INTERNATIONAL CENTRE FOR DIARRHOEAL DISEASE RESEARCH, BANGLADESH
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CENTRE
FOR HEALTH AND
POPULATION RESEARCH

TO: The Chairman, RRC

FROM: Dilara Islam, LSD D. Islam

DATE: August 13 1998

SUBJECT: Resubmission of the modified project protocol "Immune responses in children with both acute lower respiratory tract infection (ALRI) and diarrhea" (# 97-025)

Please find enclosed a modified copy of the proposal. The error pointed out in last paragraph of page 4 has been corrected. The method of diagnosis of pneumoniae has been specified according to the hospital-diagnostic procedure.

Thank you.

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TO: The Chairperson
RRC, ICDDR,B

FROM: Dilára Islam, LSD Dilara Islam

THROUGH: Division Director, LSD

DATE: August 2, 1998

SUBJECT: Resubmission of the project protocol "Immune responses in children with both acute lower respiratory tract infection (ALRI) and diarrhea (renamed of Cellular and humoral immune responses in children during acute respiratory infection (ARI) due to *Streptococcus pneumoniae* or *Haemophilus influenzae* type b)" (# 97-025) to RRC

Enclosed please find a revised-copy of the proposal entitled, "Immune responses in children with both acute lower respiratory tract infection (ALRI) and diarrhea" with the response to RRC's comments.

a) Time required for recruitment of subjects: To our consideration time frame is:

3-4 months to get reagents and necessary supplies to start the study. Next 18 months (month' 5th - 22th) to recruit 100 patients from ICCDDR,B and another 50 from Dhaka Shishu Hospital, and to followed them up for two months (based on our previous and on-going projects one and a half year is the required time to recruit such number of patients). Within the rest 14 months, 6-8 months will be required to perform all the tests, as multiple samples from individual patient will be analyzed in a batch to reduce [illegible] variation. Last 6 months for data processing and [illegible] compilation and reporting.

b) It will be difficult to monitor patients in several hospitals, as the study will require collection of blood, NPA, urine and stool from patients and all the sample will be processed at LSD, ICDDR,B. In addition multiple sample will be collected from each patients during a period of two months. Therefore, we would like to include Dhaka Shishu Hospital only for the recruitment of cases with only ALRI. Other patient groups will be recruited from Out Patient Department of ICDDR,B.

c) According to RRC's suggestion, the hypothesis and the proposal is modified.

d) From each patient samples (blood, NPA, urine and stool) will be collected on the day of admission and day-11, day-30 and day-60 thereafter (4 times).

e) Required test numbers is also reduced and each is specified in the revised version of the proposal.

f) Total budget is also reduced from 269,557 to 169,085 (US$).

Attachment 1.
(FACE SHEET)

Date 02. 08. 1998

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Principal Investigator Dilara Islam

Trainee Investigator (if any) X

Application No. 97-025

Supporting Agency (if Non-ICDDR,B) US AID

Title of Study Immune responses in children with both acute lower respiratory infection (ALRI) and diarrhea

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

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

1. Source of Population:
   (a) Ill subjects (Yes) No
   (b) Non-ill subjects (Yes) No
   (c) Minors or persons under guardianship (Yes) No
2. Does the study involve:
   (a) Physical risks to the subjects Yes (No)
   (b) Social Risks Yes (No)
   (c) Psychological risks to subjects Yes (No)
   (d) Discomfort to subjects (Yes) No
   (e) Invasion of privacy Yes (No)
   (f) Disclosure of information damaging to subject or others Yes (No)
3. Does the study involve:
   (a) Use of records, (hospital, medical, death, birth or other) (Yes) No
   (b) Use of fetal tissue or abortus Yes (No)
   (c) Use of organs or body fluids (Yes) No
4. Are subjects clearly informed about:
   (a) Nature and purposes of study (Yes) No
   (b) Procedures to be followed including alternatives used Yes (No)
   (c) Physical risks Yes (No)
   (d) Sensitive questions Yes (No)
   (e) Benefits to be derived (Yes) No
   (f) Right to refuse to participate or to withdraw from study (Yes) No
   (g) Confidential handling of data (Yes) No
   (h) Compensation &/or treatment where there are risks or privacy is involved in any particular procedure (Yes) No
5. Will signed consent form be required:
   (a) From subjects Yes (No)
   (b) From parent or guardian (if subjects are minors) (Yes) No
6. Will precautions be taken to protect anonymity of subjects (Yes) No
7. Check documents being submitted herewith to Committee:
   NA 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)
   NA Informed consent form for subjects
   ✓ Informed consent form for parent or guardian
   ✓ Procedure for maintaining confidentiality
   NA 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.

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

Dilara Islam
Principal Investigator

Trainee

27 NOV 2004

"REVISED"

APPLICATION FOR PROJECT GRANT

TITLE OF THE PROJECT	:	Immune responses in children with both acute lower respiratory tract infection (ALRI) and diarrhea.
PRINCIPAL INVESTIGATOR	:	Dilara Islam, PhD Laboratory Sciences Division (LSD), ICDDR,B
CO-PRINCIPAL INVESTIGATOR	:	Abdullah Brooks, MD, MPH Clinical Sciences Division (CSD), ICDDR,B
CO-INVESTIGATORS	:	M. A. Salam, MBBS, CSD, ICDDR,B Rubhana Raqib, PhD, LSD, ICDDR,B Shamir K. Saha, PhD Dhaka Shishu Hospital, Bangladesh M. Ruhul Amin, MBBS, FCPS Dhaka Shishu Hospital.
CONSULTANT	:	Birger Christensson, MD, PhD Division of Pathology, Karolinska Institute (KI) at Huddinge University Hospital, Stockholm, Sweden.
BUDGET	:	Total direct cost: US $ 169,085
STARTING DATE	:	When funds are available
DURATION	:	Three years from starting date
HEAD OF PROGRAM	:	Professor V. I. Mathan Division Director, LSD, ICDDR,B

SUMMARY OF THE PROJECT

The most recent WHO estimates (1990) identified ARI as the single largest cause of death in young children, accounting for 33% of all childhood deaths in developing countries; pneumonia being the most important. About one-fourth of all children less than 5 years of age die of pneumonia. Moderate and severe lower respiratory infections account for most of the mortality due to ARI and are particularly importance in developing countries. Nearly all ARI deaths in young children are due to pneumonia. In developing countries, bacterial pathogens play a great role as a primary or secondary cause of severe lower respiratory disease (pneumonia) with *Streptoccus pneumoniae* and *Haemophilus influenzae*, the most prevalent pathogens of community-acquired pneumonia in children (49). Diarrheal disease is the second-most important cause of childhood illness, malnutrition and death in these countries. Death rates are increased where both ALRI and diarrhea occur together.

We hypothesize that children who develop both ALRI (mainly pneumonia) and diarrhea have altered (defective/reduced) immune function. In order to develop better interventions, it is important to define the immune function of children who are susceptible to both. In the proposed study, we will assess and compare cellular immune responses (characterization of lymphocyte subsets, cytokine production and intra-cellular cytokine production) in children who have both diarrhea and ALRI compared to children with only ALRI, only diarrhea and in healthy children. In addition, the influence of nutritional status on the immune functions will also be studied.

Children aged 2-36 months, who present at the Dhaka Diarrheal Treatment Centre, ICDDR,B with diarrhea plus ALRI, diarrhea alone, or only and those who present with only ALRI at the Dhaka Shishu Hospital (Children's Hospital), Dhaka will be enrolled on the basis of clinical and physical symptoms. In addition, age- matched healthy controls from the same endemic area will be studied to compare their immune responses. Children will be hospitalized for 5-7 days. Patients will be recruited.

The findings of this study are expected to improve the understanding of the development of protective immunity i.e. host defense mechanisms against ALRI, and diarrhea in children in an endemic country, and may identify immunolgical" risk factors" in children, who develop ALRI and diarrhea together. This information would be important in the development of strategies to improve management of these children and may reduce the excess mortality in these children.

Hypothesis:

The proposal is based on the following hypotheses:

Children with acute lower respiratory tract infection (ALRI) (pneumonia) become susceptible to diarrhea, or vice versa, as a result of altered (defective or reduced) immune function.

General aim:

(1) To identify "immunological" risk factors in children with ALRI (mainly pneumonia) and diarrhea by defining the immune response mechanisms compared to children with only ALRI or only diarrhea. Nutritional status of these children will also be determined to assess the nutritional influence on "immunological" risk factors. The immune response mechanisms and the nutritional status of these groups will be compared to that of matched, apparently healthy children from the same endemic area.

Specific aims:

- To define the cellular immune response by: analyzing phenotypic changes of lymphocyte sub-populations, intra-cellular cytokines of peripheral blood lymphocytes (PBL), and secreted/excreted cytokines in serum and body secretions in children with ALRI and diarrhea, with ALRI only, with only diarrhea, and in healthy controls.

- To assess nutritional status (anthropometric data) of the children.

- To characterize the systemic inflammatory response in these children (pre-albumin and nitrate/nitrite levels in blood).

Background:

ARI including pneumonia is a major public health problem in developing world and is the single largest cause of death in young children accounting for 33% of all childhood deaths in these countries, including Bangladesh (20, 45). Almost all ARI deaths in young children are due to acute lower respiratory infections (ALRI), particularly pneumonia. Studies in developing countries using lung biopsy indicate that most severe pneumonia in children is caused by bacteria, usually *Streptococcus pneumoniae* (Spn) or *Haemophilus influenzae*. Over 4 million children die of ARI (5) each year. At the Clinical Research and Service Centre (CRSC) of the ICDDR,B, where over 110,000 patients are treated each year, septicemia accounts for 21% of in-patient deaths (33). Among hospitalized patients, 11% developed signs of pneumonia within a week of hospital admission. Out of 8,000 patients

who attend the out-patient department of the Dhaka Shishu (children's) Hospital, about 10% present with ARI (mainly due to Spn and *H. influenzae* type b (Hib)) (38).

