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

LSD
2001

Memorandum

4 February 2002

To : Dr. Rubhana Raqib
Laboratory Sciences Division

From : Professor Mahmudur Rahman
Chairman, Ethical Review Committee (ERC)

Sub : **Approval of protocol # 2001-026**

Thank you for your memo of 20 January 2002 attaching the modified version of your protocol # 2001-026 entitled "Innate and adaptive immune responses in *Shigella* infection" incorporating the observations of the Committee made in its meeting held on held on 9th January 2002. The modified version of the protocol is hereby approved.

You shall conduct the study according to the ERC-approved protocol; and shall be responsible for protecting the rights and welfare of the subjects and compliance with the applicable provisions of the ERC Guidelines. You shall also submit report(s) as required under the ERC Guidelines. Relevant excerpt of the ERC Guidelines is attached for your information and guidance.

I wish you all the success in running the above mentioned study.

Copy: Associate Director
Laboratory Sciences Division

Comments on the modified protocol # 2001-026 titled " Innate and adaptive immune response in shigella infection"

The PI has made necessary modifications as per suggestion of ERC. However, PI is advised to incorporate the routine tests (e.g. bleeding time and clotting time, etc.) to exclude any bleeding disorders of the subjects before taking the biopsies.

Therefore, the protocol may be recommended for ethical clearance.

The necessary modifications have been incorporated in the revised protocol. Therefore, the protocol may be considered for ethical clearance.

29/1/02

127	PHSD	2000-026	Community-based case-management using dipstick for malaria and anti-malaria drug resistance monitoring in Bangladesh	Dr. Yukiko Wagatsuma	17-09-2000 18-10-000	916,765 USAID	1-10-2000	30-09-2003	Awaiting funds
128	LSD	2000-027 209461	Phase I/II safety and immunogenicity studies of Peru 15, a live attenuated oral vaccine candidate for *Vibrio cholerae* 01 in Bangladeshi volunteers both adults and children.	Dr. F. Qadri	17-9-2000 01-11-2000	486,783	01-01-2001	31-12-2002	Ongoing
129	LSD	2000-028 208921	Population-based evaluation of shigella infections in urban area of Dhaka, Bangladesh	Dr. Anowar Hossain *Addendum*	23-10-2000 15-11-2000 19-04-2001 03-05-2001	940,246 IVI, DOMI, WHO	01-07-2001	30-06-2003	Ongoing
130	LSD	2000-029	Study on Molecular Epidemiology of Tuberculosis and Molecular Mechanism of Drug Resistance of Mycobacterium tuberculosis in Bangladesh.	Dr. Sayera Banu	23-10-2000 16-11-2000	93,854	1-12-2000	30-11-2002	Awaiting funds
131	CSD	2000-030	Controlled trial of typhoid Vi vaccine in Dhaka urban slum residents 2-60 years old: a demonstration project	Dr. W. Abdullah Brooks	23-10-2000 11-01-2001	2,029,,354 DOMI	3 years		Awaiting funds
132	PHSD	2000-031 308771	Surveillance of Dengue viral disease in Bangladesh	Dr. A. K. M. Siddique	10-01-2001 28-03-2001	744,444 USAID/W	01-05-2001	30-04-2004	Ongoing
133	CSD/ PHSD	2000-032 101081	An open, randomised clinical trial comparing the efficacy and safety of a single dose of ciprofloxacin with erythromycin administered 6-hours for 3 days in children with cholera	Dr. Wasif Ali Khan Dr. Hafizur Rahman Chowdhury *Addendum*	22-10-2000 29-11-2000 10-01-2001	223,084 NEMC, USA	15-05-2001	14-05-2003	Ongoing
134	PHSD	2000-033	Adolescents' Reproductive Health in Rural Bangladesh: Socio-cultural and Gender aspects.	Ms. Lulfa Begum	20-11-2000 10-12-2000	152,250 <NLG>	1-01-2001	1-01-2002	Ongoing
135	PHSD	2000-034 7-838-6	Adolescents' Reproductive Health in Rural Bangladesh: The impact of experiences in childhood.	Ms. Alinda Bosch	20-11-2000 10-12-2000	No cost is required from ICDDR,B	1-01-2001	31-12-2002	Ongoing
136	LSD	2000-035	Assessment of active tuberculosis infection by T cell responses to purified antigens in tuberculosis patients: comparative study between patients and house hold contacts.	Dr. Rubhana Raquib	11-01-2001 11-03-2001	218,689		3 years from starting	Awaiting funds
137	LSD	2000-036	Epidemiology and aetiology of sexually transmitted infections and antimicrobial susceptibility of surveillance of N. gonorrhea in Bangladesh.	Dr. Motiur Rahman	19-11-2000	712,627 USAID/W (ERID)	1-01-2001	31-12-2003	On going
138	PHSD	2000-037	Community-based interventions to reduce neonatal mortality in Bangladesh	Dr. Mathuram Santosham (Local PI: Dr.Shams El-Arifeen)	22-02-2001	1,528,304 USAID, SCF USA	1-03-2001	28-02-2004	Inprocess

