

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Principal Investigator TASNIM AZIM Trainee Investigator (if any) _____
Application No. 98-002 Supporting Agency (if Non-ICDDR,B) _____
Title of Study IMPORTANCE OF NORWALK-LIKE VIRUS (✓) Project status:
CHILDHOOD DIARRHOEA IN BANGLADESH () 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).

- Source of Population:
- (a) Ill subjects ☒ Yes ☐ No
 - (b) Non-ill subjects ☐ Yes ☐ No
 - (c) Minors or persons under guardianship ☒ Yes ☐ No
- Does the study involve:
- (a) Physical risks to the subjects ☒ Yes ☐ No
 - (b) Social Risks ☐ Yes ☒ No
 - (c) Psychological risks to subjects ☐ Yes ☒ No
 - (d) Discomfort to subjects ☒ Yes ☐ No
 - (e) Invasion of privacy ☐ Yes ☒ No
 - (f) Disclosure of information damaging to subject or others ☐ Yes ☒ No
- Does the study involve:
- (a) Use of records, (hospital, medical, death, birth or other) ☒ Yes ☐ No
 - (b) Use of fetal tissue or abortus ☐ Yes ☒ No
 - (c) Use of organs or body fluids ☒ Yes ☐ No
- Are subjects clearly informed about:
- (a) Nature and purposes of study ☒ Yes ☐ No
 - (b) Procedures to be followed including alternatives used ☒ Yes ☐ No
 - (c) Physical risks ☒ Yes ☐ No
 - (d) Sensitive questions ☐ Yes ☒ No NA
 - (e) Benefits to be derived ☒ Yes ☐ No
 - (f) Right to refuse to participate or to withdraw from study ☒ Yes ☐ No
 - (g) Confidential handling of data ☒ Yes ☐ No
 - (h) Compensation &/or treatment where there are risks or privacy is involved in any particular procedure ☐ Yes ☐ No NA
5. Will signed consent form be required:
- (a) From subjects ☐ Yes ☒ No
 - (b) From parent or guardian (if subjects are minors) ☒ Yes ☐ No
6. Will precautions be taken to protect anonymity of subjects ☒ Yes ☐ No
7. Check documents being submitted herewith to Committee:
- Umbrella proposal - Initially submit an overview (all other requirements will be submitted with individual studies).
 - ☒ Protocol (Required)
 - ☒ Abstract Summary (Required)
 - Statement given or read to subjects on nature of study, risks, types of questions to be asked, and right to refuse to participate or withdraw (Required)
 - ☒ Informed consent form for subjects
 - ☒ Informed consent form for parent or guardian
 - Procedure for maintaining confidentiality
 - Questionnaire or interview schedule *
- * If the final instrument is not completed prior to review, the following information should be included in the abstract summary:
1. A description of the areas to be covered in the questionnaire or interview which could be considered either sensitive or which would constitute an invasion of privacy.
 2. Examples of the type of specific questions to be asked in the sensitive areas.
 3. An indication as to when the questionnaire will be presented to the Cttee. for review.

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

Tasnim Azim
Principal Investigator

Trainee

APPLICATION FOR A PROJECT GRANT

1. **TITLE:** Investigation of the importance of Norwalk-like viruses in childhood diarrhea in Bangladesh
2. **INVESTIGATORS:**
 - Principal investigator:** Tasnim Azim, Laboratory Sciences Division (LSD), ICDDR,B
 - Co-principal investigator:** Goutam Podder, LSD, ICDDR,B
 - Co-investigators:** Zahid Hasan, PHSD, ICDDR,B
MA Salam, CSD, ICDDR,B
ASG Faruque, CSD, ICDDR,B
M. John Albert, LSD, ICDDR,B
 - Consultants:** Leanne Unicomb, Australia
Roger Glass, CDC, Atlanta, USA
4. **STARTING DATE:** As soon as possible
5. **ENDING DATE:** 2 years after commencement
6. **TOTAL BUDGET:** \$68,928 (Direct Cost)
7. **FUNDING SOURCE:** USAID
8. **HEAD OF PROGRAMME:** Director, LSD 
9. **ABSTRACT:**

This study will attempt to determine whether viruses that comprise the Norwalk group of viruses cause diarrhea among infants and children in Bangladesh. Norwalk-like viruses (NLVs) have been associated with outbreaks of food- and water-born diarrhea and have been implicated as causative agents of acute, endemic diarrhea among children. Since there remain diarrheal cases for which a pathogen cannot be detected and it is likely that a proportion of these cases are due to viruses, we have designed a study to determine whether NLVs are important in this age group. Bangladesh (and ICDDR,B) is an ideal site to address this question since there is a high burden of diarrheal disease, there is evidence from a previous study that NLVs infect children and the laboratory has experience in the study of viral diarrhea.

We will first determine the age of acquisition of NLV infection by testing for antibodies among sera that comprise a serum bank. Secondly, we will determine the importance of NLVs as a cause of diarrhea among children. This will be done on 2 sets of subjects: children who comprise a birth cohort and children who present to the Clinical Research and Service Centre (CRSC), ICDDR,B for treatment of diarrhea. This will allow us to determine the proportion of NLV infections associated with diarrhea and asymptomatic infection (cohort children) and the proportion who seeks treatment for diarrhea. Based on the figures from studies conducted elsewhere, we have proposed to study 280 diarrheal patients who present to the CRSC for treatment of diarrhea. We plan to collect enrollment stool and blood from patients who take part in the routine surveillance of the CRSC and subsequently will collect a further blood sample 3 to 4 weeks after enrollment. Since the method for detection of NLVs in stool is laborious and expensive, we will test sequential sera (from 105 cohort children) and paired sera (from CRSC patients) for IgG, IgA and IgM antibodies to recombinant antigens of representative genogroups of NLVs. Patients who seroconvert to any of the strains will have their stool samples tested for the presence of NLV by RT-PCR and Southern hybridization.