In rural Bangladesh, children experience an average of 3-5 ARI episodes/year are 3-4 episodes of diarrheal cases each year (7, 36). Although a substantial proportion of these episodes are the less serious and self-limiting upper respiratory infections of viral origin. In developing countries, bacterial pathogens have a greater role as a primary or secondary cause of severe lower respiratory disease (49). The annual incidence of moderate and severe lower respiratory infections (ALRI), which account for most of the mortality due to ARI, is of particular importance in developing countries (49). The proportion of pneumonia relative to total ARI has also been reported to be very high (17). More than half of these infections occur before the second birthday, and children 6 to 12 months of age have the highest risk of disease (44). ARI is usually diagnosed clinically, however, the clinical distinction between the various syndromes is difficult. There are a number of difficulties in establishing an etiologic diagnosis of pneumonia, although in well-conducted studies pneumococcus is the most common bacterial cause of pneumonia in children and adults worldwide (3). Blood cultures are positive in only 20-30% of adults and in <10% of children with pneumococcal pneumonia. Sputum examination may be helpful, however a reliable sample is difficult or impossible to obtain especially from young children (32). It has been suggested that a definitive diagnosis of pneumonia can be made based on X-ray findings, culture of lung aspirates and measurement of blood oxygen saturation (20) . The collection of pulmonary aspirates via bronchoscope or transtracheal route is associated with some traumatic for patients, and cannot be routinely done in developing countries.

While ARI usually occurs alone, it is often in association with other infections including malnutrition, diarrhea, or other chronic illness. A post-mortem study of-patients who died with diarrheal illness at Dhaka Treatment Centre of ICDDR,B, identified pneumonia as the underlying cause of death in one-third of children <5 years of age (10). The case-fatality rate among children <5 years of age with diarrhea and ARI was reported to be 14% (36). A respiratory tract pathogen was identified in 30% of these children, and a diarrrheal pathogen was identified in 34% (36). The etiology of pneumonia and the significance of its association with diarrheal illness remains unknown (6, 36). It has also been reported from India that, pneumonia was present in 39% of children with persistent diarrhea (42).
Spn and Hib are the most important bacterial pathogens of ALRI in Bangladesh, and are frequently isolated from children with concomitant diarrhea (36). A hospital based study among Bangladeshi children suggested a pronounced increase in the incidences of *H. influenzae* meningitis, and typing of the strains revealed that 98% of the strains were Hib (37). Despite an abundance of polymorphonuclear leukocytes and specific antibodies, *H. influenzae* is not eradicated from the lower respiratory tract. Studies in animals and humans

suggest that the presence of excessive mucus, disruption of the epithelium and dampening of ciliary function, all sequelae of the common cold, can predispose to pneumococcal pneumonia (21). Among the 84 serotypes of *S. pneumoniae,* 7 serotypes account for ~80% of infections in children (41). A strong correlation between the magnitude of Spn and Hib bacterimia and the type and severity of disease has also been observed (46).

The complex interactions between nutrition, infection and immunity are still poorly understood and the precise relative role of each factor is difficult to ascertain. It is known that children with severe undernutrition are increased risk of developing and dying from pneumonia. The malnutrition-infection synergism appears to be a physiological vicious cycle and has been recognized as important for public health policy in developing countries (35). Infection itself induces profound metabolic alterations resulting in negative nitrogen balance. PEM, whatever its origin, results in an immune deficiency characterized by impaired delayed cutaneous hypersensitivity, low production of lymphokines, low T cell counts, decrease T cell response to specific or non specific mitogen stimuli. In Bangladesh, children age and nutritional status are significant factors affecting the severity of diarrhea (8). It has been estimated that 39% of children aged 6-60 months in developing countries weigh less than 80% of the reference median weight-for-age (22). The severity of infection depends on the interplay between host resistance, including immune competence of the host, and the virulent properties of the pathogen. However, the host immune defense mechanisms during a natural course of ARI in children has not been well-studied.

MATERIALS AND METHODS:

Study design & clinical management:

Children with (i) ALRI (pneumonia)+diarrhea, (ii) pneumonia alone, or (iii) diarrhea alone will be enrolled into the study ward of ICDDR,B for atleast 5 days or in Dhaka Shishu (children's) Hospital, (pneumonia only). On admission, a detailed medical history will be obtained, a thorough physical examination will be performed, and the clinical progress and outcome will be recorded during hospitalization and at follow-up period. Children will receive standard treatment in accordance with established practices of the hospital, including appropriate antibiotic therapy, from the day of admission.

Definitions: ALRI(pneumonia): Pneumonia cases will be defined based on the WHO clinical classification for pneumonia (50). Severe pneumina cases will be identified with three criteria; 1) Tachypnoea (RR 50/min in 2-12 months, and RR 40/min in 13-59 months, 2) abnormal auscultatory findings (crepitations, bronchial breath sounds or decreased air entry) and 3) one or more of the following four danger signs: chest indrawing, cyanosis, inability to feed, lethargy. Ppneumina cases will be identified with two criteria; 1)

Tachypnoea (RR 40/min in 2-12 months, and RR 30/min in 13-59 months, and 2) no danger sign. Patients may or may not have nasal flaring, retractions, and abnormal ausculatatory findings. All patients admitted for pneumonia will receive chest X-ray.

Diarrhea: WHO clinical classification for diarrhea will be used (43). In brief, diarrhea will be defined as the passing of 3 or more abnormally loose or watery stools in a 24-h period, irrespective of etiology. At ICDDR,B an etiological diagnosis of diarrhea is not possible in about 20% of cases. Dehydration will be assessed and treated in accordance with WHO-guidelines (1). Severity of diarrhea will be assessed by- duration, stool frequency/24 h, presence of fever, presence and frequency of vomiting, etc. Diarrheal pathogens frequently isolated at ICDDR,B include enterotoxigenic *Eschericia coli* (ETEC), rotavirus, *Shigella, Salmonela, Vibrio cholerae* O1 or O139 and *Campylobacter jejuni.*

Schedule for enrollment of study patients:

	ICDDR,B		Shishu Hospital	Community
Age limit (2-36 months)	Pneumonia + diarrhea	Only diarrhea	Only ALRI	Healthy subjects
Group	Group A	Group B	Group C	Group D
Number	(n=50)	(n=50)	(n=50)	(n=50)

Fifty apparently healthy children of age limit 2-36 months from the same endemic area and with the same socio-economic status will serve as controls. For inclusion of each case in three different groups a concurrent control will be included in the study (3:1). Samples from controls will be collected only once to monitor the constancy of the research parameters in the healthy children and to compare the findings with those in patients. Children (controls or patients) will not be included in the study if they have experienced ARI in the 3 months prior to inclusion in the study (in rural Bangladesh, 3-4 ARI episodes/child (<5 years old)/year (36)). Patients with additional infections or other major disease will not be included in the study.

Study materials and preparation:

Venous blood (5 ml), nasopharyngeal aspirate (NPA), urine and stool samples will be collected on the day of admission and on day (D)- D-11, D-30 and D-60. Monitoring of specific antibody responses and lymphocyte population levels during the course of the disease will provide essential information on the kinetics of elicited immune responses. Analyses on samples and the sequence of work are shown in study flow chart-I.

a) Blood will be drawn from the median cubital vein in heparinized vacuum tube (Becton Dickinson (BD), San Jose, CA, USA), and plasma and peripheral blood

mononuclear cells (PBMC) will be separated by density gradient centrifugation of blood on Ficoll-Paque (Pharmacia, Uppsala, Sweden). The cells will be frozen in 1 ml ampoules containing (1-5)x10^6 cells, in RPMI media containing 20% FBS and 10% dimethylsulfoxide and will be stored at -196°C (Liquid N_2) until analyzed (at Karolinska Institute, Sweden).

b) NPA sample will be collected by a flexible, plastic suction catheter through nostril into the nasopharyns (flushing 0.5 ml normal saline) and then applying gentle suction with a syringe when the child coughs. The specimen will be transferred into a polypropylene microtube containing 0.5 ml of PBS. Fresh NPA will be cultured for identification of bacterial spp. and a part will be kept frozen for PCR analysis.

c) Urine samples will be frozen after centrifugation and filtration through a 0.22 μm-pore-size filter, and will be used for antigen detection.

d) Fresh stool samples will be subjected to microscopic examination and culture. In addition, fecal sample will be extracted as previously described for measurement of total s-IgA (27) and excreted cytokines In brief, 1g of feces will be mixed with 2 ml of phosphate buffered saline (PBS) containing soybean protein inhibitor (1 mg/ml) (Sigma Chemical Co., St Louis, Mo.), phenylmethylsulfonyl fluoride (1 mg/ml) (Sigma) and 0.05% Tween 20. The mixture will be vortexed and centrifuged, and the supernatant will be collected and will be filtered through a 0.22-μm-pore-size filter and will be stored at -20C until used.

All processed/unprocessed samples will be kept in -70°C until assays except PBMC.

NOTE: Sputum can not be collected from this age group children, broncho alveolar lavage collection will not be permitted from pediatric patients by local ethical committee, and CSF collection can only be done from children suspected to have maningitis.

Methodology:

1-a. Isolation and identification of the pathogen by routine culture procedure:

Culture of fresh venous blood and NPA will be done for the identification of bacterial pathogen (s) for ALRI cases, and stool culture will be done for the identification of diarrheal pathogen (s). Blood will be cultured according to our routine blood culture procedure. In brief, blood will be collected in Wampole Isolator microbial tubes (containing saponin, polypropylene glycol, and sodium polyanetholesulfonate) (Wampole Laboratories, Carter-Wallace, Inc., New Jersey), and will be plated on i) blood agar plate, McConky and Chocolate agar plate) and then will be identified by standard methods (12). After collection, NPA will be plated similarly as blood sample.

All the fecal samples will be analysed both macroscopically and microscopically. Stool samples will be cultured for enteric pathogens, such as *Salmonella, Shigella,* and *Vibrio cholera* spp. (14, 51). ETEC strains will be detected by culturing fecal samples on

colonization factor antigen (CFA) agar with and without bile salts . The suspected colonies will be tested for the presence of CFAs by slide agglutination with a panel of monoclonal antibodies (15, 16). Rotavirus will be detected with the Rotavirus ELISA Kit (IDEIA ™ Rotavirus, Dakopatts AB, Denmark). This Kit will detect rotavirus in fecal sample by double antibody sandwich ELISA. Parasites (*Entamoeba histolytica* and *Giardia lamblia*) will be identified by microscopy.