To,
Chairman
ERC, ICDDR,B
Dhaka.

26.01.2002

From : Prof. J. Ashraful Haq
Department of Microbiology, BIRDEM

Comments on the modified protocol # 2001-026 titled " Innate and adaptive immune response in shigella infection"

The PI has made necessary modifications as per suggestion of ERC. However, PI is advised to incorporate the routine tests (e.g. bleeding time and clotting time, etc.) to exclude any bleeding disorders of the subjects before taking the biopsies.

Therefore, the protocol may be recommended for ethical clearance.

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Memorandum

15 January 2002

To : Dr. Rubhana Raqib
Laboratory Sciences Division

From : Professor Mahmudur Rahman
Chairman, Ethical Review Committee (ERC)

Sub : **Protocol # 2001-026**

Thank you for your protocol # 2001-026 entitled "Innate and adaptive immune responses in *Shigella* infection", which the ERC considered in its meeting held on 9th January 2002. After review and discussion, the Committee made the following observations on the protocol:

a) On the ERC Face Sheet item # 2(c) and 2(e) should be marked YES instead of ~~NO~~ and item # 5(b) should be marked NO.

b) Starting date of the protocol on the RRC Application form be revised.

c) In the Bangla consent form, the protocol title and other relevant information be written in Bangla.

d) Adequate precaution should be taken to avert infection during taking the biopsy.

You are, therefore, advised to modify the protocol incorporating the above observations and submit the modified version of the protocol for consideration of the Chair.

Thank you.

Copy: Associate Director
Laboratory Sciences Division

Attachment I

Date:

(FACE SHEET)

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Principal Investigator: Rubhana Raqib

Application No. 2001-026

Title of Study: Innate and adaptive immune responses in Shigella infection.

Trainee Investigator (if any): _____

Supporting Agency (if Non-ICDDR,B) _____

Project Status: _____

[✓] New Study

[ ] Continuation with change

[ ] No change (do not fill out rest of the 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) Minor or persons under guardianship — Yes (No)

2. Does the Study Involve:
   - (a) Physical risk to the subjects — (Yes) No
   - (b) Social risk — 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 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 the study — (Yes) No
   - (b) Procedures to be followed including alternatives used — (Yes) No
   - (c) Physical risk — (Yes) No
   - (d) Sensitive questions — (Yes) NA
   - (e) Benefits to be derived — (Yes) NA
   - (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 parents or guardian (if subjects are minor) — 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 with 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. Example 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 Committee for review

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

Rubhana
Principal Investigator

Trainee

ICDDR,B: Centre for Health & Population Research **RRC APPLICATION FORM**

RESEARCH PROTOCOL

Protocol No.: 2001-026

FOR OFFICE USE ONLY

RRC Approval: √Yes/ No Date: 6-12-2001

ERC Approval: Yes/No Date:

AEEC Approval: Yes/No Date:

Project Title: Innate and Adaptive Immune Response in *Shigella* Infection.