The findings of this study will allow us to determine the importance of NLVs as a cause of diarrhea in children, the nature of antibody responses and the clinical signs of the infection.

10. HYPOTHESIS:

Norwalk-like viruses (NLVs) are a significant cause of diarrhea among Bangladeshi infants and children.

11. OBJECTIVES:

- a) To determine the age of acquisition of NLV infection in Bangladesh
- b) To determine the prevalence and genogroups of NLVs causing diarrhea and asymptomatic infection
- c) To examine the seroresponse to NLVs (in relation to illness)
- d) To determine the clinical signs associated with NLV diarrhea

12. BACKGROUND INFORMATION:

Even though oral rehydration solution has made significant inroads towards lowering mortality due to diarrhea in children in developing countries, the morbidity continues to be staggering. It has become imperative that ways of preventing diarrhea be pursued.

Improved domestic and personal hygiene has been stressed, however it can go only so far in poor countries where the massive outlays needed for infrastructural improvements can be ill-afforded. It has also become obvious from the experience of developed countries that viral agents of diarrhea such as rotavirus cannot be contained even with a high standard of hygiene. As a result development of appropriate vaccines against major enteric pathogens has become a priority for both developed and developing countries. Unfortunately, we cannot fully attain the goal of substantial reduction of diarrheal morbidity and mortality unless we know the relative contributions of different etiological agents, such as Norwalk-like viruses (NLVs), to the overall diarrhea burden.

Using techniques such as electron microscopy and immune-electron microscopy (using clinical material from human volunteers), NLVs were found to be a common cause of outbreaks of non-bacterial diarrhea (Greenberg *et al.* 1979) which have been associated with contaminated food and water. The inability to cultivate NLVs had hampered the preparation of reagents for sensitive and practical detection methods. Cloning of the genome of members of the NLV group has allowed the production of recombinant NLV capsid protein using the baculovirus expression system. Furthermore, sequence analysis of different NLV strains has resulted in the preparation of primers for use in RT-PCR and by using such primers, the wide variability in strains has been identified (Ando *et al.* 1995, Vinje and Koopmans, 1996). New studies using recombinant antigen in sensitive immunoassays and primers for use in RT-PCR have resulted in the epidemiological investigation of the role of NLVs as the causative agents of endemic as well as epidemic gastroenteritis (Lew *et al.* 1994a, Ando *et al.* 1995, Vinje and Koopmans, 1996).

Members of the NLV group belong to group B of the Caliciviridae family. Group B caliciviruses are subdivided into 2 genogroups based on the sequence of the RNA polymerase region of the genome. Genogroups 1 and 2 are further subdivided based on genetic and antigenic criteria into G1P1-A (prototype is Norwalk virus), G2P1-B (prototype is Taunton virus), G2P2-A (prototype is Toronto virus) and G2P2-B (prototypes are Snow mountain agent and Hawaii agent) (Noel *et al.*, in press). Outbreaks have been caused by both genogroups and endemic diarrhea has been attributed to Norwalk virus (G1P1-A) (Lew *et al.* 1994a) and Toronto virus (G2P2-A) (Lew *et al.* 1994b) so far. It is possible that NLVs cause endemic diarrhea in developing countries but little is known about the genogroups that may be involved.

In a previous serological survey conducted on specimens from Bangladesh more than 10 years ago using less sensitive tests, it was found that up to 80% of children under 5 years of age had serological evidence of exposure to NLVs (Black *et al.* 1982). The new generation of antibody tests have been found to be 16-40 times more sensitive (Green *et al.* 1993) suggesting that NLV infections in Bangladesh may be more prevalent in younger age groups than estimated by Black *et al.* (1982). Furthermore, since that study examined antibodies to a single genogroup of NLV (Norwalk virus, G1P1-A) it is probably an underestimate of infection with NLVs of all genogroups. A study conducted on antibodies to Norwalk virus (G1P1-A) among children in Japan showed that acquisition of infection occurred among older (school age) children and adults from Japan

and Southeast Asia (Numata *et al.* 1994) but the prevalence of possible infections with other genogroups in Asia is not known.

The role of serum antibodies in NLV infection is not clearly understood. It appears that the presence of antibodies at the time of infection is associated with illness rather than protection. For example, Lew *et al.* (1994a) found that the antibody levels among infants with and without diarrhea did not differ. Studies of adult volunteers have shown that pre-existing serum antibodies do not provide protection (Graham *et al.* 1994) and stool IgA antibody levels at the time of challenge were higher among volunteers who became ill than those who remained well (Okhuysen *et al.* 1995). We will be able to address the question of antibody levels and illness in this investigation.

In this study we will test sera from a serum bank for the presence of antibodies to recombinant proteins from 3 strains of NLV to ascertain the age of acquisition of infection. We will also test sequential sera (from children who comprise a birth cohort) and paired sera from children presenting with diarrhea to the CRSC to determine seroconversion to the different NLVs. Among the seroconverters we will test relevant stool samples for the presence of NLVs, which has not been done before. This will allow us to examine the relative importance of NLVs as causative agents of diarrhea in this setting and also to determine the nature and extent of the antibody response that is mounted.

13. METHODS:

A: Age of acquisition of NLV infection in Bangladesh:

We plan to perform a cross-sectional serosurvey of specific antibodies for NLVs to enable us to determine the age of acquisition of infection since this has been found to vary widely among different countries.