1-b. DNA amplification technique- polymerase chain reaction (PCR), and Latex agglutination assay for identification of ALRI pathogen in serum, NPA and urine:

In vitro DNA amplification by polymerase chain reaction (PCR) is a technology that has considerable implication for diagnosis of bacterial infections because of its potentially precise specificity and sensitivity and the method is being increasingly used in the etiological diagnosis of microbial infections. PCR has the potential for having a much higher sensitivity and specificity for identification of an infectious agent since the method does not require presence of intact (culturable) pathogens.

A PCR assay based on the amplification of pneumolysin gene fragments of spp. Spn in patient's sera and NPA will be used for the etiologic diagnosis of Spn (39). Pneumolysin is a species-specific protein toxin produced intracellularly by all clinically relevant pneumococcal strains and its virulent properties are well known, pneumolysin plays several roles *in vivo* including determination of inflammation (9, 11) A demonstration of pneumolysin DNA is thus expected to indicate pneumococcal involvement in the disease, regardless of their serotype. Two pairs of oligonucleotide primers will be used in this nested PCR, which will amplify a 348-bp and a 208-bp fragment of the pneumolysin gene. Amplified products will be analyzed by agarose gel electrophoresis. This nested PCR can detect as few as 10 organisms (39). Bacterial DNA will be extracted from patient's sera by phenol-chloroform extraction of the DNA. The limitation of PCR is that only bacteremic cases can be identified.

For detection of Hib, type b specific capsular gene will be amplified in sera and NPA according to the previously reported procedure (18). A region of the chromosome, termed *cap*, contains the gene cluster necessary for capsule expression in *H. influenzae*. The *cap* loci in all six capsular types share a common organization consisting of three regions. For isolation of Hib capsular type b specific primers will be used. Primers are of approximately 20-bp in length and will amplify a 480 bp region.

At ICDDR,B, PCR assay has already been applied for the detection of diarrheagenic bacteria in fecal samples such as, *Shigella* and demonstrated to have a higher sensitivity than conventional culture technique (25).

We will also attempt to detect antigen of Spn and Hib in serum, NPA, and urine by latex agglutination assay (Biomeriux, France) using type-specific antiserum for both Spn and Hib,

according to the previously described procedures (4). Bahl et al. have shown that, blood culture was positive in only 10 of the 110 children who had features of ARI, whereas by latex agglutination assay on serum and urine 52 cases were positive. In addition, by Bactogen-latex agglutination assay (Wampole Laboratory), polyribitol phosphate (PRP), the capsular polysaccharide of Hib will be detected in serum and urine samples (12), Bactogen detected antigen in 19 patients with clinical illness compatible with Hib infection but in whom bacteriological confirmation was lacking. It has been reported (from Dhaka Shishu Hospital by Dr. S. K. Saha) that, antigen detection test for the culture negative ARI cases provided evidence about the predominance of Hib (71.4%). (Since the sensitivity of blood culture is low, other available methods such as PCR and latex agglutination assay will be performed to increase the sensitivity of the ARI diagnostic procedure. In invasive lung infection, bacteria may enter the circulation in low numbers, making their identification not possible by conventional blood culture due to killed by host defense mechanisms or antibiotics. In these cases, PCR or latex agglutination test could be a sensitive method for the specific etiologic diagnosis).

1-c. Detection of respiratory syncital virus (RSV) in NPA:

Human RSV in NPA will be detected by ELISA using commercially available antibodies.

2. Measurement of inflammatory cytokines in plasma and fecal extract:

Cytokines are soluble proteins involved in communication between cells of immune and hematopoietic systems. Cytokines appear to play an important role in the pathogenesis of severe infections (13, 47) and has also been involved in pneumonia and meningitis (23, 34, 48). They are produced by several different types of cells such as lymphocytes, monocytes, macrophages, endothelial cells and fibroblasts. Most cytokine are secreted, but some can be expressed on the cell membrane. The biological functions exerted by cytokines are mediated through specific receptors expressed on the surface of the target cells (19). Most cytokines produced by T cells function in a paracrine fashion. Pro-inflammatory cytokines {interferon-γ (IFN-γ), interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-6, IL-8 and IL-10} are increased in active inflammation . Inflammatory cytokine will be measured in plasma samples in ALRI cases, and in plasma and fecal extract in diarrhea cases. The level of inflammatory cytokines will be determined with solid-phase double ligand ELISAs (R&D Systems, British Biotechnology, and Medgenix Diagnostics). according to the manufacturer procedures.

3. Immunophenotypic characterization and detection of intracellular cytokines of peripheral blood lymphocytes (PBL):

Flow cytometric immuno-phenotyping has become a standard method for characterizing lymphoid cell populations in clinical as well as experimental medicine. By multicolor analysis, the expression of individual cell-surface molecules can be defined on sub-population of lymphocytes. Immunophenotyping methods have been used to understand B

and T cell differentiation, maturation and functional heterogeneity. Immunophenotyping of PBL may reflect the character and magnitude of the cellular immune response in disease conditions. The regulated expression of cell surface molecules plays a crucial role in lymphocyte activation, tissue homing and cell to cell interactions. Not only the presence, but also the magnitude of expression of a particular molecule may determine the functional competence of a cell. Recently, the use of flow cytometric analysis has facilitated the identification of individual cytokine producing cells using anti-cytokine antibodies in conjunction with cell-permeabilisation techniques. These reagents will be used in conjunction with FITC, PE and PerCP conjugated antibodies to cell-surface markers to facilitate precise identification of cytokine producing cells using multi-color flow cytometry. Complete set of samples (5/patient) from each patient will be analyzed in a batch to reduce inter assay variation.

3-a. Cell preparation and staining. The methods for cell preparation, staining, and flow cytometric analysis will be used as described previously (24, 26). At the time of analysis (at Karolinska Institute (KI)), the frozen lymphocytes will be thawed at 37°C and immediately washed twice in RPMI 1640, and the viability of cells will be checked by trypan blue exclusion procedure. Thawed PBMC will be dispensed into tubes containing saturating concentrations of triple combinations (FITC, PE and PerCP conjugated) of monoclonal antibodies (MAbs). After a 30-min incubation at 4°C, the cells will be washed in PBS containing AB serum. For simultaneous identification of cell type and intracellular cytokines (IFN-γ, IL-1β, TNF-α, and IL-6 and IL-10), cell will be stained with two conjugated antibodies to cell-surface markers, and then cells will be permeabilized (using permeabilisation kit, Serotec, UK) and intra-cellular cytokine will be stained with the specific antibody (Serotec, UK). Cells will then be resuspended in PBS containing 1% paraformaldehyde until analysis. Negative controls will be included for both direct and indirect staining in each experiment.

3-b. Flow cytometric analysis. Three color fluorescent analysis will be performed on a FACSort flow cytometer (BD). Prior to data acquisition, instrument parameters will be checked and optimized using CaliBRITE Beads (BD). Data acquisition will be made using the Lysys II software (BD). Optimization of color compensation will be checked each time as the samples are stained with MAbs against CD3, CD56, and CD20. For each sample, 3,000 lymphocytes will be acquired using log-amplified fluorescence, and linearly amplified side and forward scatter signals. The data will then be analyzed with the Paint-A-Gate$^+$ software (BD). All samples will be analyzed by setting appropriate FSC/SSC gates around the lymphocyte population using back-gating on CD45$^+$CD14$^-$ cells. Multiple samples from individual patients will always be stained and run at the same time.

Analysis of selected cell surface markers:

i) Maturation of CD4 and CD8 lymphocyte subsets will be analyzed by measuring the expression of CD45RA and CD45RO on CD4$^+$ lymphocytes and CD8$^+$

lymphocytes as markers of antigenically naive- and memory T lymphocytes, respectively. The major immunoregulatory functions described for $CD4^+CD45RA^+$ cells involve suppression of immune responses, either directly or via the induction of suppressor activity by $CD8^+$ cells. In contrast, $CD4^+$ $CD45RO^+$ cells have been implicated as "helper inducers".

ii) Activation of CD4 and CD8 lymphocyte subsets will be analyzed by measuring the expression of CD25 (IL-2R) and HLA-DR on $CD4^+$ lymphocytes and $CD8^+$ lymphocytes, respectively. The early stages of T cell activation is associated with the expression of a receptor for the T cell growth factor-IL2 necessary for their autocrine growth regulation. The expression of HLA-DR on T cells is considered as a later activation marker.

Activation of lymphocytes may not only change the relative distribution of T, B and NK cell subsets but also induce emergence or disappearance of surface molecules of importance for cellular effector functions, cellular co-operation and tissue homing. An activated lymphocyte has a distinct set of surface molecules, reflecting it state of activation, cell-lineage and differentiation and its capacity to home to tissue compartments, and may also govern the role of the cell in different phases of an immune-mediated response.

iii) Expression of markers (CD56, CD57) associated with cytolytic T and NK cells: In experimental models NK cells and their subsets are important for the innate immune response following infection of the naive host.

4. *In vitro* stimulation of PBMC:

PBMC from healthy controls will be stimulated in 24-well costar plate in the presence of ARI-antigens, or OKT3 in HEPES bufered RPMI 1640 (GIBCO) (supplemented with 10% inactivated pooled human serum, 2 mM L-glutamine, 1 mM Na-pyruvat, 100U/ml penicilin and 100µg/ml streptomycin), and with media only. Supernatant from stimulated and unstimulated cells will be collected on day 1, 2, 3 or 4 and will be kept at -70°C until analyses for inflammatory cytokine levels. Stimulated and unstimulated cells will be characterized for immunophenotyping and intracellular cytokines will be detected as mentioned above.

PBMC from patients and healthy controls will be cultured in supplemented RPMI 1640 culture medium in U-bottom microtitre plates (10^5 cells/well, in triplicate) for thymidine incorporation. PBMC will be stimulated in the absence or presence of T-cell mitogens: phytohemagglutinin A (PHA, M form; Gibco BRL, Life Technology, NY; 10 µg/ml) or OKT3 (50 ng/ml) for 72 h at 37°C in a 5% CO_2 incubator. To measure cell replication, cells will be pulsed with ^{3}H-thymidine (Amersham, spec. act. 5mCi/mmol, 0.5µCi/well) 18 h before harvest. Labeled cells will be collected on filter paper by using a cell harvester and the ^{3}H-thymidine incorporation will be counted as count per minute (cpm) with a beta counter (LKB, Wallac, Pharmacia). The result will be expressed as mean stimulation index of triplicates(cpm of stimulated cells/cpm of unstimulated cells).

