially in human studies. Studies pertaining to nutritional status and immunity must be well-controlled. In large part, earlier studies were based on retrospective observations lacking in appropriate experimental design.

Methods. Details are lacking. Where will the RT-PCR be performed? There is no international collaborator listed. The viability of PBM, and thus reliability of CMI assays, is compromised with frozen samples. The experimental protocol should accomodate analysis of fresh specimen. How does one test for RV neutralizing antibodies? That is not obvious per Coulson et al., 1989. It is useless to include nutritional effects as an objective in this study when measurements are based solely on transferrin levels, quantitated in such an imprecise manner (turbidimetry).

The significance and immediate relevance of this study (as written), particularly as pertains to vaccine development, is not obvious.

Review # 2

Proposal #6

***Cellular and humoral immune responses to rotavirus infection in Bangladeshi infants and relevance to rotavirus vaccine studies.***

*Tasnim Azim and Leanne Unicomb*

Goals The measurement of cellular immune responses is of interest but may not yield any practical correlate of protection. Lack of knowledge of published correlates of protection raise concern that proper analyses of the data will be performed.

Design Adequate.

Appropriateness Literature that deals with correlates of protection Shunzo Chiba, Kim Green, David Matson, and Miguel O'Ryan are not cited or discussed. The weaknesses of the analysis by Bernstein in the 1995 paper are not recognized.

Timing and budget Timing: good. Budget: no information.

Ethics Addressed.

Background The proposal is poorly put together. In particular, many references in the body of the proposal are not included in the reference citations. The authors appear unfamiliar with the literature.

→ one IV

Other None.

R3-D,I

**Proposal 6:** "Cellular and humoral immune responses to rotavirus infection in Bangladeshi infants and relevance to rotavirus vaccine studies"

Review # 3

**Principle Investigator:** T. Azim

**1. Goals.** The aims of this proposal are to determine the best immunological markers of rotavirus (RV) infection and to assess the role of malnutrition in the outcome of RV disease.

**2. Design.**

RV-specific humoral and cellular responses will be measured in well-nourished and malnourished children (7-24 months of age) comparing those with RV diarrhea ("cases") with those with diarrhea due to other causes ("controls"). Two nutritional status groups will be determined by weight-for-length ≤75% and >90%. Laboratory evaluation for micronutrients will include only a transferrin level. An isotype-specific ELISA (IgG, IgA, and respective subclasses) will be used to determine antibody levels against RV; an RV viral neutralizing assay will also be used. Cellular responses will be determined by lymphocyte proliferation responses to the infecting RV G and P serotypes.

I really don't know how this study design will answer either of the questions given in the hypothesis (p. 2). First, the nutritional assessment based upon one parameter (weight-for-length) is inadequate. (See general comments on nutritional assessments above.) Only one biochemical marker is used, and there are no measurements of zinc or vitamin A, which are subjects of particular imunological interest in several other proposals from these investigators. Second, the "control" group consists of children with diarrhea due to other causes; this may provide an adjunctive control group that might be expected to have some similarities with the cases regarding to immune function. However, a more reasonable primary control group would consist of otherwise healthy children who fall into the proposed nutritional groups (with additional nutritional assessments to provide for later stratification if necessary). Since responses to RV (ELISA and lymphocyte stimulation) are likely to be influenced by recent exposure or acute infection with RV, neither of the control groups would be expected to be comparable to the cases. For this purpose, responses to unrelated antigens would be more suitable. For example, they might consider giving Hib or varicella vaccine, since standardized immunologic tests are readily available. They would also need to account for the possibility that children with diarrhea may have normal antibody responses but have low antibody levels because of acute or ongoing protein losses from the gut. Another possible study might be a comparison of vitamin A/zinc supplementation with respect to RV responses in children infected with RV, analogous to Proposal 2 on cholera. Or they could follow their own model in Proposal 7 on shigellosis.

**2.1 Definitions of key concepts and variables.** The definitions of nutritional status are inadequate.

**2.2 Study populations, sample size, and sampling strategy.** Don't know.

**2.3 Clarity of analysis plans.** Inadequate.

**2.4 Feasibility of proposed methods.** No.

**2.5 Adequacy of laboratory methods.** See above.

**2.6 Adequacy of record abstract forms (data collection).** No information to review.

R2-M
R3-D,I

**3. Appropriateness.** Not applicable.

**3.1 Potential for improving child health care.**

**3.2 Scientific significance.**

**4. Timing** (and budget).

**5. Ethics.**

**6. Background.** This is interesting and informative. They make a good case for the study of nutritional effects on RV disease but do not provide a rationale for the proposed study design.

**7. Other.** This is a poorly conceived study that would not be expected to yield much useful information in the present form.