Sample collection: We will collect single serum samples from approximately 200 subjects less than 10 years of age from leftover samples from the Clinical Biochemistry Laboratory of ICDDR,B and will compile information on age and gender of patients from whom samples will be obtained. Forty samples will be collected from the age groups: 0-6, 7-12, 13-24, 25-60 and 61-120 months of age. The sample size is based figures from Black *et al.* (1982) who found that the lowest rate of detection of antibodies to Norwalk virus in any age group was 7%. We have taken this as a conservative estimate and if we test 40 samples from any age group we can expect to detect at least 2 positive samples. Based on figures from a study of seroprevalence among Australian aborigines (Parker *et al.* 1994), at approximately 40% of sera and above are likely to be antibody positive. Including 40 samples in each age group is likely to be ample.

Laboratory tests: The serum samples from the above group will be tested using an ELISA for antibodies to NLV (antigens given below) as described by Monroe *et al.* (1993).

Briefly, microtiter plates will be coated with 1 µg/ml of the listed recombinant antigens and incubated overnight at 4°C. After washing, sera will be added (serial 3-fold dilutions) and incubated at 37°C followed by addition of horseradish peroxidase conjugated anti-human IgA or IgG or IgM and substrate. The titer will be determined using a computer program ('MULTI', DataTree Inc. Watthams, MA, USA).

NLV	genogroup
Norwalk	G1P1-A
Toronto	G2P2-A
Hawaii	G2P2-B

B: Determination of the association of NLVs with diarrhea and asymptomatic infection and the genogroups responsible:

We wish to determine whether NLV infection in the absence of other enteropathogens is associated with diarrhea. In order to do this we plan to detect NLVs among seroconverters. A previous study of antibodies in sequential sera used antibody rise as an indicator of infection and speculated that a diarrheal episode falling within the time period covered by the seroconversion which had no known enteropathogen detected in the stool, was probably due to NLV (Lew et al. 1994a). Detection of viruses in stool specimens of children with seroconversion has not been performed. In this study we will also consider a NLV infection as one in the absence of any co-pathogen.

Two groups of subjects will be used for this purpose: children from a birth cohort and children who present to the CRSC with diarrhea. Among the cohort children from whom sequential sera have been collected, we can detect symptomatic and asymptomatic infections and among children who present to hospital we can determine the prevalence of NLVs in diarrhea that is severe enough to warrant a hospital visit. Generally, case-control studies are conducted to determine the importance of putative new agents of disease, however, since the detection method for NLVs is laborious and expensive it will only be performed on stool samples which are likely to contain NLVs (i.e. those from seroconverters). Therefore we would have to collect paired serum samples from patients without diarrhea who are difficult to recruit. Moreover, it is thought that NLV infections occur frequently in Bangladesh (Black et al. 1982) yet it is not known whether these infections result in diarrhea. If it is found that a significant proportion of diarrheal cases are associated with NLVs then a cases-control study would be justified. We will still be able to determine the frequency with which asymptomatic infections occur from examination of specimens from cohort children.

Sample collection:

1. Cohort children: The samples from the children who comprise the birth cohort (N=235) have already been collected (study entitled 'Epidemiology of diarrhea and ARI in a cohort of newborns in rural Bangladesh', Dr Z. Hasan PI). Children were enrolled at

birth and followed for 2 years; monthly stools, diarrheal stools, and four 6-monthly blood samples (among other samples) were collected and stored at -20°C . If we test samples from all subjects who have a complete set of serum samples ($N=105$) then we would expect 50% to seroconvert ($n=52$) and of those, 16% are likely to be symptomatic infections in the absence of other pathogens ($n=8$) and 44 to be asymptomatic based on figures from Lew et al. (1994a). These comprise a smaller than optimal number for analysis of the role of antibody in protection from disease (see page 12), therefore we will test sera from all of the the 105 children.

In the cohort study, diarrhea was defined as the passage of at least 3 liquid stool in 24 hours. Diarrheal stools were tested for a number of enteropathogens and two weekly diarrhea surveillance (active surveillance) was performed (information on consistency and frequency of bowel movements was recorded, ORS packet were given, information on diarrhea management was given and if the child was found to be 'moderately or severely ill' was referred to the diarrhea clinic of a local hospital).

2. Diarrhea patients from the CRSC: We will determine the most appropriate age range from the 'age of acquisition' section of the study by determining the age at which the proportion of children with antibodies plateau. We plan to collect samples from 280 diarrheal patients. We would like to detect a minimum of 28 NLV cases so that we can combine these cases with the 8 cohort children resulting in 36 symptomatic NLV infections (see 'Seroresponse in relation to illness' section for calculation of sample size). Based on the rate of NLV among children from Finland of approximately 10% we would have to test paired samples from 280 patients. Given that approximately 100,000 patient present to the CRSC, 2000 are part of the surveillance and approximately 40% will be children less than 2 years of age (a total of 800 children). We envisage that it will take one year to enroll the patients required considering at least a 30% drop-out to follow-up.

Children younger than the 'plateau' age group will be the target for this section of the study and enrolled based on the following criteria:

- The guardians consent for the patients to participate
- diarrhea defined as the passage of 3 or more liquid stools in 24 hours
- They are part of the routine 2% surveillance of the CRSC. The surveillance system, which was established at the CRSC in 1979, currently enrolls every 50th patient who presents to the hospital with diarrhea. Routine demographic, clinical and etiological information is obtained and specimens are tested for enteropathogens namely *V. cholerae* O1, *V. cholerae* O139, *Shigella* spp, other vibrios, rotavirus, *Salmonella*, diarrheagenic *E. coli*, *Campylobacter jejuni*, *Amoebae*, *Giardia lamblia* and helminths.