Theme: ***(Check all that apply)***

- ☐ Nutrition
- ☐ Emerging and Re-emerging Infectious Diseases √
- ☐ Population Dynamics
- ☐ Reproductive Health
- ☐ Vaccine evaluation
- ☐ Environmental Health
- ☐ Health Services
- ☐ Child Health
- ☐ Clinical Case Management
- ☐ Social and Behavioural Sciences

Key words: Shigellosis, rabbit model, antibacterial peptides, LL-37, HBD-1

Principal Investigators: Rubhana Raqib , Jan Andersson
Division: LSD **Phone:** 2404 **Email:** rubhana@icddrb.org
Addresses: Immunology, LSD, ICDDR,B, Mohakhali, Dhaka-1212
And
Division of Infectious Diseases, Dept of Medicine, Karolinska, Institutet, Huddinge University Hospital, S-141 86 Stockholm

Co-Investigator(s):
Dr Birgitta Agerberth, Professor Gudmundur Gudmundsson, KI
Firdausi Qadri, Pradip K Bardhan, K Zaman, Hamidur Rahman, David Sack, ICDDR,B

Collaborating Institute(s): Department of Medical Biochemistry and Biophysics (MBB), Karolinska Institutet (KI), Microbiology and Tumorbiology Center (MTC)

Population: Inclusion of special groups (*Check all that apply)*:

Gender
- ☐ Male √
- ☐ Females

Age
- ☐ 0 – 5 years
- ☐ 5 – 9 years
- ☐ 10 – 18 years
- ☐ 18-45 years √
- ☐ > 65

- ☐ Pregnant Women
- ☐ Fetuses
- ☐ Prisoners
- ☐ Destitutes
- ☐ Service providers
- ☐ Cognitively Impaired
  - ☐ CSW
- ☐ Others (specify _____)
- ☐ Animal √

Project / study Site (*Check all the apply)*:

- ☐ Dhaka Hospital √
- ☐ Matlab Hospital
- ☐ Matlab DSS area
- ☐ Matlab non-DSS area
- ☐ Mirzapur
- ☐ Dhaka Community
- ☐ Mirsarai
- ☐ Patyia
- ☐ Other areas in Bangladesh Kamlapur
- ☐ Outside Bangladesh, name of country: _____
- ☐ Multi centre trial

❒ Chakaria
❒ Abhoynagar

(Name other countries involved) _____

Type of Study *(Check all that apply)*:

❒ √ Case Control study
❒ Community based trial / intervention
❒ Program Project (Umbrella)
❒ Secondary Data Analysis
❒ Clinical Trial (Hospital/Clinic)
❒ Family follow-up study
❒ Cross sectional survey
❒ Longitudinal Study (follow-up)
❒ Record Review
❒ Prophylactic trial
❒ Surveillance / monitoring
❒ Others √ (Animal study)

Targeted Population *(Check all that apply)*:

❒ No ethnic selection (Bangladeshi) √
❒ Bangalee
❒ Tribal groups
❒ Expatriates
❒ Immigrants
❒ Refugee

Consent Process *(Check all that apply)*:

❒ Written √
❒ Oral √
❒ None
❒ Bengali language √
❒ English language √

Proposed Sample size: Total sample size: Human, 38; Rabbit, 72 ❒

Sub-group Human, 19 x 2; Rabbit, 12x6 ❒

Determination of Risk: Does the Research Involve *(Check all that apply)*:

❒ Human exposure to radioactive agents?
❒ Fetal tissue or abortus?
❒ Investigational new device? (specify_____)
❒ Existing data available from Co-investigator
❒ Human exposure to infectious agents?
❒ Investigational new drug
❒ Existing data available via public archives/source
❒ √ Pathological or diagnostic clinical specimen only
❒ Observation of public behaviour
❒ New treatment regime

Yes/No

❒√ ❒ Is the information recorded in such a manner that subjects can be identified from information provided directly or through identifiers linked to the subjects?

❒ ❒ √ Does the research deal with sensitive aspects of the subject's behaviour; sexual behaviour, alcohol use or illegal conduct such as drug use?

Could the information recorded about the individual if it became known outside of the research:

❒ ❒ √ a. place the subject at risk of criminal or civil liability?

❒ ❒√ b. damage the subject's financial standing, reputation or employability; social rejection, lead to stigma, divorce etc.

Do you consider this research *(Check one)*:

- ☐ √ greater than minimal risk
- ☐ no more than minimal risk
- ☐ no risk
- ☐ only part of the diagnostic test

Minimal Risk is "a risk where the probability and magnitude of harm or discomfort anticipated in the proposed research are not greater in and of themselves than those ordinarily encountered in daily life or during the performance of routine physical, psychological examinations or tests. For example, the risk of drawing a small amount of blood from a healthy individual for research purposes is no greater than the risk of doing so as a part of routine physical examination".