#6
R1-M

EVALUATION OF CHILD HEALTH RESEARCH PROPOSAL

Name of proposal: Cellular and humoral immune responses to rotavirus infection in Bangladeshi infants and relevance to rotavirus studies

Review #4

Name of proposed investigator: Azim et al.

Date of review: July 4, 1997

Review:

Hypothesis 1: should clarify 'a more reliable reflection of the immune response...' than what?

Objectives: The objectives should clarify better which comparison groups will be used for each objective stated (based on table p. 6). For example, objective 1 probably will compare cases (a+b) vs controls (c+d), whereas objective 3 will compare only malnourished cases (a) vs well-nourished cases (b). Objective 3, for instance should state that it will test the difference between well-nourished and malnourished children in the outcome of illness from RV.

Review # 5

Proposal #6

Title: Cellular and humoral immune responses to rotavirus infection in Bangladeshi infants and relevance to rotavirus vaccine studies.

P.I.: T. Azim et. al.

Goals: The goals of the study are to investigate the cellular and humoral immune responses to rotavirus infection in well nourished and malnourished Bangladeshi infants, and to correlate the immune responses with the duration of viral secretion.

Design: The project is designed as a case control study with 4 arms, i.e. well nourished cases and controls and malnourished cases and controls. More cases than controls (170 vs. 30) will be enrolled in each group in order to have sufficient sample size to investigate illness outcome (i.e. duration of viral shedding?). Comments on design issues are:

1. As in the other proposal by the same investigators, diarrhea cases with co-pathogens should not be included in the rotaviral case group. Evaluation for co-pathogens is not described.

2. Sample size calculations for the evaluation of cases is not shown, although the sample of 170 per group appears reasonable. Although the expanded sample was chosen to compare "outcome of illness", the outcome variables to be studied here are not defined. The sample size of 30 for evaluation of immunologic data appears to be a standard sample at ICCDRB, but as in several other proposals the justification for this sample size is not provided. A clear outline of outcome variables (clinical, virologic, and immunologic) would reassure the reviewer that the investigators have a clear plan for analysis and that sample selection is appropriate. As it stands, only immunologic parameters are specifically laid out as outcome variables.

3. Rotaviral antigen is detected by a locally produced ELISA, but background on this test (including sensitivity and specificity) is not given. As this assay is crucial for detection of cases and for determining length of viral excretion, further information on the assay should be provided.

4. The wording of the hypotheses and objectives is not precise in a couple of instances, i.e. 1) Hypothesis #1: "more reliable reflection of the immune response" than what?; and 2) Objective #3: associations between malnutrition, prolonged viral excretion, and altered immune function cannot be used to make any statement about causality from the studies performed.

5. A case definition for diarrhea should be given in inclusion criteria.

Proposal #6, continued

Appropriateness: Rotaviral vaccines are potentially an important public health intervention in developing countries, and this study could add new information that will assist in the interpretation of vaccine-induced immune responses in malnourished children.

Timing: No timetable is laid out to justify the 2.5 year timeframe.

Ethical concerns: No concerns

Background: Appropriate

R3-M

Child Health Research Proposals

Review #6

Reviewer's name:

Name of Proposal: Cellular and humeral immune reponse to rotavirus...

Name of proposed investigator: Azim

Date of review: 6/12/97

For CHR project staff only

No rationale for controls in specific aims/objectives.

Investigating nutritional status + immune response (second part of obj. 3) will require sample >> 30.

R3'1.

Child Health Research Proposals

Name of Proposal: Cellular + humeal response to rotavirus

Name of proposed investigator: Azim

Basic study proposal is sound, but needs some modification.

1) Goals: Clear, for most part, altho' objective #3 should describe "corellation" n "associatn" rather than "influence" - cannot assess causality in this design.

2) Design:
Control groups not justified in objectives.
Sample size for obj #3, pt 2 re: correlates with immune response likely to be quite insufficient.
Why not all pts given the sample size determined to assess clearance.
Inclusion/exclusion criteria fairly slight. How to ascertain if this is primary RV infection

3) Appropriateness
Is an important study given magnitude of problem related to RV, and relative lack of knowledge.

R3/M

Name of Proposal: Cellular + humoral response to rotavi

4) Timing ~~and Budget~~ from available information

Do not agree that recruitment will take 2 years, but there may be factors that have not been defined.

5) Ethics

No problems identified.

6) Background

PIs & team appear to have needed experience + training to accomplish study.

7) Other:

# RESPONSE TO REVIEWERS COMMENTS

**TITLE OF PROPOSAL:**
Cellular and humoral immune response to rotavirus infection in Bangladeshi infants and relevance to rotavirus vaccine studies.

**NAMES OF PROPOSED INVESTIGATORS:** Tasnim Azim and Leanne Unicomb

**Reviewer #1:**

Hypotheses and Objectives:
These have been reworded in order to clarify that the aim is to determine which immune response correlates with infection and not "protection from infection".