From these patients we will collect a recruitment stool (this routinely comes to the Virology lab for determination of rotavirus) and blood (5ml). We will ask them to come back 3 to 4 weeks later and will collect a further blood sample. Blood samples will be centrifuged and sera will be stored at -20°C until tested and stool samples will also be frozen until tested.

Laboratory tests: Samples from both the Cohort and Surveillance collections will be tested similarly. We will test sequential and paired sera for NLV-specific IgG, IgA and IgM antibody titers in an ELISA and using the antigens described on page 6. A seroconversion will be defined as a rise of ≥ 4 fold of IgG or IgA between consecutive samples or serum pairs. Stool samples from surveillance children and from the relevant time period from Cohort children may be tested for NLV-specific IgA antibodies.

Among seroconverters from both collections, relevant stool samples will be taken to the Viral Gastroenteritis Section, CDC, Atlanta and tested for the presence of NLV using RT-PCR and southern hybridization as described by Ando *et al* (1995) and primers/probes to be used are given in appendix I. Samples proving negative by southern hybridization among seroconverters will have their PCR product sequenced for comparison to sequences of prototype strains. Samples from which a PCR product is not detectable will be examined by immune-EM with the patients positive serum. If NLVs are detected, RNA from such stool samples will be tested by RT-PCR using the primers described by Vinje and Koopmans (1996).

C: Seroresponse in relation to illness:

NLV disease will be defined as an episode of diarrhea that falls within the period bound by the seroresponse and when no other pathogen is detected during the diarrheal episode. As described above, we will test sera from cohort children and from children who present to the CRSC for anti-NLV IgA, IgG and IgM antibodies. We would like to compare the antibody levels prior to/at the time of a diarrheal episode (acute specimen) among children with symptomatic vs. asymptomatic infections. Based on the findings of Lew *et al.* (1994a) when they showed that 48% of children with low antibody levels to NV had subsequent NV infections compared to 15% with higher antibody levels, we have calculated that with 95% confidence and 80% power (using the Epi info version 5 program), to detect a similar difference we will need a sample size of 36 in each group (8 from cohort children and 28 from surveillance children). If this is attained we will examine the possible role of preexisting antibodies and severity of diarrhea.

We will describe the proportion of children who mount a response of each isotype to the different NLV genogroups.

D. Clinical signs of NLV infection:

We will compare the clinical signs and demographic features of the NLV infected and non-infected CRSC patients. The following information is collected as part of the routine surveillance and will be examined:

- demographic features: age, gender, socioeconomic status, family size
- treatment prior to hospitalization: antibiotic therapy, fluid replacement
- environmental factors: source of drinking water, place of defecation
- clinical signs: temperature, duration of diarrhea prior to hospitalization, duration of hospitalization, stool character, abdominal pain, vomiting, respiratory signs, dehydration

14. ANALYSIS PLAN:

We will:

1. determine the age of acquisition of NLV infections by describing the proportion of children with NLV infections defined by the presence of NLV-specific antibodies among Bangladeshi children of different age groups and determine the age at which the proportion with antibodies plateau.
2. describe the prevalence of symptomatic and asymptomatic NLV infection in children less than 5 years of age.
3. compare the titer of the different antibodies in acute samples between symptomatically and asymptotically infected children (using the Mann-Whitney test) to determine whether pre-existing antibodies play a role in protection and compare the proportion of children with antibody responses (IgG vs. IgA vs. IgM) using the chi-squared test.
4. compare the symptoms and demographic features of NLV infected vs. non-infected children with diarrhea using the chi-square and chi-square for trend tests on stratified and categorical variables. This will allow us to describe NLV disease.

15. STATEMENT OF SIGNIFICANCE AND POTENTIAL IMPACT ON POLICY:

In order to formulate strategies for diarrhea prevention and treatment, it is optimal to know the cause of infection. A proportion of cases are caused by organisms either low in numbers or for which detection methods have not been developed. Based on findings from 2 developed countries and a study conducted in Bangladesh more than 10 years ago, it is likely that diarrhea among Bangladeshi infants and children may be caused by NLVs. If NLVs prove to be a common cause of diarrhea, further studies to determine the burden

of infection would be warranted to suggest the necessity of the introduction of interventions such as vaccination. Vaccine development for NLVs has been initiated.

16. ETHICAL CONSIDERATIONS:

In this study, we will compile a serum bank (as given above) which will consist of 'anonymous' samples which are about to be discarded from the Clinical Biochemistry lab of ICDDR,B. We will collect information on gender and age only.

We will also recruit patients who have diarrhea. These patients will be part of the routine surveillance of the Clinical Research and Service Centre (CRSC). A stool sample will be collected as part of the routine surveillance which comes to the Virology lab for rotavirus testing. We will have a study physician who will look for surveillance patients who fall in the appropriate age category (<5 years of age) and will approach them for enrollment into the study. The physician will read the consent form to acquaint the guardians with what is involved in the study. After the guardian has given written consent (or a thumbprint impression), a blood sample (5ml) will be obtained by venepuncture. The blood sample will be sent to the Virology Lab for processing and will be stored at -20°C until the time of testing. Guardians will be asked to return 3-4 weeks later for collection of a further blood samples and will be reimbursed for the return fare at the time.

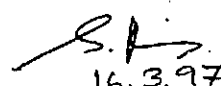
To protect confidentiality, all information collected will be kept in a locked cabinet only accessible to the investigators. Patients will be informed that if they refuse to participate in the study they will continue to receive routine clinical care and that they can withdraw from the study at any time (see consent form).

