Attachment 1. Date May 03, 1988

ETHICAL REVIEW COMMITTEE, ICDDR,B.

96

Principal Investigator Dr. Nazrul Islam Trainee Investigator (if any) _____

Application No. & Dr. Mahbubur Rahman PCC/2/88 Supporting Agency (if Non-ICDDR,B) _____

Title of Study An evaluation of Coagglutination, Reverse Passive Haemagglutination and Enzymelinked Immunosorbent Assay(ELISA) for diagnosis of rotavirus diarrhoea & their correlation with clinical illness.

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 NA
   (d) Sensitive questions Yes No NA
   (e) Benefits to be derive (Yes)
   (f) Right to refuse to participate or to withdraw from study (Yes)
   (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.

(PTO)

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

Principal Investigator

Trainee

SECTION I - RESEARCH PROTOCOL

1. TITLE : AN EVALUATION OF COAGGLUTINATION, REVERSED PASSIVE HAEMAGGLUTINATION AND ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR DIAGNOSIS OF ROTAVIRUS DIARRHOEA AND THEIR CORRELATION WITH CLINICAL ILLNESS.

2. PRINCIPAL INVESTIGATORS : DR. NAZRUL ISLAM
Associate Professor, Department of Virology
IPGM&R

DR. MAHBUBUR RAHMAN
ICDDR,B

CO-INVESTIGATORS: ICDDR,B: DR. A.N. ALAM
DR. ANOWAR HOSSAIN

IPGM&R: DR. M. HAQUE

3. CONSULTANT : DR. IVAN CIZNAR

4. STARTING DATE : Shortly after receipt of the fund

5. COMPLETION DATE : One and a half year from the starting date of the protocol.

6. TOTAL DIRECT COST: US$ 27,254

7. SCIENTIFIC PROGRAM HEAD :

This protocol has been approved by the Laboratory Science Division Head Ivan Ciznar

Date: April 20 1988

8. ABSTRACT

Rotavirus is a major cause of acute diarrhoea in infant and young children with a marked morbidity and mortality in Bangladesh. At present no simple laboratory method is available in Bangladesh to diagnose rotavirus diarhoea and differentiate it from other diarrhoea for proper management. To establish a simple, low cost and rapid diagnostic technique for rotavirus; coagglutination (COAG), reversed passive haemagglutination (RPHA)

will be applied to detect rotavirus antigen in stool of 250 rotavirus positive patients and 250 non-diarrhoeal controls. The same samples will be tested by enzyme-linked immunosorbent assay (ELISA) and the results will be compared with those of COAG and RPHA tests. All the reagents for COAG and RPHA tests will be prepared at IPGM&R or ICDDR,B. In ~~24~~ rotavirus positive patients the tests will be repeated on 3rd, 6th, 9th, 14th and 20th day after attending the hospital to determine the efficacy of the tests at the different stages of illness and faecal rotavirus excretion period. Demographic and clinical information of all rotavirus positive patients will be recorded.

9. REVIEW

(a) Scientific Review Sub-Committee(SRC)

(b) Ethical Review Committee

Approved
A Chowdhury
2/5/88
Director,
Institute of Postgraduate Medicine
Research, Dhaka.

SECTION II - RESEARCH PLAN

A. INTRODUCTION

1 Objective

To set up coagglutination (COHG) and reversed passive haemagglutination (RPHA) tests for detecting rotavirus antigen in stool and rectal swab of diarrhoeal patients and compare with ELISA results. This study will also help to define clinical illness, risk factors of rotavirus infection and to determine the efficacy of the tests during the course of illness.

2. Background

Diarrhoeal diseases occupy the leading position amongst the causes of mortality and morbidity in Bangladesh and other developing countries. Walsh and Waren [1] found diarrhoea to be responsible for the highest number of mortality and morbidity followed by respiratory tract infection in worldwide estimate. They estimated 3-5 billion cases of diarrhoeal episodes with an annual mortality figure of 5-10 million.