Yes/No

√ ☐ ☐ Is the proposal funded?

If yes, sponsor Name:
Sida/SAREC

Yes/No

☐ ☐ Is the proposal being submitted for funding ?

If yes, name of funding agency: (1) NA

(2) _____

Do any of the participating investigators and/or their immediate families have an equity relationship (e.g. stockholder) with the sponsor of the project or manufacturer and/or owner of the test product or device to be studied or serve as a consultant to any of the above?

IF YES, submit a written statement of disclosure to the Director.

Dates of Proposed Period of Support	**Cost Requested for the Budget Period ($)**			
(Day, Month, Year - DD/MM/YY)	*a. Ist Year*	*2nd Year*	*3rd Year*	*Other years*
Beginning date February 03, 2002	95,381 $	93,783 $	84,533 $	
End date 31 Dec, 2004	*b. Direct Cost :*		*Total Cost :*	273,698 US$

Approval of the Project by the Division Director of the Applicant

The above-mentioned project has been discussed and reviewed at the Division level as well by the external reviewers.
The protocol has been revised according to the reviewer's comments and is approved.

(A G B Nair)

Name of the Division Director	Signature	Date of Approval
	Oct 2, 2001	Oct 2, 2001

Certification by the Principal Investigator
I certify that the statements herein are true, complete and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

Signature of PIs

1. Rubhana Raqib Rubhana

2. Jan Andersson

Professor
Jan Andersson
Infectious Diseases
Huddinge Univ. Hospital
SE-141 86 Huddinge, Sweden
Phone: +46 8 585 822 34

Date: 3/10/2001

Name of Contact Person (if applicable)

Table of Contents

ABSTRACT SUMMARY

Shigellosis is one of the major causes of morbidity and mortality in many developing countries. The annual worldwide mortality rate from acute shigellosis is approximately 1 million 94 thousand. Although available antibiotics effectively treat shigellosis, prevalence of antibiotic resistant strains is very high and is increasing at an alarming rate. Thus, there is a continuing great interest in the future possibilities of using alternative medicines.

Antimicrobial peptides are effectors of immediate defense in innate immunity and are induced during infection and inflammation. A mixture of these antibacterial peptides drenches the mucosal epithelial surfaces that may participate in the regulation of normal flora and are induced for eliminating invading microorganisms. The known antibacterial peptides expressed by human epithelial cells are LL-37, the β-defensins-1 and –2 and the α-defensins-5 and –6. The peptide LL-37 belongs to the cathelicidin family and exerts its antibacterial activity extracellularly. Recently it was shown that *Shigella* down-regulates the expression of LL-37 in the colon of adult patients during acute shigellosis thereby facilitating its proliferation in the gut. The purified peptide was shown to inhibit the growth of various microbes including *S. dysenteriae* type 1, *S. flexneri*, and *S. boydii*. This down-regulation can be an important immune escape mechanism for pathogens avoiding potent mucosal effector molecules. Initial experiments indicate that bacterial DNA might be a potential mediator for the down-regulation in cell lines. Down-regulation of LL-37 and HBD-1 was also seen in watery diarrhea caused by pathogens other than *Shigella*. Thus, it is conceivable that pathogenic bacteria down-regulate our front line defenses as a part of their invasive process.

Interestingly, we have noted up-regulation of the gene encoding LL-37 in cultured epithelial cell lines when treated with butyrate and indeed butyrate has been shown to decrease the severity of *Shigella* infections in a rabbit experimental model system. In addition, we found that IFN-γ stimulates the secretion of LL-37 in PBMC and enriched T and NK cells. The down-regulation of the gene encoding LL-37 may be controlled by IFN-γ. We have observed down-regulation of IFN-γ (both at the transcriptional and translational level) in the rectal tissues and in stool of patients with acute shigellosis. The clinical relevance is that if the down-regulation of LL-37 or IFN-γ can be blocked then the clinical recovery may be enhanced as with antibiotics. Understanding these basic regulatory mechanisms will help develop alternative methods in combating infectious bacterial pathogens that become resistant to classic antibiotics.