5. Measurement of nitrate/nitrite in plasma:

To investigate the role of nitric oxide (NO) in the pathophysiology of ALRI, level of the end products of NO (nitrate & nitrite) will be measured in plasma and urine by a colorimetric method (28). NO is a short-lived free radicle produced by a variety of cell types and is involved in the pathophysiologic processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation, and regulation of cell mediated cytotoxicity (2, 31). NO production results from the conversion of L-arginine to L-citrulin by the enzyme NO synthase (NOS). An inducible calcium-independent NOS known to produce NO after stimulation with cytokines and LPS. The inducible-NOS isoform generates large amount of NO that may have harmful cytotoxic effects on the host.

6. Assessment of nutritional status:

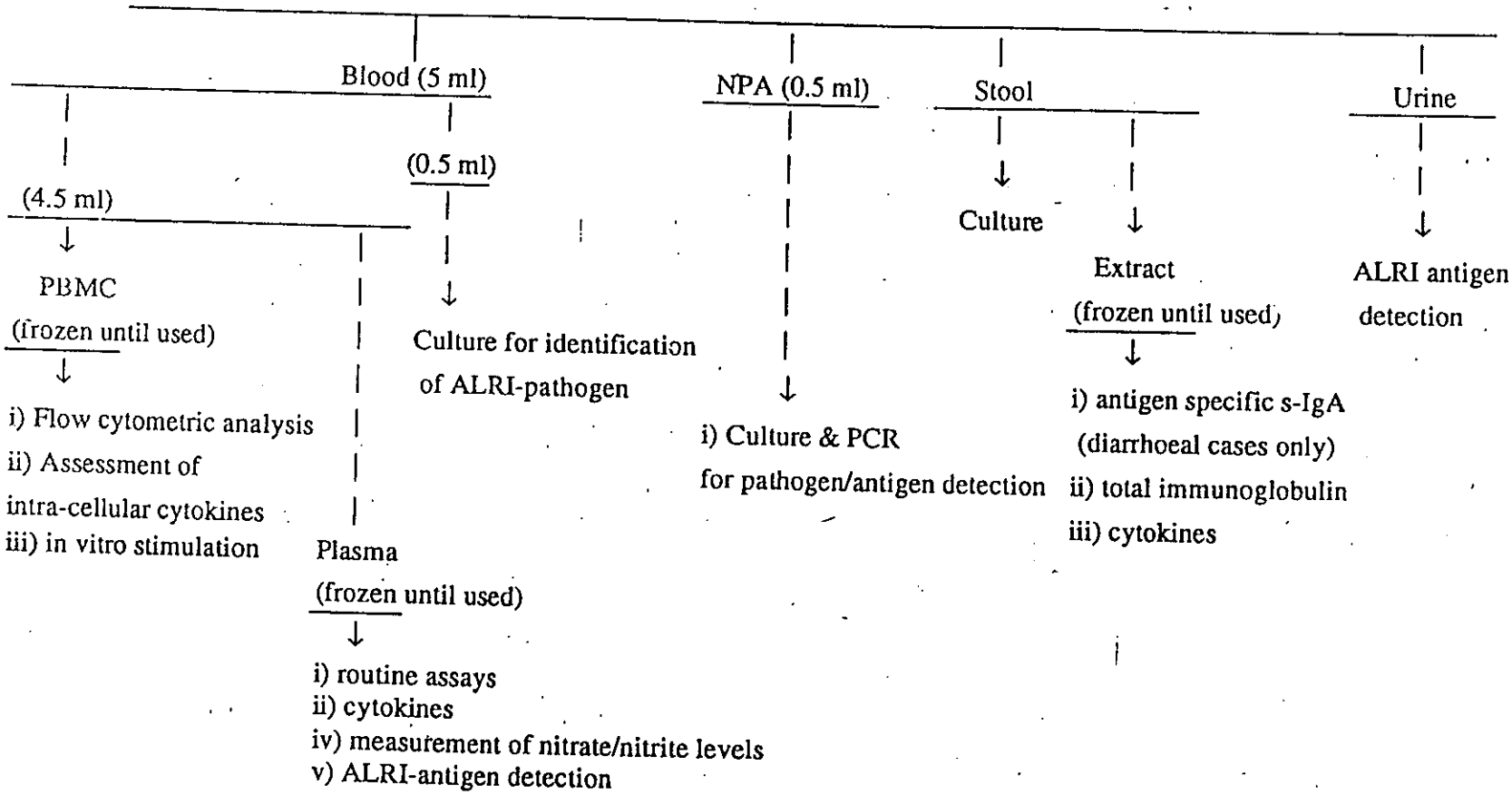
Anthropometric data: weight, height, age, and midupper arm circumference will be recorded halfway between the elbow and the shoulder of the left arm. Triceps skinfold will be measured with a Holtain cailper and read to the nearest 0.1 mm 2-3s after its application. All measurements will be taken independently three times and their mean value will be recorded. The anthropometric measurments will be compared with standard values of the National Center for Health Statistics and will be expressed as Z-scorees: height for age (HAZ), weight for age (WAZ), and weight for height (WHZ) Z-scores. The cut-off point chosen for weight for age, height for age and weight for height Z-scores will be -2s.d. of the reference median. The cut-off point chosen for MUAC will be 13.5 cm. Children will be classified as "satisfactory nutritional status "if WHZ -2 and serum albumin concentration 35g/l and with no biological sign of infections.

7. Routine laboratory assays:

Total and differential leukocyte count of peripheral blood; oxygen saturation and Hb% in blood;pre-albumin, albumin, total IgA, IgG and IgM in plasma; chest X-ray. The routine tests will be performed in the CRSC at ICDDR,B.

Sequence of work with collected samples:

Collection of samples from pediatric patients with- i) ALRI+diarrhea, ii) ALRI, iii) diarrhea and iv) controls
Venous blood (from median cubital vein), NPA, stool and urine



Flow Chart for sequence of tasks within time frame (October1998 to September 2001):

October'98-September '99

i) Procurement of supplies and standardization of methods

ii) Recruitment of patients and controls in the study

iii) Processing and storage of samples & recording of clinical information

iv) The following procedures will be carried out at the time of patient enrollment: detection of pathogen by various techniques, and routine tests such as: TC, DC and oxygen saturation levels in blood

↓

October'99-September '2000

Continuation of ii, iii and iv part of the work

v) Collected lymphocytes (only full set of collected samples (on admission (D-0), D11, D30 and D-60) from patients, and single sample from controls during the first one and a half year will be transported to Stockholm for phenotypic characterization by flowcytometry under the supervision of Birger Christensson at Karolinska Institute (KI). vi) Analyses of cytokines in plasma and secretion (only in samples with full-set)

↓

October'2000-September '2001

vi) Completion of assays (at KI and ICDDR,B), compilation and analysis of data and reporting the outcome of study in several articles

Time frame: To recruit the desire number of study children 2 years of time will be required and the third year for completion of assays and compilation and analyses of data.

Technical expertise and collaborative arrangements:

The immunology laboratory at LSD, ICDDR,B has required expertise to perform assays for this study. However, due to lack of Flowcytometer, immunophenotypic characterization and intra-cellular localization of cytokines of PBL can not be carried out at ICDDR,B. PBMC in liquid nitrogen carrier will be transported to KI for analysis, as has been done for a number of collaborative studies in past.

Sample size calculation:

The minimum required sample size in all the study groups is calculated on the basis of the available information (30, 40). The primary outcome measures of the study is cellular responses in the groups with ARI+diarrhea, with only ALRI, with only diarrhea and healthy controls. By assuming 85% of patients with only ALRI will elicit 3-4 fold increase in cellular

response, 60% of patients with ALRI+diarrhea will have that level of cellular response, and 5% of controls, by using the formula

$$n = \frac{P_1(100 - P_1) + P_2(100 - P_2)}{(P_1 - P_2)^2} \times f(\alpha, \beta)$$

where, P_1 =85, P_2=60, $f(\alpha,\beta)$= 7.9 considering level of significance 95% and power 80%; calculated n= 46

Therefore 50 individuals will be considered for each group considering 5% dropout rate for followup.

Since, to our knowledge, there are no related reference values to consider, therefore, we assumed those P_1, P_2 values based on ours' and others' previous studies. To find out the actual sample size, we will be recalculate sample size based on the findings of first 20 cases in each group.

Statistical analysis:

Both multivariate and univariate analyses will be performed to assess the changes among the groups (29). The JMP software (SAS Institute Inc., NC, USA) program will be used for statistical analysis. Data will be summarized and patient groups will be compared to each other as well as with the control group. Statistical analyses which will be included are- descriptive as well as analytical methods, arithmetic mean and standard deviation if the distribution is normal; median and ranges if the distribution is not normal. If the outcome is expressed as categorical variable, the significance of differences will be evaluated by chi-square test. When the main outcome measures are continuous variables multiple analysis of variance for repeated measurements using Bonferroni/Dunn statistics will be performed. If necessary equivalent nonparametric tests will be performed because asymmetric distribution of measurements may occur.

SIGNIFICANCE:

In this study, we will make efforts to describe immunological responses in three patient groups: children with both diarrhea and ALRI, with ALRI alone, with diarrhea alone and those , to ascertain whether there are differences in the responses. A healthy matched-control group will be required to obtain base-line information in an endemic area. Therefore, the results of this study may help us to understand: i) the immune defense mechanisms of children with ALRI, with diarrhea, and with ALRI+diarrhea in Bangladesh, a country where ARI and diarrhea are both endemic; ii) changes in the immune function which makes ALRI infected children to predispose to diarrheal diseases or vice versa, and iii) the kinetics of the

induction of specific B and T cell responses against important pathogens. The study may benefit other emerging areas of interest. For example, the study may provide needed insight into the pattern of distribution of pathogens associated with ALRI in this patient population. This in turn can provide the development of appropriate treatment and management protocols, which in turn is important to the Centre as it is embarks on the creation of a "Center of Excellence" for Integrated Management of Childhood Illness (IMCI), of which ALRI case management is an important component.