Background: No response is required for these comments.

Methods: Appendix I has now been added which provides details of the method for determination of neutralising antibodies, RV typing by ELISA and RT-PCR. The RT-PCR will be done at ICDDR,B. The method has been established through another study by Leanne Unicomb which involves typing of rotavirus strains in Bangladesh. This was done with the help of Dr. R. Glass, Center for Disease Control, Atlanta who was a consultant in the study.

It is true that the viability of frozen peripheral blood mononuclear cells (PBMs) are compromised to some extent compared to fresh PBMs. However, experience from previous studies on shigellosis using fresh and frozen PBMs have shown that either may be used. Islam et al, 1995 (referenced in the reference section on pg. 13) carried out assays on frozen PBMs. We get approximately 80% recovery when PBMs are frozen in foetal bovine serum with 10% DMSO.

Nutritional measures: This has been changed and expanded so that in addition to anthropometric measures of weight-for-length, biochemical measures of albumin, retinol binding protein (RBP), retinol and zinc in serum (pg. 6, para 3) will be included. However, as a result of addition of these assays, the budget has to be increased. The budget breakdown for these assays is shown below:

| | US$/sample |
|---|---|
| RBP | 12 |
| Albumin | 4 |
| Retinol | 5 |
| Zinc | 3.5 |
| Total | 24.5 |

With 350 children the cost is 350 x 24.5 x 2 = 17,150. The budget is shown on pg. 11 and the budget justification is shown on pg. 12, #4.

Significance: this has been rephrased (pg. 10).

**Reviewer #2:**

Goals, Appropriateness and Background: The Background section has been expanded to include several other references which show good correlation between protection and neutralising antibody levels and this includes references of Chiba et al, 1986 and Matson et al, 1993 (pg. 3, para 2). At the same time, data that do not show a good correlation between the two have also been cited (pg. 3, para 2).

One reference (that of Matson et al, 1993) that had been cited in the text was accidentally omitted from the References list. This has been corrected. The comment that we are unfamiliar with the current literature is in direct contrast to those of reviewers #1 and 5.

**Reviewer #3:**

1. Design:
The study design has been modified as suggested by this reviewer as follows:

Nutritional parameters: This has been increased as shown in response to comments of reviewer #1 (pg. 6, para 3).

Control group: we have excluded a control group from the study as suggested by reviewer #6. This has been shown on pg. 5 under "Study population".

The reviewer's comment regarding measurement of the immune response to an unrelated antigen in order to assess whether acute infection may be lowering the overall immune response, is a very relevant one. As most children will have received the DPT vaccine by 7 months of age, i.e. our youngest age of enrolment, they should have a positive delayed type hypersensitivity (DTH) response when these antigens are introduced into the skin. We will therefore apply a commercially available DTH kit into the skin of the paravertebral region of the backs of children on enrolment (pgs. 8-9, under "tests for cellular immune responses"), in order to assess whether the overall immune response is lowered during the acute infection. Introduction of a vaccine such as Hib or varicella vaccine, which is not a part of the national programme of immunisation, will be difficult.

2.1. The definition of nutritional status has been expanded as discussed for reviewer #2.

2.2. The study populations are children $\leq$75% and >90% weight-for-length of the NCHS median with acute diarrhoea due to rotavirus only (pg. 5, under "study population").

The section on sample size calculation has been expanded (pgs. 6-8) to show how the calculations were done based on each outcome variable. The sample size has been revised considerably and the number of children is 175 in each nutritional group for all parameters to be tested.

2.3. The analysis plans have been broken down into sections to show how each objective will be analysed (pgs. 9-10).

2.4. Feasibility of proposed methods: the study design has been modified as discussed above.

2.5. Adequacy of laboratory methods: this has also been modified as discussed above.

Overall, the main criticism of this reviewer is that the study design has to be modified in order to answer the stated objectives. We have considerably modified the design of the study as suggested by the reviewer.

**Reviewer #4:**

Hypothesis: hypothesis 1 has been rephrased (pg. 2).

Objectives: The analysis plans based on each objective are shown on pgs. 9-10.

**Reviewer #5:**

Design:

1. Children with rotavirus infection only will be included. If other enteric pathogens are present, those children will be excluded as shown in the exclusion criteria on pg. 6, para 1. Other enteric pathogens will be determined by stool culture as described by WHO (1987) and by identification for *E. coli* which will be done by probe hybridisation as described by Faruque et al (1992). These have been shown on pg. 8 under Laboratory Tests.