1. Our question is: Is there a common denominator in all *Shigella* sero groups that is responsible for the down-regulation of IFN-γ and LL-37? **(A)** In human, we aim to (1) determine potential *Shigella*-derived antigens' capacity to down-regulate mediators of central importance in peripheral blood cells from human (2) reconstitute *Shigella* specific immunity *in vitro* via pharmacologicical up-regulation of *Shigella* specific endogenous peptide (LL-37) by sodium butyrate which is a potential anti-inflammatory drug that reduces the bacterial load as well as inflammation. (3) study quantitatively, the expression and synthesis of endogenous antibacterial peptides LL-37, beta defensins (HBD-1) *in vivo* at the acute and convalescent stages of shigellosis in rectal mucosal cells. **(B)** In rabbit model, we aim to (1) study quantitatively the expression and synthesis of endogenous antibacterial peptides, CAP18 *in vivo* directed against *Shigella* bacteria. (2) reconstitute *Shigella* specific immunity *in vivo* via pharmacologicical up-regulation of *Shigella* specific endogenous peptide (CAP18) by sodium butyrate therapy. The study in human will be carried out to understand the relationship between the quantity of endogenous antimicrobial peptide produced to severity and clinical outcome of shigellosis. In vitro study in human will help to figure out possibilities of pharmacological reconstitution of *Shigella* specific immunity. Concurrently, the rabbit model of shigellosis will be initiated because the results may help us to generate a complete new treatment

strategy thereby reducing the risk for future antibiotic resistance development and potentially shortening the duration and severity of disease.

2. Since sigmoidoscopy will be performed at three time points in a patient, bleeding, though seldom and usually minor, may occur from the site of biopsy. There is a rare possibility of perforation during sigmoidoscopy, even though the procedure will be performed by a trained and well-experienced clinician. While drawing blood, momentary pain and a very small chance of bruising at the site of insertion of the needles may occur.

3. To prevent complications, every possible precaution will be taken, and the patient will be kept under observation for 3-4 hours after the sigmoidoscopic examination for any untoward effect if it were to occur. Necessary treatment will be provided and appropriate measures will be taken to avoid introduction of any infection during the procedure. To minimize the chance of infection, aseptic precautions will be taken and disposable, sterile syringes and needles will be used for drawing blood.

4. All information/data of this study will be kept confidential under lock and key in a cabinet and the keys will be with the PI of the study.

5. For inclusion of patients and healthy subjects (controls), informed consents will be obtained according to the guide lines of the local ethical committee at ICDDR,B.

6. NA

7. There will be no direct benefit to the patient or healthy controls as a result of participation in this study. However, the results of this study may help us to formulate an alternative method in combating infectious bacterial pathogens especially shigellosis that become resistant to classic antibiotics. This in the long run will thus benefit the society.

8. For the study, peripheral blood, stool and rectal tissue samples will be required from the study subjects.

DESCRIPTION OF THE RESEARCH PROJECT

Hypothesis to be tested:

Concisely list in order, in the space provided, the hypothesis to be tested and the Specific Aims of the proposed study. Provide the scientific basis of the hypothesis, critically examining the observations leading to the formulation of the hypothesis.

1. Purified antigens (invasion plasmid antigen (Ipa) proteins or lipoproteins (LP)) from *Shigella* induce down-regulation of both innate and adaptive immune responses by down-regulating IFNγ and inhibition of LL-37 production.
2. Butyrate, a potential anti-inflammatory drug reduces the bacterial load as well as inflammation by enhancing LL-37 production in the epithelial cells, granulocytes and lymphocytes .

Specific Aims:

Describe the specific aims of the proposed study. State the specific parameters, biological functions/ rates/ processes that will be assessed by specific methods (TYPE WITHIN LIMITS).

Humans:
(i) To determine potential *Shigella*-derived antigens' capacity to down-regulate mediators of central importance (IFN-γ and LL-37 synthesis) in cells from peripheral blood (*in vitro*).

(ii) To reconstitute *Shigella* specific immunity *in vitro* via pharmacologicical up-regulation of *Shigella* specific endogenous peptide (LL-37) by sodium butyrate.

(iii) Quantitative study of expression and synthesis of endogenous antibacterial peptides LL-37, beta defensins (HBD-1) *in vivo* at the acute and convalescent stages of shigellosis in rectal mucosal cells.