Amongst the non-bacterial causes of diarrhoea, rotavirus is the most important aetiologic agent of gastroenteritis in the infants and young children in both the developed and the developing countries [2]. Bishop et al [3], in the year 1973, first reported the presence of a virus in the duodenal mucosa in a case of gastroenteritis. Since then its association with gastroenteritis has been reported from different parts of the world. On a global estimate rotavirus accounted for 500 million of diarrhoea cases annually (10% of all diarrhoea cases) [4]. It was

also estimated to be the second most important killer agent in the world after malaria parasite.[4]

It is now known, rotavirus enteritis is generally a disese of infants and young children in the age limits of 6 to 24 months with a peak incidence betwen 9-12 months though rotavirus enteritis occurs in adult.[2] In a number of hospital based studies rotavirus has been detected from the faecal samples of approximately 20-50% of diarrhoeal patients.[4]

Rotavirus is a major cause of acute diarrhoea in infant and young children in Bangladesh with a marked morbidity and mortality.[5,6,6a] It has been isolated from 19-24% of diarrhoeal cases seeking treatment to ICDDR,B Dhaka hospital and from 4% of patients in the community in a rural area.[5,6,6a] Information about rotavirus diarrhoea in Bangladesh is insufficient primarily due to the lack of facilities for the aetiological diagnosis. The existing diagnostic techniques for rotavirus identification are expensive and not readily available in this part of the world. Moreover, the well-equipped laboratory and skilled personnels are prerequisite for performing the tests.

So far a number of tests have been employed for detection of rotaviral antigen in stool for diagnosis of rotavirus diarrhoea as large numbe
detected i
differential ce
counterimmunoel
latex agglutin

enzyme immuno assay[14]. The limitation of EM is well known for its cost demand of skill and special facilities [illegible] its regular [illegible] complicated procedure [illegible] sensitive test but it is [illegible] inexpensive for the high cost of buying the [illegible] visible radiation source and other [illegible] labelled expensive immuno-chemical reagents [illegible] the current test of choice for the diagnosis of [illegible] and it is not readily available here for its need expensive immuno chemical reagents, skilled personnel and costly [illegible] equipment. The latex agglutination test is relatively simple, inexpensive and of good specificity but suffers from usually low sensitivity in comparison with that of ELISA. SPPA showed good sensitivity and specificity in comparison with ELISA in the study[13]. Coagglutination test using Staphylococcus aureus Cowan 1, coated with specific antibody has been used to detect rotavirus antigen in stool[16]. The study was performed in a relatively small number of fecal samples and the results were either equally or slightly less sensitive than ELISA but very specific.

In the perspective given above, the two last-mentioned inexpensive tests namely the reversed passive haemagglutination (RPHA) and coagglutination (COAG) stands the best chance to select as methods of choice for the diagnosis of rotvirus diarrhoea in Bangladesh as well as other developing countries. In present study we aim to set up and evaluate these tests

using locally prepared reagents and standardise them specifically in reference to ELISA using faecal samples. Large number of samples spreading over one year will be tested to compare the effectiveness of coagglutination test and RPHA with ELISA.

3. Rationale

The present study will be able to establish simple and inexpensive RPHA and COAG tests for the diagnosis of rotavirus diarrhoea which is the commonest cause of diarrhoea in children in Bangladesh. A Prompt diagnosis of the disease using these tests will assist to render correct treatment of children suffering from the disease and avoid unnecessary use of antibiotics with their associated hazards. This appropriate technology may be transferred to other developing countries.

The work will be done in confirmity with the findings reported in the Third Meeting of the Scientific Working Group of WHO on viral diarrhoeas. (1-2 February; 1984. Geneva).

B. SPECIFIC AIMS

1. To set up and identify a simple, inexpensive and quick test for diagnosis of rotavirus diarrhoea.
2. To correlate laboratory results with clinical illness.
3. To establish a collaborative linkage between the Department of Virology, IPGM&R and ICDDR,B through Bangladesh Medical Research Council.

C. METHODS OF PROCEDURE

1. Study Period

The study period will extend for a period of one and a half year from the starting date of the protocol.

2. Study Population

Group A will include diarrhoeal patients from ICDDR,B hospital. Every 50th patient from surveillance study (2-3/day) of ICDDR,B hospital will be selected for the study. Demographic informations, medical history and physical examination findings will be recorded. A stool or two rectal swabs will be collected from all patients.

Group B will comprise selected diarrhoeal patients from IPGM&R. Every day 1 to 2 outpatients or inpatients (from paediatric and Neonatal units) will be enrolled in the study. A physician will record the demographic and medical history and physical examination findings. A stool or two rectal swabs will be collected from all patients.