17. BUDGET (in US\$):

	1st year	2nd year	Total
1. Personnel			
Dr Goutam Podder (NOA, 50%)	6,074	6,474	12,548
Study doctor (100%)	2,040	2,040	4,080
Subtotal	8,114	8,514	16,628
2. Laboratory supplies (reagents, plasticware, etc.)	14,500	13,400	27,900
3. Interdepartmental	7,000	5,200	12,200
4. Print and publication	1,000	2,000	3,000
5. Equipment*	2,200		2,200
6. International travel**	3,500	3,500	7,000
TOTAL (direct cost)	36,314	32,614	68,928

*8- and 12-channel pipettes (one each) and -20°C freezer

**Travel: 1) Melbourne-Dhaka-Melbourne and living costs for 1 month in Dhaka to initiate the study; 2) Dhaka-Atlanta-Dhaka and living costs for 1 month at CDC, Atlanta to perform RT-PCR and Southern hybridization.


16.3.97
Shamima Moln
Controller, Budget & Costing

15. REFERENCES:

Ando T, Mulders MN, Lewis DC, Estes MK, Monroe SS, Glass RI. Comparison of the polymerase region of small round structured virus strains previously classified in three antigenic types by solid-phase immune electron microscopy. Arch Virol 1994;135:217-226.

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Green KY, Lew JF, Jiang X, Kapikian AZ, Estes MK. Comparison of the reactivities of baculovirus-expressed recombinant Norwalk virus capsid antigen with those of the native Norwalk virus antigen in serologic assays and some epidemiologic observations. *J Clin Microbiol* 1993;31:2185-91

Greenberg HB, Valdesuso J, Yolken RH, Gangarosa E, Gray W, Wyatt RG, Konno T, Suzuki H, Chanock RM, Kapikian AZ. Role of Norwalk virus in outbreaks of non bacterial gastroenteritis. *J Infect Dis* 1979;139:564-8

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Vinje J, Koopmans MPG. Molecular detection and epidemiology of Small Round-Structured viruses in outbreaks of gastroenteritis in the Netherlands. *J Infect Dis* 1996;174:610-5

16. Publications of Principal Investigators (from 1993 till data):

Tasnim Azim:

1. Qadri F, Azim T, Hossain A, Islam D, Mondol G, Faruque SM, Albert MJ. A monoclonal antibody to *Shigella dysenteriae* serotype 13 cross-reacting with Shiga toxin. *FEMS Microbiol Lett* 1993; 107:343-348.
2. Qadri F, Haque MA, Hossain A, Azim T, Alam K, Albert MJ. Role of *Shigella dysenteriae* type 1 slime polysaccharide in resistance to serum killing and phagocytosis. *Microbial Pathogen* 1993; 14:441-449.
3. Qadri F, Azim T, Chowdhury A, Hussain Z, Sack RB, Albert MJ. Production, characterisation and diagnostic application of monoclonal antibodies to *Vibrio cholerae* O139 synonym Bengal. *Clin Diagn Lab Immunol* 1994; 1:51-54.
4. Qadri F, Azim T, Hossain A, Chowdhury A, Albert MJ. Monoclonal antibodies specific for *Shigella dysenteriae* serotype 13. *Diagn Microbiol Infect Dis* 1994; 18:145-149.
5. Qadri F, Chowdhury A, Hossain J, Chowdhury K, Azim T, Shimada T, Islam KMN, Sack RB, Albert MJ. Development and evaluation of a rapid monoclonal antibody-based coagglutination test for direct detection of *Vibrio cholerae* synonym Bengal in stool samples. *J Clin Microbiol* 1994; 32:1589-1590.
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7. Islam LN, Ferdous A, Azim T, Qadri F, Rahman ASMH, Islam MS, Tzipori S. Peripheral blood granulocytes and mononuclear cell responses in monkeys with experimental shigellosis. *J Diarrhoeal Dis Res* 1994; 12:97-102.
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10. Azim T, Islam LN, Halder RC, Hamadani J, Khanum N, Sarker MS, Salam MA, Albert MJ. Peripheral blood neutrophil responses in children with shigellosis. *Clin Diagn Lab Immunol* 1995; 2:616-622.
11. Qadri F, Mohi MG, Hossain J, Azim T, Khan AM, Salam MA, Sack RB, Albert MJ, Svennerholm A-M. Vibriocidal antibody response in cholera due to *Vibrio cholerae* O139 Bengal: a comparison with response in cholera due to *Vibrio cholerae* O1. *Clin Diagn Lab Immunol* 1995; 2:685-688.
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International Centre for Diarrhoeal Disease Research, Bangladesh Consent Form

Investigation of the importance of Norwalk-like viruses in childhood diarrhea in Bangladesh

Your child is suffering from watery diarrhea. Sometimes the cause of diarrhea cannot be ascertained and we suspect that the diarrhea could be due to some viruses that are not usually tested for. We are conducting a study to see whether these viruses are causing diarrhea. For this purpose we would like to perform some special tests on your child's stool and blood. If you agree to the participation of your child in this study, we will collect stool and 5 ml (approximately one teaspoonful) blood from the forearm of your child on enrollment and three weeks later. Other than temporary pain due to the needle stick, drawing of this amount of blood will not cause any harm. For the second sample we will request you to bring your child to the hospital.

It is you who will decide whether your child participates in this study, and you may withdraw your consent any time during the study. Your child will receive the good care of this hospital if you do not include your child in this study, and also if you withdraw your consent during the study.

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

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

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

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

Thank you for your co-operation.

Signature of the investigator:

Signature/LTI of
parent/guardian:

Signature of the witness:

Date: _____

Date: _____

Date: _____

APPENDIX I

Description of oligonucleotide primers and probes for the detection of NLVs (from Ando et al. 1995).