Rabbit model of shigellosis:
(i) Quantitative study of expression and synthesis of endogenous antibacterial peptides, CAP18 *in vivo* directed against *Shigella* bacteria.

(ii) To reconstitute *Shigella* specific immunity *in vivo* via pharmacologicical up-regulation of *Shigella* specific endogenous peptide (CAP18) by sodium butyrate therapy.

Background of the Project including Preliminary Observations

Shigellosis is one of the major causes of morbidity and mortality in many developing countries. It is estimated that approximately 165 million people are infected with *Shigella* species every year throughout the world, of which about 163.2 million are from the developing world [1]. The annual worldwide mortality rate from acute shigellosis is approximately 1 million 94 thousand. Approximately 61% of all *Shigella*-related deaths are in the developing world in children younger than 5 years who are the most susceptible and 0.2% in industrialized countries. Persistent diarrhea, a

common sequel to shigellosis accounts for an additional death toll in the hundreds of thousands annually. Out of the four pathogenic *Shigella* species, *S. dysenteriae* type 1 and *S. flexneri* are of major importance for dysentery in developing countries. Although available antibiotics effectively treat shigellosis, prevalence of antibiotic resistant strains is very high.

In endemic areas, prevention of shigellosis could be achieved by two basic strategies, i.e. by improvements in housing and sanitation thereby eliminating the possibility of ingestion of contaminated food and water and through vaccination. Preventive strategies to reduce exposure are important but difficult to implement, since in many developing countries the costs for efficient infrastructure improvements cannot be met. Population burden makes it inadequate to effectively control shigellosis in these developing countries. Therefore, the vaccination approach is seen as a rational alternative and there is a continuing great interest in the development of efficacious vaccines. For a rational vaccine program development, an in depth understanding of the immune response elicited in natural and induced shigellosis is required.

Our previous studies:

We published a series of papers based on certain aspects of cellular and humoral responses in natural *Shigella* infections in adults and children. This work has also been summarized in a doctoral thesis defended at the Karolinska Institute by the prinicipal investigator [2-6]. After the completion of PhD studies, the principal investigator has joined ICCDR,B and collaborative studies on shigellosis has continued between the groups at Huddinge Hospital, Karolinska Institutet and at ICDDR,B. Outcome of this continued collaboration (since 1996): five papers have been published [7-12] and two manuscripts submitted and two in preparation. In brief: During the acute stage of shigellosis, a marked inflammation in the rectum was associated with increased infiltration of granulocytes, T-lymphocytes, macrophages and NK-cells [2]. Extensive production of pro-inflammatory cytokines (IL-1-α, IL-1-β, TNF-α, TNF-β, TGF-β, IL-6 and IL-8) was observed at the local site that persisted up to a month after the onset of disease [5]. Analysis of cytokine producing cells at the single cell level showed that increased frequencies closely correlated to the histological grading of severity. A concomitant production of Th1 (IFN-γ, TNF-α, TNF-β) and Th2 (IL-4, IL-5, IL-10) types of cytokines was seen during shigellosis. In the early phase of the disease, 100 times higher cytokine concentrations were found in stool than in plasma [4]. Increased stool concentrations of cytokines correlated to the severity of inflammation in the gut as well as to clinical markers of disease severity.

Down-regulation of IFN-γ. Production of IFN-γ was significantly down-regulated during the acute stage of the disease and progressively increased during convalescence [4]. In contrast, healthy controls had significantly higher concentrations of IFN-γ in stool and plasma than the patients reflecting a protective role of IFN-γ. A progressive entrapment and binding of IFN-γ to its specific receptor was observed at the local site during recovery from shigellosis that was comparable to the constitutive level of expression in the healthy subjects [3]. Several fold higher frequencies of cytokine mRNA expressing cells were observed for most cytokines than the corresponding protein producing cells at the local site during the course of *Shigella* infection [8]. A selective immunosuppression of IFN-γ protein and mRNA in peripheral circulation was also observed during acute shigellosis, in line with the findings at the local site [10]. Shiga toxin, known to inhibit protein synthesis at the level of translation could not account for the discrepancy. Which factor causes down-regulation of IFN-γ in acute shigellosis is not known. The study by Sing Sing Way *et al* showed that IFN-γ was essential for innate resistance to primary *Shigella* infection and IFN-γ deficient mice were highly susceptible to virulent *Shigella* strain [13]. The source of IFN-γ was found to be NK cells. Thus, down-regulation of IFN-γ may be a strategy applied by *Shigellae* to evade the front line immune defense mechanisms.