Group C will include 24 rotavirus positive patients; 12 patients of 2 yrs, 6 patients of 3-12 yrs and another 6 patients of >12 yrs age. They will be selected from Group A and B and stool or rectal swab will be collected on 3rd, 6th, 9th, 14th and 20th day after attending the hospital. Home visits will be made to collect the samples.

Group D will comprise non-diarrhoeal controls. They will be enrolled in the study from IPGM&R outpatients or inpatients matching the age and sex with the cases. A control will not suffer from diarrhoea in preceding 60 days and will be selected

on the same or next day of selection of a case. A stool or rectal swab will be collected from each patient.

3. Sample Collection

Stool samples will be collected in a clean container. Two rectal swabs will be collected in a single phosphate buffered saline container. The samples will be transported quickly to the laboratory. One swab will be inoculated for bacterial isolation and other with PBS stored at -20 C untill tested for antigen detection.

4. Microbiological Studies

Fecal samples of diarrhoeal patients will be cultured for Salmonella, Shigella, Vibrios, etc. and identified by standard methods.

5. Preparation of fecal suspensions

Approximately 1g of stool will be suspended in 10 ml of PBS, homogenised over a vortex and incubated for 2-3h. It will be centrifuged at 5000 rev/min for 10 mins. The supernatant will be taken off in another conical centrifuge tube and the process will be repeated again. The supernatant will be used for the detection of viral antigen using each of 3 tests. Rectal swabs (two only) will be collected in 1 ml of PBS and the supernatant after centrifugation will be tested.

6. Reagents (preparation) and test procedures

a) Detection of antigen by ELISA

ELISA (Dakopath, Denmark) diagnostic kit will be used.

The tests will be carried out as described in the company literature

b) Reversed Passive Haemagglutination (RPHA)

RPHA will be performed according to the method of Nakagomi et al[13].

b.1) Preparation of anti-Wa (Wa-strain of human rotavirus) IgG

The Wa strain of human rotavirus will be grown in rhesus monkey kidney cell line, MA 104 cells. When approximatley 90% of the cells will exhibit a cytopathogenic effect, the rotavirus will be obtained from 25 ml of the cell culture by sonic disruption of the infected cells. The cell debris will be removed by centrifugation (800Xg for 10 min). The process will be repeated twice to remove all debris. Finally, rotvirus will be collected in a pellet by centrifugation at 240000 X g for 30 minutes at 4 °C. The pellet will be suspended in phosphate buffered saline (PBS) and stored at -70 °C until used. For partial purification, the virus suspension will be added to equal volume of fluorocarbon and mixed vigorously. The mixture will be centrifuged at 10000 x g for 5 minutes and the virus will be collected in pellet. The pellet will be suspected in 8 ml of PBS and be used to immunize rabbits. An amount of 0.25 ml of partially purified virus suspension mixed with an equal part of complete Freund adjuvant will be injected intramuscularly at various sites. The second injection, the booster, will be given two weeks after the first injection. Serum titre will be checked weekly until bleeding. Immunoglobulin IgG fraction will be separated from hyperimmune serum by gel filtration [illegible] Sephadex G-200 (Pharmacia Fine Chemicals).

Commercial rotavirus antibody (Welcome) will also be used to prepare reagents.

b.2) Preparation of anti-rotavirus IgG sensitised Sheep red blood cell (SRBC)

Method of Nakagomi[13] will be used to sensitize the red blood cell. A solution of anti-Wa IgG (2mg/1ml in saline) will be prepared and 0.1 ml will be added to 0.55 ml of piperazine buffer (0.27M, pH.6.5). The antibody solution will be added to 0.1 ml of packed sheep red blood cells mixed well. Freshly prepared 0.15 ml of chromic chloride solution (0.85 mg/ml saline) will be added to above suspension and the mixture will be incubated for 5 minutes at 30 °C. The sensitized cells will be washed three times with saline. Finally the Pellet cells will be suspended in 20ml of PBS (00.067M, pH 7.2) containing heat inactivated fetal calf serum (3% v/v)

b.3) Test Proper

RPHA will be carried out by microtitre method using V-bottom microtitre plates

Serial two fold dilution of specimen will be made in duplicate using 25 ul volume diluent consisting of PBS containing 2% normal rabbit serum