Recent strategy to prevent ARI emphasizes the use of vaccines. To achieve this objective, it is important to understand the host immune mechanisms, so that vaccination schedules mimicking immune response to natural infection can be developed. T cells are important not only as mediators of cell mediated immune response, but also for the development of high affinity antibody responses. Antigen-specific T cells orchestrate many specific and non-specific immune functions. The nature of bacterial antigens eliciting specific T cell responses in ARI are virtually unknown. Efficient vaccine development requires successful design of a vaccine which in turn requires efficient antigen presentation as well as specific T cell activation. Effective T cell responses mediate both the humoral and cell-mediated immune responses to pathogens. Little is known about T cell activation and influence of the modes of antigen presentation for T cell function in ARI in human. The current study will characterize antigen specific T cells that may provide help for both types of immune reactions. Thus, findings of this study may help design/develop/define a successful vaccine against ARI suitable for use in Bangladesh and other countries where similar health problems are seen.

By comparing the results of PCR assay and culture method it will be possible to define efficiency of PCR for the detection of ARI pathogens in blood, and thus improve diagnosis as well as facilitate specific interventions.

In summary, knowledge gained from the results of the proposed study may help better understand the development of host defense mechanisms against ALRI mainly pneumonia, and diarrhea in children in an endemic country, and may identify "immunolgicall risk factors" in children, who are getting ALRI and diarrhea together, thus may help in better management of these children *ie*, may greatly reduce death in children. Furthermore, it may strengthen other areas of Centre-activity, namely IMCI, as it provides insight that can be used to formulate ALRI treatment strategies.

ETHICAL CONSIDERATIONS:

The proposed study involve: (i) management of diagnosis and patients with ALRI and diarrhea. (ii) sampling of blood, NPA, urine and stool.

The proposal will be reviewed by the Ethical Review Committee (ERC) of ICDDR,B. Apart from known complications of blood drawing which are generally minor and include mild discomfort and potentials for bruising at the site of venipuncture this involves no substantial risks. All sampling procedures will be performed by well-experienced personnel. Informed

consent will be obtained from the parents of all participating children as well as the controls. Confidentiality of data will be maintained. The subjects will have the right to refuse participation in this study, and also to withdraw from the study at any time during the study. The decision of withdrawing from the study will not influence subsequent care of any children in the hospital. Study children will receive clinical care and treatment free of charges which would be of similar type had they not been enrolled in the study.

BUDGET FOR THREE YEARS (1998-2000):

		Amount in US$ yearly budget			
		1st year	2nd year	3rd year	TOTAL
1. Personnel:	% of time				
Principal Investigator	50	7950	8,348	8,765	25,063
A. Brooks (Co-PI)	15	nil	nil	nil	nil
Rubhana Raqib (Co-i	5	580	609	nil	1,189
M. A. Salam (Co-inv	5	960	1,008	nil	1,968
S. K. Shaha (Co-inve	5	nil	nil	nil	nil
Medical Officer	50	4,000	4,200	nil	8,200
Research Officer	100	4,000	4,200	4,410	12,610
Laboratory technicia	100	2,000	2,100	2,205	6,305
Lab attendant	100	2,200	2,310	nil	4,510
TOTAL PERSONNEL COST		**21,690**	**22,775**	**15,380**	**59,845**
2. Capital equipment:					
Liquid nitrogen tank		8,000			8,000
Water bath & shaker		3,000			3,000
Centrifuge		4,000			4,000
Computor		5,000			5,000
TOTALEQUIPMENT COST		**20,000**	**0**	**0**	**20,000**
3. Pateint costs:					
Hospitalisation cost ($30/day x 5 days		8,000	7,000	0	15,000
Wage loss for follow-up ($10/visit x 3		2,000	3,000	0	5,000
Medicines as required		500	500		1,000
TOTAL PATIENT COST		**10,500**	**10,500**	**0**	**21,000**
4-a. Laboratory investigations:					
Blood culture, routine tests of blood, urine and stool. Total Immunoglobulin types, sub-types and CRP, pre-albumin measurment in		4,000	5,000		9,000
4-b. Immunological assays:					
PCR-detection of pathogen		3,000	5,000		8,000
Measurement of antigen specific antibodies in ser			4,000	2,000	6,000
Immunophenotyping of PBL		5,000	12,000	8,000	25,000
4-c. Laboratory supplies:					
Plastic ware, glassware and office sup		2,000	2,000	1,000	5,000
TOTAL LABORATORY COST		**14,000**	**28,000**	**11,000**	**53,000**

	yearly budget			
	Ist year	2nd year	3rd year	TOTAL
5. Other services:				
Printing and publication, library charges, communication charges and maintena	740	1,500	3,000	5,240
6. Travel:				
Trip to KI to perform immunophenoty by flowcytometry	5,000		5,000	10,000
TOTAL COST	71,930	67,775	29,380	169,085

BUDGETJUSTIFICATION:

Md. Bozlur Rahman
Senior Budget & Cost Officer
ICDDR, B, Mohakhali
Dhaka-1212, Bangladesh

1) Salary of involved personnel will require about 35% of the total cost.

2) Small equipments, which will be required essentially for this study will cost 10% of the total budget

3) Expenditure for 150 patients, who will be hospitalized atleast for 5 days, either at ICDDR,B or Children's hospital will cost 11% of the total budget

4) To perform all the mentioned routine tests, which will be necessary to monitor patients clinical condition, and required immunological assays will cost 37% of the total budget, since most of the reagents for these tests/assays are expensive.

5) In this study on type of sample (PBMC) will be transported to Karolinska Institute, Sweden to perform immuno-flowcytometric analyses.
For this purpose travel (twice) to Sweden will be required.

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INTERNATIONAL CENTRE FOR DIARRHOEAL DISEASE RESEARCH, BANGLADESH

"ALRI & Diarrhea"

CONSENT FORM FOR PEDIATRIC PATIENTS

Your child have both acute lower respiratory infection (ALRI) and diarrhea. ARI is the single largest cause of death in young children, accounting for 33% of all childhood deaths in developing countries like Bangladesh. It is hypothesize that children with ALRI and diarrhea are likely to have altered (defective/reduced) immune function. In order to understand more about this illness and how to increase immunity, we are conducting a study. The results of this study may help us to understand the immune defense mechanisms of children with ALRI+diarrhea, only ALRI, only diarrhea and changes in the immune function which makes ALRI infected children to predispose to diarrheal diseases or vice versa, thus may help in better management of these children with ARI and diarrhea in Bangladesh, a country where ALRI and diarrhea are both endemic.
For this purpose, we would like your permission to enroll your child in this study. Participation of your child in this study may help to pursue the study.

During the study period, your child will be examined thoroughly and will receive necessary treatment of this hospital. Your child will have to stay in the hospital and will be discharged based on the clinical condition of the child. You will be able to stay with your child in the hospital. The hospital costs and therapy will be free of charge. About 5 ml of blood (one tea-spoon full) will be collected on the day of admission and 11, 30 and 60 days after (4 times) that from your child (median cubital vein). You will be requested to bring your child for follow-up visits on days 11, 30 and 60 after discharge. Stool, urine and saliva samples will also be collected on these days.

It is completely your decision whether your child should be enrolled in this study or not. After initial participation in the study, you have the right to withdraw your child from the study at any time point at your will. Your child will receive the standard care and treatment of this hospital whether he/she is enrolled in this study or not. We will compensate for any wage loss and travel costs that you may incur for each follow-up visit. All information/data of this study will be kept confidential and will be provided to you upon your request.

If you agree to let your child participate in this study, please sign or put your left thumb imprint at the specified space below.

Thank you for your co-operation.

Signature / left thumb impression of the guardian	Date
Signature of the investigator	Date
Signature of a witness	Date

DHAKA SHISHU HOSPITAL

"ALRI"

CONSENT FORM FOR PEDIATRIC PATIENTS

Your child has acute lower respiratory infection (ALRI). In Bangladesh, the average ARI episodes/year irrespective of severity, is 3 to 5. More than half of these infections occur before the second birthday, and children 6 to 12 months of age have the highest. The severity of infection depends on the interplay between the host resistance, including immune competence of the host, and the virulent properties of the pathogen. However, the host immune defense mechanisms (understanding of the development of natural immunity) during a natural course of ALRI in children has not been well-studied. In order to understand more about this illness and how to increase immunity against this disease, we are conducting a study. Results obtained from this study may help to understand protective immunity and may provide information on better therapeutic interventions and management of this disease. For this purpose, we would like your permission to enroll your child in this study. Participation of your child in this study may help to pursue the study.

During the study period, your child will be examined thoroughly and will receive necessary treatment of this hospital. Your child will have to stay in the hospital and will be discharged based on the clinical condition of the child. You will be able to stay with your child in the hospital. The hospital costs and therapy will be free of charge. About 5 ml of blood (one tea-spoon full) will be collected on the day of admission and 11, 30 and 60 days after (4 times) that from your child (median cubital vein). You will be requested to bring your child for follow-up visits on days 11, 30 and 60 after discharge. This will not be harmful to your child in any way.

It is completely your decision whether your child should be enrolled in this study or not. After initial participation in the study, you have the right to withdraw your child from the study at any time point at your will. Your child will receive the standard care and treatment of this hospital whether he/she is enrolled in this study or not. We will compensate for any wage loss and travel costs that you may incur for each follow-up visit. All information/data of this study will be kept confidential and will be provided to you upon your request.

If you agree to let your child participate in this study, please sign or put your left thumb imprint at the specified space below.

Thank you for your co-operation.