Healthy controls constitutively expressed cell-surface cytokine receptors in the rectum. In contrast, during the acute stage of shigellosis a loss of cytokine receptors (IL-1 type I, TNF type I, IL-3, IL-4, IFN-γ and TGF-β type I) was observed [3]. These findings suggested that the loss might occur as a consequence of internalization and/or shedding of the receptors following ligand-binding. In patients, the levels of soluble cytokine receptors in plasma were 100 fold higher than the corresponding cytokines. In contrast, soluble receptors in stool were 4-6 folds lower than that of the corresponding cytokines at the acute stage. Counter-regulatory actions of soluble receptors at the local site were thus overcome by excessive local production of cytokines thereby promoting immune activation as well as tissue-damage. The expression and secretion of cytokines and cytokine receptors in the acute and convalescent stages were differentially regulated in order to modulate systemic immune activation.

In adults with acute *S. dysenteriae* 1 infection, massive apoptosis in T cell, macrophages and granulocytes was paralleled by an increased expression of Fas/Fas-L , perforin/Granzyme A and a down-regulation of Bcl-2 and IL-2 in the rectal mucosa (submitted). At the late recovery stage, a significantly enhanced expression of Bcl-2 and IL-2 was accompanied by reduced numbers of apoptotic and necrotic cells.

We hypothesized that increased morbidity and mortality seen in children in comparison to adults were due to a lack of adequate specific and nonspecific immune response development in children during the course of natural *Shigella* infection. To test the hypothesis, we studied humoral mediators of the innate defense system in stool and plasma of adult and pediatric patients with shigellosis and in healthy matched controls [11]. Increased concentrations of lactoferrin (Lf), myeloperoxidase (MPO), prostaglandin E_2 (PGE_2), leukotriene B_4 (LTB_4) and 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) in stool during acute shigellosis in both children and adults indicated that activated cells of the innate defense system at the local surface were secreting the mediators. Increased concentration of MPO and 8-iso-$PGF_{2\square}$ and lower levels of superoxide dismutase (SOD) activity in stool during acute *Shigella* infection suggested increased formation of reactive oxygen species, free radical-catalyzed peroxidation of membrane lipids and decreased scavanging of the reactive oxygen radicals leading to increased oxidative stress. Consistent expression of SOD genes in the rectum, lower tissue expression of SOD with severe inflammation and lower levels of SOD activity in stool and persistently high levels of lactoferrin even after clinical recovery suggested a restriction in translation of SOD genes in children and persistence of inflammation. The results indicated that some aspects of innate immunity against shigellosis in children were different than in adults. Antigen specific immune responses were studied in pediatric patients with shigellosis and compared with those of adult patients. Peak frequencies of antigen (invasion plasmid coded antigen B (Ipa B), lipopolysaccharide (LPS)) specific IgM-ASC (antibody secreting cells) were seen within 3-5 days after the onset of diarrhea (1^{st} time-point) in children while peak IgA- and IgG-ASCs were obtained 8-10 days later (2^{nd} time-point) as seen in adults. Antigen-specific ASC responses in children ranged between 2-4% of the total ASC responses in contrast to 8-15% in adults. When arranged in decreasing order of concentrations, LPS-specific serum IgG subclass pattern in younger patients (2.5-5 yrs) exhibited a different kinetics (IgG1>IgG2>IgG4>IgG3) than seen in older group (6-8 yrs) (IgG2>IgG1>IgG3>IgG4) or in adult patients. Secretory IgA levels in stool peaked 8-10 days after onset in both adults and children. However, a rapid induction of stool LPS specific-IgA, -IgA1 and -IgA2 occurred in adult patients at the 1^{st} time-point while in children, peak levels were attained at the 2^{nd} time-point. Similarly, higher number of TNF-α and IFN-γ expressing cells were seen in adult patients in response to antigens (LPS and Ipa B) at the 1^{st} time-point in contrast to pediatric patients. Healthy Bangladeshi adults have significantly higher titers of LPS specific IgG antibodies in serum than Bangladeshi infants (6-10 months) and children and Swedish adults thus

showing a lower risk of developing shigellosis. Thus, pediatric patients with shigellosis have reduced and delayed adaptive immune responses compared to adult patients.