Primer or probe set	Identifi- cation	Sequence	Polarity
Primers			
G1,G2 ^a	SR33	tgt cac gat ctc atc atc acc	-
G2 ^b	SR46	tgg aat tcc atc gcc cac tgg	+
G1 ^b	SR48	gtg aac agc ata aat cac tgg	+
G1 ^b	SR50	gtg aac agt ata aac cac tgg	+
G1 ^b	SR52	gtg aac agt ata aac cat tgg	+
Probes			
P2-B ^c	SR47d	atg tca ggg gac agg ttt gt	-
P2-A ^c	SR61d	atg tcg ggg cct agt cct gt	-
P1-A ^c	SR63d	aca tca gga gag tgc cca ct	-
P1-A ^c	SR65d	aca tca ggt gat aag cca gt	-
P1-B ^c	SR67d	aca tct ggt gag aga cct ga	-
P1-A ^c	SR69d	aca tcg ggt gat agg cct gt	-

Nucleotide positions: a-4856-4876; b-4754-4773; c-4804-4823.

EVALUATION OF CHILD HEALTH RESEARCH PROPOSAL

Review 1

Reviewer's name:

Name of proposal: Investigation of the importance of new viral agents of diarrhea.

Name of proposed investigator: Leanne Unicomb

Date of review: June 25, 1997

This proposal addresses the role of Norwalk-like viruses as a cause of diarrhea in Bangladeshi infants and children. The abstract is nicely written, the hypothesis is straightforward and testable, and the objectives are within reason; the title of the proposal is non-descript. The Background section is comprehensive and discusses new technologies for virus identification and classification, and the association of serum antibodies with clinical disease.

Methods. The study is well-designed, first employing a cross-sectional, seroepidemiological survey to obtain age-prevalence data, and then utilizing a case-control and/or cohort design. Serology will be carried out using recombinant antigens in an ELISA format. Stools from seropositive children will be transported to the CDC for molecular epidemiologic analyses. This is an excellent resource and demonstrates good use of collaborating institutions.

This study is significant in that it is a classical epidemiologic investigation that prospectively uses immunological and molecular tools to develop a sound strategy for prevention and treatment of diarrhea. Overall, an excellent proposal.

Proposal #5

Review 2

*Investigation of the importance of new viral agents of diarrhea**Léanne Unicomb and Goutam Podder*

Goals This is a study to identify patterns of antibody acquisition and identification of new strains of caliciviruses as a cause of diarrhea in Bangladesh. The authors will collect a stratified set of sera samples in children up to 10 years of age. The investigators also will analyze stool specimens collected from a cohort of children monitored during the first two years of life or they will collect stool specimens from patients with diarrhea who are younger than 5 years of age. They will collect stool and paired sera from these children.

Design

1. On page 9, the authors indicate that the sera would be tested for IgG, IgA, and IgM antibody titers "in a ELISA as described above". No ELISA was described above. Because the investigators, including the U.S. collaborator at the CDC, have not developed any ELISAs for caliciviruses we are uncertain what ELISAs are being discussed and what will be the availability of reagents for ELISAs that have been published. What arrangements have the authors made to have reagents for the ELISAs available?
2. It is unclear how the authors will interpret the results of the antibody tests in order to choose primers. No information is provided about what primers will be chosen and what the specificity of the primers is. For example, one of the available ELISAs is for viruses within the G2P2-B viruses related to Snow Mountain viruses. Volunteers effected with G1P1-A viruses (Norwalk virus) seroconvert in assays using G2P2-B viruses as antigen. [If this is not true, the evidence should be in the proposal.] Primer specificities tend to be significantly narrower than antibody specificity in these ELISAs.
3. It may be better ^{to} test the age-stratified serum collection before deciding what age range of diarrhea patients admitted to the CRSC will be enrolled. Five year-old children may be too old because a plateau of antibody prevalence may be reached by age 3 or 4. Few infections or few first infections may be occurring in children 4 or 5 years of age. In that case many of the samples in the collection from diarrhea patients at the CRSC would not be fruitful.
4. It is also unclear what criteria would be used to recognize a new strain of calicivirus.
5. It is questionable whether a large enough number of identified infections will be yielded to determine the role of pre-existing antibody protection and even if particular virus strains are identified it would be difficult for the authors to make conclusions about the role of the detected antibody in protection against infection by a particular strain. This study has a high risk of a negative result.
6. The proposal has no sample size calculations.

#5 cont'd

Appropriateness It is surprising that the stool samples will be sent to the CDC for investigation rather than transferring the technology for that investigation to the laboratory in Bangladesh.

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

Ethics Minimal risk.

Background Little apparent awareness of publications in the field. —

Other None.

Proposal 5: "Investigation of the importance of new viral agents of diarrhea"

Review 3

Principle Investigator: L. Unicomb

1. Goals. Determine if Norwalk virus (NV) and Norwalk-like viruses (NLV) are a significant cause of diarrhea in Bangladesh.

2. Design.

This is a natural history study of Norwalk and related viruses of the calicivirus group which, except for outbreaks that have occurred worldwide, have gone largely undiagnosed because of difficulties in culture and detection. This proposal aims to gain a better understanding of disease caused by these viruses using improved serologic and RT-PCR detection methods. The investigators will determine the age of acquisition of NLV in a cross-sectional serologic survey of 200 children < 10 years of age. They will determine the prevalence of NLV and their various genotypes by RT-PCR/Southern hybridization (to be done by R. Glass at the CDC) in children < 5 years of age who are seroconverters and are negative for other diarrheal pathogens. They plan to look at the isotypic serologic responses in relation to illness using an ELISA with recombinant NV antigens on a baculovirus expression vector. Previous studies suggest that an antibody rise is a reasonable indicator of NV/NLV infection but does not seem to be related to clinical outcome. Only one previous study has looked at the role of mucosal (stool) antibody in protection (Okhuysen, 1995). No mucosal antibodies are proposed currently. Presumably this can be done on stored stool sample or in a new study if results of the proposed work do not point to a clear relationship between serum antibody and protection. One would expect to see some similarities with the rotavirus story.