Signature / left thumb impression of the guardian

Date

Signature of the investigator

Date

Signature of a witness

Date

INTERNATIONAL CENTRE FOR DIARRHOEAL DISEASE RESEARCH, BANGLADESH

"DIARRHEA"

CONSENT FORM FOR PEDIATRIC PATIENTS

Your child has diarrhea. Diarrheal diseases is the second important cause of childhood illness, malnutrition and death in Bangladesh. On the otherhand acute respiratory infection (ARI) is the single largest cause of death in young children, accounting for 33% of all childhood deaths in developing countries like Bangladesh and the average ARI episodes/year irrespective of severity, is 3 to 5. More than half of these infections occur before the second birthday, and children 6 to 12 months of age have the highest. The severity of infection depends on the interplay between the host resistance, including immune competence of the host, and the virulent properties of the pathogen. Death rate are increased where acute lower respiratory infection (ALRI) and diarrhea occur together. It is hypothesize that children with ARI and diarrhea are likely to have altered (defective/reduced) immune function. In order to understand more about this illness and how to increase immunity, we are conducting a study, where children with ALRI+diarrhea, ALRI and diarrhea will be included. Thus the results of this study may help us to understand the immune defense mechanisms of children with ALRI+diarrhea, ALRI and diarrhea, and changes in the immune function which makes ALRI infected children to predispose to diarrheal diseases or vice versa, thus may help in better management of these children with ALRI and diarrhea in Bangladesh, a country where ARI and diarrhea are both endemic.
For this purpose, we would like your permission to enroll your child in this study. Participation of your child in this study may help us to pursue the study.

During the study period, your child will be examined thoroughly and will receive necessary treatment of this hospital. Your child will have to stay in the hospital and will be discharged based on the clinical condition of the child. You will be able to stay with your child in the hospital. The hospital costs and therapy will be free of charge. About 5 ml of blood (one tea-spoon full) will be collected on the day of admission and 11, 30 and 60 days after (4 times) that from your child (median cubital vein). You will be requested to bring your child for follow-up visits on days 11, 30 and 60 after discharge. This will not be harmful to your child in any way. Stool, urine and saliva samples will also be collected on these days.

It is completely your decision whether your child should be enrolled in this study or not. After initial participation in the study, you have the right to withdraw your child from the study at any

time point at your will. Your child will receive the standard care and treatment of this hospital whether he/she is enrolled in this study or not. We will compensate for any wage loss and travel costs that you may incur for each follow-up visit. All information/data of this study will be kept confidential and will be provided to you upon your request.

If you agree to let your child participate in this study, please sign or put your left thumb imprint at the specified space below.

Thank you for your co-operation.

Signature / left thumb impression of the guardian	Date
Signature of the investigator	Date
Signature of a witness	Date

INTERNATIONAL CENTRE FOR DIARRHOEAL DISEASE RESEARCH, BANGLADESH

"CONTROL"

CONSENT FORM FOR CHILDREN

Acute respiratory infection (ARI) is the single largest cause of death in young children, accounting for 33% of all childhood deaths in developing countries like Bangladesh and the average ARI episodes/year irrespective of severity, is 3 to 5. More than half of these infections occur before the second birthday, and children 6 to 12 months of age have the highest. The severity of infection depends on the interplay between the host resistance, including immune competence of the host, and the virulent properties of the pathogen. Diarrheal diseases, on the other hand, is the second important cause of childhood illness, malnutrition and death in these countries. Death rate are increased where acute lower respiratory infection (ALRI) and diarrhea occur together. It is hypothesize that children with ARI and diarrhea are likely to have altered (defective/reduced) immune function. In order to understand more about this illness and how to increase immunity, we are conducting a study. The results of this study may help us to understand the immune defense mechanisms of children with ALRI+diarrhea, ALRI and diarrhea, and changes in the immune function which makes ALRI infected children to predispose to diarrheal diseases or vice versa, thus may help in better management of these children with ALRI and diarrhea in Bangladesh, a country where ARI and diarrhea are both endemic. Healthy subjects are needed to be examined in order to monitor the constancy of the research parameters in the healthy children and to compare the findings with those in patients. For this purpose, we would like your permission to enroll your child in this study. Participation of your child in this study may help us to pursue the study.

For this purpose, your child will be examined by a qualified physician for a routine check up. For our study, we will collect venous blood, nasopharyngeal aspirate, urine and stool samples only once from your child. Approximately 5 ml (one tea-spoon full) of blood will be taken from your child (median cubital vein). This will not be harmful to your child in any way.

It is your decision to let your child participate in this study. All information/data of this study will be kept confidential and will be provided to you upon your request. We will compensate for any wage loss and travel costs that you may incur while your child participates in this study.

If you agree, please sign or put your left thumb imprint at the specified space below.

Thank you for your co-operation.

_____ _____

Signature / left thumb impression of the guardian

Date

_____ _____

Signature of the investigator

Date

_____ _____

Signature of the witness

Date

R1-I

Review #1

EVALUATION OF CHILD HEALTH RESEARCH PROPOSAL

Reviewer's name:

Name of proposal: Cellular and humoral immune responses in children during acute respiratory infection (ARS).

Name of proposed investigator: Dilara Islam

Date of review: June 25, 1997

The principal aims of this proposal are to study basic cellular and humoral immune responses in children with acute respiratory infections and diarrhea as relates to establishment of protective immunity. However, the specific aims are not focused as one aim deals with improved diagnostic methods. The study design is adequate. The Materials section lacks detail. For instance, in part d it is stated that "fecal samples will be extracted as previously described (21)". This technique is never described. The respective advantages, disadvantages and uses of PCR and latex agglutination tests are not stated. PCR has high specificity, latex agglutination has much lower specificity. PCR is lab-based, latex agglutination is more rapid and versatile. No description has been give. However, considerable detail was given to the immunophenotyping section; it is not evident where these analyses will be carried out [Sweden is indicated in the timetable]. No direct cellular function assays are contemplated, i.e., lymphocyte proliferation and antigen-induced cytokine expression/production. Without these assays, it would be difficult to make conclusions as to specific cell-mediated immunity to enteric pathogens. The flow charts of techniques and the timetable is helpful.

The proposal fails to address specific cellular immune responses as stated in the Significance section. The section mentions T cell activation and T cell responses and antigen presentation. These aspects are not characterized in the present proposal.

Proposal #8 Review # 2

Cellular and humoral immune responses in children during acute respiratory infection.

Dilara Islam

Goals Good, except see "appropriateness" section below.

Design Appropriate.

Appropriateness An efficacious vaccine for H. influenzae B is available. The Background section of the proposal does not provide an argument for continuing to study the immune response and other components of H. influenza B disease. What will we learn from this study that would improve the H. influenzae B immunization program?

On the other hand, characterizing differences in criteria for diagnosis and response to treatment and infection in patients with respiratory disease vs. respiratory disease with gastroenteritis is appropriate new investigation.

Timing and budget Timing: adequate. Budget: funds to support the liquid nitrogen tank, computer, and centrifuge should be included in years 2 and 3, on the order of $2000 per year.

Ethics Addressed.

Background Appropriate.

Other None.

R2-I'
R3-D,I

Review #3

Proposal 8: "Cellular and humoral immune responses in children during acute respiratory infection (ARI)

Principle Investigator: D. Islam, PhD

1. Goals. "To study the development of systemic immune defense mechanisms in children with ARI and diarrhea."

2. Design. This proposal seeks to compare 120 children (6-36 months of age) having ARI, diarrhea, or both simultaneously, with respect to antibody responses to pneumococcal and Hib antigens by ELISA in serum and circulating antibody secreting cells (ASC) by ELISPOT, to a variety of inflammatory markers, and to compare these responses to those in matched healthy children. To establish the etiology of ARI, they will assess new PCR-based methods for diagnosis of pneumococcal and Hib infection. Patients with ARI plus diarrhea will be enrolled at ICDDR,B and those with ARI alone will be at Shisu Hospital. (The table of patient populations on page 6 has several obvious labeling errors, so the groups and subgroups at the two sites are not entirely clear; recruitment of 60 age-matched healthy controls is stated but not described.)

Blood, nasopharyngeal aspirates (NPA), saliva, urine, and stool will be collected on days 0, 7, 15, 30, and 60. Some samples will be processed immediately, while others will be frozen until used. Finger prick serum samples will be obtained daily during hospitalization to measure CRP and endotoxin for monitoring severity of infection. NPA will be cultured and used for PCR pathogen detection and IgA antibody studies. There is no mention of blood cultures, but this is apparently part of routine clinical labs, according to Study Flow Chart I. Phenotyping of peripheral blood lymphocytes will be done at the Karoliska Institute. Nitrate/nitrite and endotoxin will be measured in plasma and urine.

2.1 Definitions of key concepts and variables. Clinical definitions of ARI will be done according to WHO classifications of ARI, but there is no mention of radiographic exclusion or confirmation of pneumonias. There is no description of assessments of severity of diarrhea or associated dehydration.

2.2 Study populations, sample size, and sampling strategy. The population size was estimated by a formula on page 15. This seems rather arbitrary, since confidence limits for most of the parametres to be tested are probably unknown. In any case, 30 per group x 4 plus 60 controls is quite a few considering the enormous number of test proposed

2.3 Clarity of analysis plans. Described in general terms (p. 15).

2.4 Feasibility of proposed methods. Two intriguing diagnostic methods are proposed (p. 8). A PCR test for pneumococcus is based upon amplification of the pneumolysin gene in serum, developed by Salo, et al. (ref. 31). The PCR was positive for 20/20 patients with pneumococcal pneumonia confirmed by positive blood cultures and had a false positive rate of about 6% among healthy elderly controls. However, this test has not been verified for detection of pneumonia in patients whose blood cultures are negative. No provisions for verifying the latter are included in the current proposal, but the test certainly deserves further scrutiny. The PCR test for Hib is based on amplification of the *cap* gene, as used for PCR typing of *H. influenzae* in reference 13. This method has been applied to organisms already grown in laboratory cultures, but it has not been applied to detection of Hib genes in clinical materials such as serum. The investigators have had success in applying similar techniques for detection of Shigella genes in fecal samples. However, it remains to be seen if either test is more sensitive than blood cultures (assuming a low rate of false positives), or if, like the less sensitive latex agglutination methods, is only positive when

#8
R3-M

Child Health Research Proposals

Name of Proposal: Cellular + humoral responses in children w/ ARI

Name of proposed investigator: Islam

Several important flaws in study as proposed, most strikingly in discordance between sampling plan + analysis plan.

1) Goals:

Well-stated, + supported although hypothesis #2 a bit vague, + not clear how it is addressed or used in study.

2) Design:

Poor sampling plan. Incorrect equation to assess differences between groups + most definitely for sub-group analysis.