In one dilution series 25 ul of PBS will be added to each well and in the other the same amount of anti-Wa serum (RPHA inhibition titre, 1:2000) will be added to each well. The mixture will be incubated for 20 minutes at 37 °C and then 25 ul

suspension of erythroicytes coated with anti-Wa IgG will be added to each well. After gentle shaking the trays will be covered, kept at room temperature or at 37°C and the pattern of agglutination will be observed after one hour.

c) Coagglutination test

The test will be carried out according to the method of Kronvall.[18]

c.1) Preparation of Coagglutination reagent

Staphylococcus aureus Cowan 1 (ATCC 12598) will be grown in Trypticase soya broth for 18 hours at 35°C on a shaker, harvested and washed three times with phosphate buffered saline (PBS). The bacteria will be suspended in 0.5% formaldehyde in PBS for three hour at room temperature. The cells are then washed three times in PBS and adjusted to a final 10% suspension in PBS(v/v). The suspension will be heated for 1 hour at 80°C and cooled quickly. For antibody-coating rotavirus antiserum (0.1 ml) is added to 1 ml of S. aureus suspension (10%), and the mixture is left at room temperature for one hour on a shaker. The suspension will be washed once with PBS, brought to 10 ml with PBS containing 0.1% sodium azide and stored at 4°C until used. A 2% (Vol/Vol)formaldehyde-stabilized, heat-treated S. aureus cell coated with normal rabbit serum and suspended in PBS with 0.1% sodium azide will be used as negative control.

c.2) Test Procedure : One drop (approximately 50 ul) of specimen supernatant will be added to each of two rectangles drawn on a glass slide and 1 drop of rotavirus antiboidy coated S. aureus

is added one rectangle and one drop of negative control reagent will be added to other rectangle, mixed thoroughly with the applicator stick, rotated manually (15 to 20 times/min) and agglutination will be noted after 2 minutes. The coagglutination test will be considered positive if agglutination as seen with coagglutination reagent but not with negative control reagent.

D. SIGNIFICANCE

The study will help to establish a relatively inexpensive, simple and rapid diagnostic test for rotavirus diarrhoea. It will also help to set up a routine diagnostic test for rotavirus diarrhoea in our labs and initiate further studies related to rotavirus.

E. FACILITIES REQUIRED

The study will be carried out at ICDDR,B and the Viroloby department of IPGM&R. Basic laboratory facilities and the instruments for carrying out various tests are available at the department. Specific reagents, chemicals as well as ELISA kits will have to be procured for the study.

F. COLLABORATIVE ARRANGEMENT

The protocol will be carried out under a collaborative arrangement between BMRC, Department of Virology, IPGM&R and ICDDR B.

REFERENCES

1. Welsh JA and Warren KS. Selective primary health care: an interim strategy for disease control in developing countries. N. Engl. J. Med., 1979;301:967-74.

2. WHO Scientific Working Group. Rotavirus and other viral diarrhoeas. Bull. WHO, 1980;58(2):183-198.

3. Bisho RF. Davidson GP., Holmes IH., and Ruck BJ. Viral particles in epithelial cells ofduodenal mucosa from children with acute non bacterial gastroenteritis. Lancet;1973;2:1281-31.

4. Butler TC. Viral Diarrhoeas. J. Diar. Dis. Res. 1984 2(3); 137-141.

5. Stoll BJ., Glass MI., Khan MU et al. Surveillance of patients attending diarrhoeal disease hospital in Bangladesh. British Med. Journal. Oct. 23, 1982;285:1185-1188.

6. Black RE., Marson MH., Rahman ASMM., et al. A two year study of bacterial, viral and parasitic agents associated with diarrhoea in rural Bangladesh. J Infect. Dis., 1980;142:660-4.

6a. Black RE, Merson MH. Huq I, et al. Incidence and severity of rotavirus and Escherichia coli diarrhoea in rural Bangladesh: implications for vaccine development. Lancet 1981; 1:141-143

7. Bishop RF, Davidson GP, Holmes IHl and Ruck BJ. Detection of new virus by electrom microscopy of fecal extracts from children with acutre gastroenteritis. Lancet, 1974;1:49.

8. Flewett TH,Brydan AS and Davies H. Diagnostic electron microscopy of faeces. 1 The viral flora of faeces as seen by electron microscopy. J. Clin. Pathol. 1974;27:603-608.