2.1 Definitions of key concepts and variables. Adequate.

2.2 Study populations, sample size, and sampling strategy. Sample sizes are estimated from prevalence data in previously published studies. It is not entirely clear which aspects of the study are to be done using archived serum and stool samples (p. 6 "Sample collection"; p. 7 "Sample collection: 1: Cohort children") or using prospectively collected specimens (p. 8).

2.3 Clarity of analysis plans. See above.

2.4 Feasibility of proposed methods. Yes.

2.5 Adequacy of laboratory methods. Methods appear to be adequate and well established at the ICDDR and CDC laboratories. However, of several references with overlapping authorship for the ELISA, no one paper was specifically cited for use on p. 9.

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

3. Appropriateness.

3.1 Potential for improving child health care. Clinical definition of NLV disease would be helpful. If NV and NLV are found to constitute a substantial proportion of severe diarrheal cases, and if a limited number of viral phenotypes are found to be involved, and if the presence of antibodies were found to be associated with protection (so far not the case), then it would be reasonable to consider developing a vaccine.

1-2-77
R3-D, I

3.2 Scientific significance. Natural history and definition of NV and NLV disease.

4. Timing (and budget). Timing appears adequate and appropriate.

5. Ethics. No ethical objections.

6. Background. The background section shows depth of understanding of epidemiologic and immunologic issues important to the studies, some of which have been major contributions by the investigators..

7. Other. This appears to be a fairly straightforward and worthwhile proposal in an area with little current clinical or scientific knowledge.

Proposal #5

Review 4

Title: Investigation of the importance of new viral agents of diarrhea

P.I.: L. Unicomb et. al.

Goals: The goal of the study is to assess the importance of Norwalk like viruses as causes of diarrheal disease in Bangladesh

Design: The study will review serological data from children in a previously followed birth cohort and from 200 children with diarrhea enrolled prospectively. Only stool samples from seroconverters will be tested for NLVs by PCR. Clinical features of NLV disease will be assessed. Sample size calculations and sampling strategy appear appropriate.

My main comment on study design is that even though diarrhea cases will be evaluated by stool culture and rotaviral assay, there is no mention of excluding cases with a documented co-pathogen when NLV+ cases are selected. Parasitologic evaluation is not mentioned, and children with diarrhea-associated parasites such as Giardia should also be excluded. Selection of cases with NLV as the only identified pathogen will result in cleaner and more reliable data. As in other proposals, a case definition for diarrhea is not given and should be supplied.

Appropriateness: The investigators put forth data suggesting that NLV may be an important cause of diarrhea in Bangladesh, and the study will provide additional data to support or disprove that hypothesis. The discussion of disease control and justification of the study in support of future vaccine development is a bit premature, but the study is important in that further information about the etiology of diarrhea with no identifiable cause may be obtained. As rapid diagnostic tests for NLV are currently not available, the study will have limited impact on current clinical evaluation or management.

Timing: Timetable is not clearly described, but the 2 year time frame appears appropriate.

Ethical considerations: No concerns

Background: Appropriate

EVALUATION OF CHILD HEALTH RESEARCH PROPOSAL

Review 5

#5
R1-M

Name of proposal: Investigation of the importance of new viral agents of diarrhea

Name of proposed investigator: Unicombe et al.

Date of review: July 4, 1997

Review:

Page 6 of this proposal is missing, which makes assessment of the methodology for the second objective difficult to evaluate.

Objective 1: sample size calculations are not presented: are 2 positive samples per age group really sufficient to determine the age of acquisition of infection? Are leftover samples representative of the population? Are these samples just a melting pot of leftovers from different studies? Please clarify.

Objective 2: Page 6 is missing and therefore, we do not have the full information on how this objective will be addressed. What is the sample size that will be used from the 'cohort children'? How was it calculated?

Sample size calculations for the 200 patients from the surveillance system are also not presented. How will the two different data sets contribute to answering the question asked?

Since the objective here is to assess the prevalence of symptomatic and asymptomatic NLV infection in children less than 5 years of age, it is important to have a representative sample of the population of under fives. This point is not discussed and it is not clear whether the two samples used have sufficient representativity to be used for population prevalence estimates.

The proposed methodology for objective 3 is fine.

Objective 4: Mention is made that the clinical signs and demographic features of the NLV infected patients will be examined. In order for this information to be more useful (at least demographic and socioeconomic information), data from non-infected patients should also be collected to compare between infected and non-infected subjects. Otherwise, the data on the NLV infected patients are purely descriptive and do not mean much.

#5
R3m

Child Health Research Proposals

Review 6

Reviewer's name:

Name of Proposal: Investigation of the importance of new viral agents of diarrhea

Name of proposed investigator: Unicom

Date of review: 6/12/97

For CHR project staff only

Good, appropriate use of preexisting samples
↓ data collection options to address
important health issue.

Rational sampling strategy

Child Health Research Proposals

Name of Proposal: Investigation/new viral agents

Name of proposed investigator: Unicom

Well-considered proposal, addressing an increasingly important global issue, with direct relevance to care.

1) Goals:

Clear & well-stated; well-supported in text

2) Design:

Good sampling plan + clear analysis plan; different populations in study well-supported & justified; ^{partial} use of pre-existing samples a strength in terms of burden on patients & collection costs to investigation.

3) Appropriateness

Relevant to primary health care of children in developing countries;

Name of Proposal: Investigation / new viral agents

4) Timing and Budget

Given the use of pre-existing samples, ~~the~~ the proposed duration appears somewhat excessive; suspect enrollment of 200 diarrhea cases could go more quickly.