Controls are irrelevant to study aims as described + inclusion not well supported. Matching by age + then analyzing by age inappropriate.

3) Appropriateness

Important disease.

Not clear how early C admission sampling will be conducted given delay in identification of causative agent.

Exclude children w/ hx of antibiotics in last 6 mos? Hx of vaccination, esp Hib or S. pneu

R1-D

Proposal #8

Review #4

Title: Cellular and humoral immune responses in children during acute respiratory infection (ARI).

P.I.: D. Islam et. al.

Goals: The goals of the study are to describe the immune responses in children with ARI due to S. pneumoniae (Spn) and H. influenzae (Hib), including groups with and without concomitant diarrhea.

Design: The study defines 3 groups, i.e. ARI + diarrhea, ARI alone, and controls, and will follow children in each group with serial blood, NP, saliva, urine, and stool cultures. The hypothesis is that immune responses to ARI are different in children who also have diarrhea, although the background data provides little justification for that hypothesis. Although the investigators intend to select for patients with Spn and Hib disease, they use the term ARI patients loosely and often do not clearly distinguish between ARI as a syndrome (which may have many viral and bacterial causes) and ARI due to the bacteria of interest. Comments about design issues are:

1. The investigators say they are selecting children "prone to ARI and diarrhea" or "prone to ARI alone", when in fact they have no historical basis from the data collected to tell if this is the case. What do they mean by "prone to ARI and diarrhea"? As diarrhea is common in Bangladesh, how do they distinguish association by chance from association due to a particular immunologic predisposition.

2. The premise of the protocol is to examine immune responses to Spn and Hib, so I am surprised that the Specific Aims do not make this distinction and focus on ARI alone. A more rigorous distinction in the presentation of experimental plans should be requested.

3. Diarrhea is not defined, and a case definition is necessary.

4. Methods for routine diagnostic testing for diarrhea and respiratory specimens are not provided.

Appropriateness: I do not believe that the investigators have clearly articulated why the data generated will add significantly to previous studies and impact public health. The data may allow us to compare immunologic responses of children in Bangladesh with those of children in developed countries, but the study groups and the rationale for selecting patients with and without diarrhea is not developed (especially since diarrhea may have many causes).

Timing: A well defined timetable is provided and appears reasonable.

Ethical considerations: No concerns.

Background: Appropriate

#8
R1-M

EVALUATION OF CHILD HEALTH RESEARCH PROPOSAL Review #5

Name of proposal: Cellular and humoral immune responses in children during acute respiratory infection (ARI)

Name of proposed investigator: Islam et al.

Date of review: July 4, 1997

Review:

The comparison groups to be included in this study are very confusing. According to the general description, it appears that there would be 3 groups: 1) ARI, 2) ARI+ diarrhea 3) Control. According to the table in page 6, there are some subgroups, which do not correspond to the description provided, and the typos at the bottom of the table just make it impossible to understand what is going on (group B1 is mentioned twice; group B2 is not mentioned).

The study also seems to be some hybrid of the case-control design, but there are 2 different typese of cases (ARI and ARI + diarrhea) and 1 control per case (of any type). This design does not make much sense.

The specific aims of the study should be formulated to indicate that the study is a comparison study, as opposed to a purely descriptive study, and the groups to be compared should be indicated in the formulation of the objectives.

Relative to the case-control design, the authors should consult the literature on case-control studies to avoid biases in selecting their cases and controls. Three main points have to be considered in case-control studies. First, relative to the selection of subjects, both the cases and the controls must be selected from a representative population of cases and controls, respectively. Secondly, the cases and controls should be matched on factors that are important determinants of the outcomes studied (and could be confounding factors if not controlled for), but that are not among the risk factors of interest in the particular study (for example, age, gender, nutritional status in this case). Finally, the analysis of the data must be 'matched', i.e. cases and controls must be paired in the analysis.

The formula used for sample size calculation is inappropriate for a case-control study and so are the analyses proposed.

#8
R3-M

Child Health Research Proposals

Review #6

Reviewer's name:

Name of Proposal: Cellular and humeral immune responses in children during ARI

Name of proposed investigator: Islam

Date of review: 6/12/97

For CHR project staff only

Sampling plan totally inadequate given proposed analysis plan by subsets of study subjects.

"Control" group irrelevant to proposed study aims & use completely unsupported in proposal.

Title of the proposal: **Cellular and humoral immune responses *in children* during acute respiratory infection (ARI) due to *Streptococcus pneumoniae* or *Haemophilus influenzae* type b**

PI: **Dilara Islam.**

Subject: **Response to the Reviewers' Comments**

Reviewer I:

A. **Specific aims:** We have dropped comparison of various diagnostic methods as a specific aim of the protocol.

B. **Fecal extraction procedure:** Have been detailed in the "Study materials and preparation" section of the revised version (page 8).

C. **Diagnosis of ARI pathogen (s):** In this study, we have planned to detect only two ARI pathogens, *S. pneumoniae* (Spn) and type b *H. influenzae* (Hib). Currently, blood cultures can detect the pathogens from only 10-20% of cases. Thus, we think it important to look for better methods for detection of the pathogens, and consider that PCR and latex agglutination assays have enough potentials to be useful. These assays will be performed in Laboratory Sciences Division, ICDDR,B.

D. **Immunophenotyping:** We actually had indicated in the original protocol (page 16 of the revised version) that due to the lack of Flowcytometer, immunophenotypic characterization of PBL can not be carried out at ICDDR,B. PBMC in liquid nitrogen carrier will be shipped to Karolinska Institute (KI), Stockholm, Sweden, for analysis". We would also like to indicate that we practiced the same for a number of earlier collaborative protocols.

E. **Cellular function analysis:** We have incorporated the suggestion of the reviewer in the protocol, and will perform assays to determine cellular functions like lymphocyte proliferation and cytokine expression, along with immunophenotyping. Similarly, cytokine production will be measured in plasma and secretion with the commercially available kits.

F. **Failure to address specific cellular immune responses:** We have incorporated in the protocol assays to determine lymphocyte proliferation, cytokine expression and

cytokine production. These, along with *in vitro* stimulation of PBMC from healthy subjects with specific antigens may help address the issue.

Reviewer II:

A. **Hib vaccine:** The reviewer raised a genuine concern, however, we do not consider that the issue has been resolved. Although the vaccine has been determined to be effective in some population, in some others the vaccine efficacy were lower. The vaccine has not been tried on Bangladeshi population. We think the efficacy of the Hib vaccine may depend on a number of factors such as (i) differences in population characteristics, (ii) differences in the degree and type of Hib exposure, (iii) differences in susceptibility to disease in various population, (iv) differences in the Hib strains, and (v) strain-specific differences in outer-membrane protein and enzyme electromorphs.

Under the circumstances, we consider it very important to determine vaccine efficacy before implementing immunization program in a country. This vaccine is not yet available in Bangladesh, a country where population characteristics are likely to be different from countries where the vaccine has been found to be effective.

Reference: (i) Eard, J., G. Brenneman, G. W. Letson, W. L. Heyward, and The Alaska H. influenzae vaccine study group. 1990. Limited efficacy of a Haemophilus influenzae type b conjugate vaccine in Alaska Native infants. N Eng J Med. 323:1393-1401; ii) Siber, G. R., M. Santosham, G. R. Reid, C. Thompson, J. Almeido-Hill, A. Morell, G. de Lange, J. K. Ketcham, and E. H. Callahan. 1990. Impaired antibody response to *Haemophilus Influenzae* type b polysaccharide and low IgG2 and IgG4 concentrations in Apache children. N Eng J Med. 323:1387-1392}. In brief, there findings are: lower Hib antibody response in Native American children than in Whites of a similar age. The efficacy of the same PRP-D vaccine was 94%, when given to Finnish infants (Eskola, J., H. Kayhty, A. K. Takala, H. Peltola, P.-R. Ronnberg, E. Kela, E. Perkanen, P. H. McVerry, and P. H. Makela. 1990. A randomized, prospective field trial of a conjugate vaccine in the protection of infants and young children against invasive *Haemophilus influenzae* type b disease. N Eng J Med. 323:1381-1387). The elicited low level of immunity in Finnish-infants may have been sufficient to protect them, as they were infrequently or less intensely exposed and who tend to have disease at older ages.

B. **Timing and budget:** For the purpose of this study, we'd require liquid nitrogen tank, computer and centrifuge right from its beginning which is the reason for their inclusion in the budget for the first year.

Reviewer III:

A. **Design:** The confusion was due to errors in labeling study population, and we now have corrected the table for better clarity. We also have incorporated procedures for blood culture under the "Methodology" section of the revised version of the protocol (page 8).

A.1 **Definitions for the key concepts and variables:** Accepting the reviewer's comments, we would now perform chest X-ray on all study children. Dehydration will be assessed using the WHO guidelines (A manual for the treatment of diarrhea. Rev. 2. WHO/CDD/SER/80.2. 1990).

A.2 **Study populations, sample size and sampling strategy**: The basis for determination of the sample size has been incorporated in the revised version of the protocol (pages 16 & 17). The sample size has now been determined to be 46 children in each group, i.e. a total of 276.

A.3 **Feasibility of proposed methods**: As indicated in reference 13 (# 21 in the revised version), identification of Hib by PCR was done only on isolated strains, however, we understand that this technique is potentially usable. We are not sure if we would be able to identify Hib from blood samples by PCR, however, detection of pneumolysin was possible (reference 31 which is reference # 44 in the revised version).
That is why we considered those two studies to follow for PCR.

A.4 Dr. S. K. Saha is a coinvestigator of this study who will be responsible for enrollment of study children at his hospital (Dhaka Shishu Hospital) as well as for collection of specimen. He will not be involved in any of the assays including PCR, all laboratory works will be done at LSD, ICDDR,B, and there will not be any overlap.

B. **Adequacy of laboratory methods**: We will follow WHO guidelines for ELISA to detect antibodies to Hib and pneumococcal capsular antigens, as suggested by the reviewer (Ref: Siber, R. G., Claudette, P., Madore, D. V. Standardization of antibody assays for

measuring the response to pneumococcal infection and immunization. 1989, Pediatr. Infect. Dis. J.8:S84-91). taking into consideration of another reviewer's (No. I) suggestion, ELISPOT will now be replaced by lymphocyte proliferation, intra-cellular cytokine expression assays.