9. Spence L, Faurrel M; Bouchard S, Babiuk L and Saunders JR. Test for reovirus like agent (letter) Lancet, 1975;2:322.

10. Spence L, Fauvel M, Retro R, Blocks. Comparioson of counterimmunoelectrophoresis and electron microscopy for laboratory diagnosis of human revirus like agent associated gastroenteritis. J. Clin. Microbiol. 1977;5:248-49.

11. Kalika AR, Purcell RH, Sereno MT, Wyatt RJ, Kim HU et. al. A microtitre solid phase radio immunoassay for detection of human reovirus like agent in stools. J. Immunol;118:1275-1279.

12. Senekata J, Yoshida Y, Oda K. Detection of rotavirus in faeces by latex agglutination. J. Immunol. Meth. 1981; 41:377-87.

13. Nakagomi O, Nakagom A, Suto T et al. Detection of human rotavirus by reversed passive haemagglutination (RPHA) using antibody against a cultivable human rotavirus as compared with electron microscopy (EM) and enzyme-linked immunosorbent assay (ELISA). Microbial Immunl1982;26:747-751.

14. Yolken RH, Kim HW, Clam T, Wyatt RG, Kalikan AR, Chanok RM. Enzyme-linked immunosorbent assay for the detection of human reovirus like agent of infantile gastroenteritis. Lancet 1977;2:263-66.

15. Doern GU, Herrmann JE, Henderson P, et al. Detection of rotavirus with a new polyclonal antibody enzyme Immunoassay (Rotazyme II) and commercial latex agglutination test (Rotalex): comparison with monoclonal antibody enzyme immuno assay. J. Clin. Microbiol 1986 23(2):226-9.

16. Skaug K, Figenschau KJ, Orstavik I. A rotavirus staphylococcal co-agglutination test. Scta Pathol Microbiol Immunol Scand (B) 1983 Jun;91(3):175-178.

17. World Health Organisation. Diarrhoeal Disease Control Programme. Report of 3rd meeting of scientific working group on viral diarrhoea: microbiology, epidemiology and vaccine development. Geneva: 1-2 Feb. 1984.

18. Kronvall G. A rapid slide agglutination test for typing pneumococci by means of specific antibody absorbed to protein A- containing staphylococcus. J. Med. Microbiol. 1973;6:187-190.

SECTION III - ESTIMATED BUDGET

1. Personnel Services:

Name	Position	Time Effort	Monthly Amount (US $)	Months (X yr)	Total (US $)
IPGM&R:					
Dr. Nazrul Islam	P.I.	20%	-	-	-
Dr. M. Huq	Coinvestigator	20%	50	12X1.5	900
To be named	Res. Physician	100%	2X150	12X1.5	5400
To be named	Res Asst (Lab)	100%	120	12X1.5	2160
To be named	Lab. Attnd	50%	33	12X1.5	594
B. ICDDR,B					
Dr. Mahbubur Rahman	P.I.	25%	-	-	-
Dr. A.N. Alam	Co-investigator	5%	-	-	-
Dr. Anwar Hossain	-do-	10%	-	-	-
To be named GS5	Res Asstt.	100%	220	12X1.5	3960
To be named GS1	Lab. Attnd	100%	105	12X1.5	1890
To be named	Secretary	-	-		150

B. SUPPLIES AND MATERIALS

a.	Glasswares etc.	1500
b.	Chemicals and media, diagnostic kits	6000
c.	Printing and Publication	700
d.	Animal Resources	400
e.	Equipments	1500
f.	Travel and Transportation	1500
g.	Transportation of materials	150
h.	Medical Illustration	200
i.	Xerox	250
	Total US $	27,254

CONSENT FORM

Institute of Postgraduate Medicine & Research and International Centre for Diarrhoeal Disease Research, Bangladesh is carrying out a study to develop a rapid, simple and inexpensive test for the diagnosis of rotavirus diarrhoea. We would like you/your child to participate in the study. The infoirmation gathered from the study will not hamper your/your child's private life and the data will be quite valuable for the development of the newer and inexpensive test. If you agree and let yourself/your child to participate in the study, you will be expected to participate in the following :

1. provide rectal swab/stool samples for the study,
2. respond to the questionnaire on socio-economic and clinical aspect, all of which will be kept confidential.

If you agree to participate in the study, please sign your name here.

_____	_____
Signature of the Investigator	Signature of the Subject
Date:_____	Date: _____