5) Ethics

None that are not addressed

6) Background

PI has experience relevant to study activities, although appears to have been removed from active research for a time (based upon publication record provided).

7) Other:

**PROTOCOL: INVESTIGATION OF THE IMPORTANCE OF NEW VIRAL AGENTS
OF DIARRHEA**

RESPONSE TO REVIEWERS COMMENTS:

REVIEWER NO. 1:

The title has been changed.

REVIEWER NO. 2:

DESIGN: POINT 1:

Sera will be tested using an ELISA that was actually 'described above' but on page 6. This confusion has been clarified on page 10. The US investigators have not only developed an ELISA for antibodies to one of the Caliciviruses but have also published this work (Monroe et al. J. Clin Microbiol 31: 2866-2872, 1993). We will be obtaining the recombinant reagents for the antibody ELISAs from CDC.

POINT 2:

We have now modified the method for determination of primers etc. based on antibody responses. This is given on page 10. Primers and their specificity have been given in appendix I.

POINT 3:

This is a very good suggestion which has been incorporated (see page 8, first paragraph).

POINT 4:

In all cases, serology will be performed before tests for the presence of viruses are initiated. Therefore it is assumed that a 'new calicivirus strain' is one from a patient that seroconverts but stool samples do not give a positive reaction with the primers and/or probes that we have described. The strategy to be taken in such a situation has been incorporated on page 10.

POINT 5:

We will be analysing samples from 'cohort' children and 'surveillance' children. Based on a study by Lew et al. (1994a), 49% of children tested for antibodies to NV only were found to seroconvert and 16% of those had symptomatic infections in the absence of infection with other enteropathogens. As we will be testing sequential sera from 105

children, approximately 50 potential seroconverters and 8 symptomatic infections will be detected. We have also estimated that we will detect 28 NLV infections among children from the hospital who present with diarrhea. Based on findings from Lew et al. (1994a), 48% of children with low antibody levels went on to have a further NV infection compared to 15% with higher antibody levels suggesting that a sample size of 36 would be required. Using this as a proxy of symptomatic vs. asymptomatic infections, it is possible that these numbers will be obtained (at least 36 symptomatic and 42 asymptomatics from cohort) but analysis of the protective role of antibodies will only be performed if sample size is met (in the protocol on page 11).

It is also possible that NLVs may not be associated with diarrhea in Bangladeshi children, a point which the reviewer made. I am assuming that this is not a criticism as such a finding will be important.

POINT 6:

Sample size justifications and calculations have been given thus: age of acquisition on page 6; number of cohort children on page 8; number of surveillance children on page 9; seroresponse and illness on page 11.

APPROPRIATENESS:

If NLVs prove to be important pathogens in this setting, total transfer of the techniques involved will be justified. We have experience with RT-PCR and will be able to obtain probes once we determine those that are appropriate for Bangladeshi strains. It is likely that sequencing will be required, a technique that is not currently available in our department. If large numbers of NLVs are found to infect the Bangladesh population, this would be justification to set up this technique at ICDDR,B.

BACKGROUND:

We have given the minimum references required to illustrate the points made in the Background. The referee has not drawn our attention to relevant recent publications that we have missed.

REVIEWER NO. 3:

DESIGN:

Firstly, the work to be done at CDC will be performed by the co-P.I., Dr Goutam Podder (a scientist at ICDDR,B). Secondly, as suggested by the reviewer, mucosal antibodies can be investigated using the stored samples and this point has been incorporated (page 10).

STUDY POPULATION ETC.

A clarification of which aspects of the study will be done on archived vs. freshly collected samples is given on page 10.

POTENTIAL FOR IMPROVING CHILD HEALTH:

I agree with the reviewer the outcome of this study is not likely to result in the development of a vaccine but may, in fact, prompt further studies to determine whether vaccine development is warranted.

The definition of NLV disease has been incorporated (page 10).

REVIEWER NO. 4:

DESIGN:

The reviewer suggests that only cases of NLV in which no other enteropathogen is found should be studied which is a very good suggestion and has been incorporated (see page 10). Among the patients enrolled from the hospital, the following parasites will be tested: *Amoebae*, *Giardia lamblia* and helminths. This information has been incorporated (see page 9). Diarrhea has now been defined (incorporated, pages 8 and 9).

APPROPRIATENESS:

At present, rapid diagnostic tests for NLV are not available. If we find significant numbers of NLVs infecting children in Bangladesh, evaluation of diagnostics would be a logical role for our group.

REVIEWER NO. 5:

OBJECTIVE NO. 1:

We have developed a conservative figure for the minimum no. of positives expected in each age group based on very old antibody detection methods therefore it is unlikely that only 2 will be found in each group. Since this is a descriptive study, we have calculated a 'justifiable number' for testing (page 6). The leftover sera are not from different studies conducted within the hospital but will be those from patients who present for diagnostic tests from outside our hospital. They are likely to represent a better socio-economic status from the general population.

OBJECTIVE NO. 2:

See the response to points 5 and 6 for reviewer no. 2.

The difference between the cohort and surveillance children is that surveillance children are representatives of an urban population and cohort children are representatives of a rural setting.

OBJECTIVE NO. 4:

We will compare non-infected and infected patients for the demographic characteristics etc., as suggested by the reviewer (incorporated, page 11).

REVIEWER NO. 6:

TIMING:

Since we will be collecting paired samples from surveillance patients, we will rely on them to return to the hospital for the second collection. Experience from other studies has shown a dropout rate of at least 30% in studies where follow-up requiring blood collection have been conducted. The time frame we have given is therefore reasonable.

BACKGROUND:

I think there is some confusion. The PI has had one or more publications each year since the cutoff of 1993.