We thank the reviewer in helping identify a place (and person) for getting pneumolysin, and will communicate with JC Paton or GJ Boulnois (North Adelaide, Australia) with our request.

C. **Questionnaire:** There is a standard questionnaire for ARI, and diarrhea studies in ICDDR,B, which will be used.

D. **Potentials for improving child health care:** The findings of this study will not directly help in the antimicrobial therapy. Trials have not been done to determine efficacy of the available Spn and Hib vaccines in Bangladesh or in any Asian countries. Since there has been reports on lesser efficacy of the vaccines in different population, studies are necessary to determine suitability of available vaccines on population before their introduction. In this study, we will make efforts to describe immunological responses in two patient groups: children with ARI alone and those with both diarrhea and ARI, to ascertain whether there are differences in the response. A healthy matched-control group will be required to obtain base-line information in an endemic area. We hope that the results of this study will help determining (i) development of systemic immune defense mechanisms against disease along with a better understanding of the development of protective immunity (i.e. host defense mechanisms) against ARI due to Spn and Hib, and diarrhea in children in an endemic country like Bangladesh. Thus, this study may help development of an effective vaccine for use in countries like Bangladesh. Additionally, we may also determine a more sensitive method for diagnosis of infections due to Spn and Hib in ARI, and thus in better management of such patients.

E. **Others:** We will use various samples for different purposes. For example, NPA will be used mainly for isolation of pathogens and measurement of s-IgA; saliva and urine will be used to measure mainly the s-IgA, and urine will also be used to identify antigens of the pathogens under consideration; stool will be used for identification of diarrheal pathogen, and measurement of total and antigen-specific s-IgA. A number of immunological assays will be performed with plasma and PBMC. Previously, we have done similar studies in patients with infections due to *Shigella*. We are confident that all of the works indicated in the protocol can be performed well and within the time frame.

Reviewer IV:

A. Design:

A.1 Definition of ARI and ARI Pathogen: ARI are classified under "disease of the respiratory system". Acute bronchitis and broncholitis, influenza and pneumonia are grouped under ARI (Garenne M., Ronsmans C., and Campbell H. 1992. The magnitude of mortality from acute respiratory infections in children under 5 years in developing countries. Wld hlth statist quart., 45: 180-192). In this study, we'll use the WHO clinical classification for ARI. Children of either sex, aged 6-36 months, who have symptomatology indicative of ARI (clinical parameters to define ARI cases) such as cough, fever, sore throat, breathing difficulty, stridor, and/or wheezing, with or without diarrhea will be enrolled in the study. Final inclusion in the study will be done only when either *S. pneumoniae* or Hib have been isolated.

Compared to the developed countries, bacterial pathogens play a far greater role as primary as well as secondary cause of severe ARI, and *S. pneumoniae* and *H. influenzae* are the two most prevalent bacterial agents of community-acquired pneumonia in children in the developing countries (WHO. 1986. Acute respiratory infections: A guide for the planning, implementation, and evaluation of control programs within primary health care. WHO/RSD/86.29[PAHO]). Moreover, these are the two pathogens for which vaccines are currently available. These are the reasons for our study to focus specifically on ARI caused by these pathogens.

A.2 About ARI + diarrhea group: Our hypothesis ("children with ARI are prone/susceptible to diarrhea (or vice versa) as a result of altered immune function") is based on existing information. In the developing countries, ARI often occur in association with other infections, such as diarrhea and malaria. A previous study at ICDDR,B (where 401 children <5 years with ARI studied) has shown that, the symptoms of ARI and diarrhoea developed simultaneously or within 48 hours in 53% of the children, and that the case-fatality was 14% in children who had bacterial pneumonia as well as diarrhea. (Rahman M., Huq F., Sack DA., Butler T., Azad AK., lam A., Nahar N., and Islam M. 1990. Acute lower respiratory tract infection in hospitalized patients with diarrhea in Dhaka, Bangladesh. Rev Infect Dis, 12(Supplement):S899-S906). A post-mortem study of patients who had diarrhoea and died in the Dhaka Hospital of the ICDDR,B, has identified pneumonia as the underlying cause of death in one-third of the children <5 years of age (Butler T., Islam M., Azad AK., Islam MR., and Speelman P. 1987. Causes of death in

diarrheal diseases after rehydration therapy: an autopsy study of 140 patients in Bangladesh. Bull WHO. 65:317-323). However, those studies were conducted in hospital settings. There is lack of information of the incidence of diseases caused by these pathogens in different communities or different population in Bangladesh. Although, the association between ARI and diarrhea remains to be elucidated, it will be unlikely that they occur purely by chance. It is possible that the association is due to immunological predisposition. Therefore, we considered that comparison of the cellular and humoral immune responses in children with only ARI with those having ARI plus diarrhoea may provide useful clue in this direction.

The design of the study is based on our principal hypothesis that the immune response in children with only ARI and children with ARI plus diarrhoea are different. Our hypothesis can only be tested by comparing immune responses in these two groups of children, and in the revised version of the protocol we have described our plan quite elaborately.

We'll use the WHO definition and classification of diarrhea (1990. A manual for the treatment of diarrhea Rev. 2. WHO/CDD/SER/80.2). In brief, diarrhea will be defined as passage of 3 or more, abnormally loose or watery stools in a 24 hour period. Dehydration will be assessed and managed as per recommended WHO-guidelines.

In this study, we will have two groups of cases: (i) children of either sex, aged 6-36 months, who only have symptomatology suggestive of ARI such as cough, fever, sore throat, breathing difficulty, stridor, and/or wheezing; and (ii) those who have ARI as well as diarrhoea. Final inclusion in the study will be done only if Spn or Hib are isolated from the cases. A healthy matched-control group will be included in the study to get the base-line information in that endemic population to which results of the two groups of cases can be compared.

A.3 Methods for routine diagnostic testing: Culture of venous blood and freshly collected NPA will be done for the identification of ARI pathogen(s), and stool will be culture for the identification of diarrheal pathogen (s). These procedure are described in details under the methodology section of the revised version of the protocol (pages 8 & 9).

A.4 Appropriateness: This has been described in an earlier paragraph of this response. We are hopeful that the findings of this study will provide us useful information as well as better understanding of the development of protective immunity (i.e. host defense

mechanisms) against ARI due to Spn and Hib, and diarrhea in children in an endemic country. the findings may also be helpful in the designing of vaccines, and in vaccination program.

The findings of this study may help in antimicrobial therapy since we'll use methods for identification of the pathogens with better sensitivity which are not available for routine use. There is also indication (please see response to reviewer II, A) that a vaccine that is useful in protection of its users in one population may not be as effective in another setting. Thus, it will be important to understand the specific immune responses against naturally occurring ARI as well as base-line information from the population/community concerned, especially in countries where the problems are acute. The findings of this study will help developing vaccination programs against Spn and Hib in Bangladesh, and may also be helpful for development of similar programs in other countries which share similar health problems.

Reviewer V:

A. **Study groups:** This error has been corrected in the revised version of the proposal.

B. **Study design:** We have indicated earlier (please see response to reviewer IV, A:2) that there will be two groups of cases in the study- those with only ARI and those with ARI + diarrhoea. For every case there will be a matched control. This design will allow us to have information on immune responses among children with ARI, ARI + diarrhea, and in healthy children and their comparison between the groups.

C. **Specific aims:** The aim of this study is to describe and compare specific immune responses in children who have only ARI with those who have ARI plus diarrhoea, and their comparison with the responses in healthy children. These information have now been incorporated in the revised version of the proposal.

D. **Representativeness of the study population:** This will be a hospital based study. The Clinical Research and Service Centre (CRSC) of ICDDR,B treats around 110,000 patients each year, about 60% of whom are <5 years of age. This is the only diarrhoea treatment hospital in Dhaka city. Dhaka Shishu (children's) Hospital, the only Children's hospital of the country, has 350 beds and admits about 1,300 children in its inpatient units each month. Both of these hospitals serves mainly the population of the Dhaka city and its

adjoining area. People who attend these hospitals are similar to those who will attend other hospitals of the country. Thus, the population will be representative of Bangladesh.

Inclusion of the control group is essential to test our hypothesis. The inclusion criteria of the control group has now been described in detail under the section on 'schedule of the study population' (page 7) of the revised version of the proposal.

E. **Sample size:** The sample size has been determined to be xx in each of the three groups. The basis for determination of the sample size has been provided in the revised version of the proposal (pages 16 & 17).

Reviewer VI:

A. **Sampling plan:** There will be three groups: with only ARI (due to Spn or Hib), with ARI+diarrhea and matched controls. There will be no other sub-group. This has been described earlier (please see response to reviewer V, B and D).

B. **Inclusion of a control group:** The importance for inclusion of this groups has been described earlier (please see response to reviewer V, D).

C. **Sampling and analysis plan:** We have responded to this issue earlier.

D. **Proposal hypothesis #2:** We already have responded to this issue (please see response to reviewer IV, A:2).

E. **Sample size calculation:** We have responded to this issue earlier (please see response to reviewer V, E).

F. **Exclusion criteria:** In Bangladesh children of the age group to be studied frequently suffer from infectious diseases. Thus, it will be difficult to exclude children who have received antibiotic during the previous 6 months. Moreover, antibiotics are available over the counter, and people do not preserve medical information. Thus, it will be extremely difficult to obtain a history of what drugs a patient has actually taken. Therefore, we will only exclude children who have a history suggestive of ARI during last 3 months. vaccines against Hib and Spn are not yet available in Bangladesh.

G. **Additional sample collection site:** We have already responded to this issue in an earlier section of this response [his will be a hospital based study, since both the patient groups will be recruited from hospitals. From ICDDR,B and Dhaka Shishu hospital patients will be recruited. The Clinical Research and Service Centre of the ICDDR,B are treating about 100,000 patients per year. This is the only hospital in Dhaka city, where a large number of patients with diarrhea are treated. Dhaka Shishu (children's) Hospital, the only children's hospital (at national level) in Bangladesh, is a 350 bed teaching hospital with a catchment population of 16 million from Dhaka and adjoining districts. The hospital admitted about 1300 children per month.