2. Sample size calculation has been expanded to show how the figures for each outcome variable are derived (pgs. 6-8). The outcome variables to be measured include (pg. 6, para 4):

- determination of the best immune correlate of infection - RV-specific antibody responses or/and lymphocyte responses
- kinetics of immune response in children with RV infection
- comparison of viral excretion between well nourished and malnourished children
- comparison of RV-specific humoral and cellular responses between well nourished and malnourished children

The initial calculation for the numbers of children needed to determine viral excretion was shown to be 170 children in each group. However, on recalculation this was found to be an underestimate and the calculation shown here is 175 in each group. A similar number of children will be used to investigate immune responses (pg. 6-8).

3. The locally produced ELISA for detection of rotavirus antigen is now being evaluated under an external quality control scheme. This involves comparing our results with a commercially available kit from Dakopatts, calibration of all equipment at regular intervals and exchange of material between Northfield Laboratories Pty Ltd, Adelaide, Australia and ICDDR,B (pg. 8).

4. Both Hypotheses and Objectives have been rephrased (pg. 2).

5. Diarrhoea is defined as three or more loose stools in 24 hrs. and has been added to the inclusion criteria on pg. 5.

Appropriateness: no response is required here.

Timing: a time table has been added on page 10 under "Time frame". The justification for a 2.5 years time frame has been provided on pg. 8, para 3, where the surveillance figures of 1995 show that only 300 children ≤75% weight-for-length with our inclusion criteria attended the CRSC. Although we need 175 children in each group, we estimate that two years will be needed to enrol these numbers for the following reasons:

- a drop-out rate of 30%,
- many of these children will have other enteric pathogens or a history of measles
- competition with other ongoing studies on rotavirus for patient recruitment
- lack of compliance by the guardians

Ethical concerns and Background: no response is required to these comments.

**Reviewer #6:**

1. Goals: The wording of Objective #3 has been changed.

2. Design: Controls have been dropped as suggested.

A sample size of 175, and not 30, will be required for comparison of the immune response between nutritional subgroups as shown on the section on Sample Size Calculation (pgs. 6-8).

Inclusion/exclusion Criteria have been expanded on pgs. 5-6.

It will not be possible to ascertain whether the RV infection is primary or secondary from this study and that is not the objective of this study.

3. Appropriateness: no response is required here.

4. The reason why patient recruitment will take two years has been explained above for reviewer #5. The figures behind this calculation have been added to the section on pg. 8, para 3.

5. Ethics: no response is required here.

6. Background: no response is required here.

## ABSTRACT SUMMARY FOR THE ETHICAL REVIEW COMMITTEE

Group A rotavirus (RV) accounted for 35% of all diarrhoeal cases in children below 2 years attending the Clinical Research and Service Centre (CRSC) of ICDDR,B in 1996. Control of RV infection will, therefore, have a great impact on morbidity and mortality associated with diarrhoeal diseases in Bangladesh and vaccination is now considered the best method for control. The vaccine candidates tested so far have been based on studies of antibody responses but correlation between humoral responses and protection has not always been observed. The best correlates of infection and protection remains unknown. Recently, it has been shown that the cellular immune response is necessary for viral clearance. We are aiming to study the humoral and cellular immune responses in children with RV infection and to correlate these responses with illness from RV infection. Also, reports from vaccine trials show reduced vaccination efficacy in children from developing countries compared to those from developed countries. The cause(s) have not been investigated. As protein energy malnutrition and micronutrient deficiency are major problems in children from developing countries, nutritional status may be a determining factor in reduced vaccine efficacy observed in children from developing countries. Therefore, this study will also determine whether malnutrition is associated with prolonged RV excretion and lower RV-specific immune responses in RV infected children. For this purpose, the study groups will include children, 7-24 months of age, admitted to the CRSC of ICDDR,B with RV infection only in the extreme categories of nutritional status (weight-for-length $\leq$75% and >90% of the National Centre for Health Statistics median). This study will provide baseline information on the nature and kinetics of RV-specific immune responses and determine the best immune correlate of RV infection as well as the association of nutrition in viral clearance and RV-specific immune responses. Such information is pertinent for future vaccine studies.

1. The high morbidity and mortality associated with rotavirus infection is observed in young children below 2 years of age. In Bangladesh, children are often malnourished and their immune response to rotavirus infection compared to that in well nourished children has not been described.

2. The risks of the study will include the pain while drawing blood and possible bruising.

3. Not applicable.

4. Anonymity will be maintained by using only identification numbers during analysis.

5.

a) A signed consent form will be obtained, and the Bengali consent form will be read out for the parents/guardians who are unable to read.

b) Information will not be withheld.

c) This has been mentioned in the consent form.

6. Interview will be carried out for obtaining medical history and clinical examination will be performed. These procedures will take approximately 20 minutes.

7. The subject will get the usual, high standard medical care and treatment that the CRSC reputed for. The study will not directly benefit the subject and it will not put the subject at any risk.

8. The study will use hospital records, blood and stool samples.