These studies indicated that both the specific cell-mediated immune response as well as the innate defense system was important in adults infected with *shigella* for local disease development and recovery.

Antimicrobial peptides in *Shigella* infections

Ten human antibacterial peptides have been characterized, nine of them belong to the defensin family with three disulfide bridges, six α-defensins (HNP 1-4, HD 5-6) (reviewed in [14]), three β-defensins (HBD 1-3) [15, 16] and the amphipatic helical peptide LL-37, that we characterized originally [17]. The peptides are stored in vacuoles of granulocytes ready for activation upon stimuli or secreted directly on to mucosal surfaces by epithelial cells. It is evident that induced expression of the peptides is frequently an integral part of the inflammatoy response [18, 19]. In addition, some of the peptides are chemotactic, as the α-defensins are to neutrophils and T cells [20], and LL-37 to granulocytes and CD4 T cells [21]. The human β-defensin 2 (HBD-2) is a chemoattractant for dendritic- and memory CD4 T cells, a response that is mediated through the chemokine receptor CCR6 [22]. This suggests a role for these peptides in the crucial link between innate and adaptive immunity. Thus, the peptides have acquired new functions during evolution apart from the bactericidal activity [23].

Recently we have observed epithelial downregulation of LL-37 and human β-defensin-1 (HBD-1) in connection to certain severe bacterial pathogens like *Shigella dysenteriae* type I and other bacteria causing diarrhea [24]. This down-regulation can be an important immune escape mechanism for pathogens avoiding potent mucosal effector molecules. Furthermore, we could repeat the down-regulation *in vitro* by using the cell lines U937 of monocyte origin and HT-29 of epithelial origin, infected with *Shigella* [24]. This is the first time that down-regulation of antibacterial peptides at transcriptional level are shown in connection with infections.

Further aim is to characterize the mechanism for this down-regulation i.e. the bacterial effector and the affected signal pathways. The initial experiments indicate that bacterial DNA might be a potential mediator for the down-regulation. Since antibacterial factors line up mucosal surfaces and we also have detected down-regulation of LL-37 and HBD-1 in watery diarrhea caused by other pathogens than *Shigella*, these results bring up an interesting motive connected to serious infections. It is conceivable that pathogenic bacteria down-regulate our front line defenses as a part of their invasive process. The clinical relevance should be obvious, especially if the down-regulation can be blocked. Interestingly, we have noted up-regulation of the gene encoding LL-37 in cultured epithelial cell lines when treated with butyrate and indeed butyrate has been shown to decrease the severity of *Shigella* infections in a rabbit experimental model system [25]. Thus, a connection between the severity of *Shigella* infections and mucosal level of antibacterial peptides seems probable. In addition, we found that IFN-γ stimulates the secretion of LL-37 in PBMC and enriched T and NK cells [21]. The down-regulation of the gene encoding LL-37 may be controlled by IFN-γ. Down-regulation of IFN-γ (both at the transcriptional and translational level) was evident in the rectal tissues as well as in stool and plasma in patients with acute shigellosis and down-regulation of LL-37 in the acute stage was also evident in adult patients [4, 8-10].

Regulation of the gene encoding LL-37

The signal pathways and necessary transcription factors for expression of genes encoding antibacterial peptides in mammals have not been defined. These effector peptides are likely key components of immediate mucosal defenses and therefore one may argue that understanding their expression and induction is of importance. Based on previous research, their regulations seem to be

included in the differentiation program of neutrophil stem cells [17], regulated through pattern recognition receptors on epithelial cells [26] and affected by cytokines in specific lymphocytes [21].

We have analyzed several cell lines with respect to expression of the gene encoding LL-37. These cell lines might be valuable tools to identify the signal pathway(s). In the initial phase we will use a luciferase reporter system for gene expression and the human epithelial cell line HT-29. A 500 bp region upstream of the transcription start site has been cloned into a vector coding for the luciferase reporter gene, which is frequently used for promoter studies [27, 28]. This construct will be transfected into HT-29 cells. It is likely that the 500 bp upstream of the transcription start site contains the necessary cis-regulatory elements. Deleted version of the promoter region and mutated versions of potential binding sites for transcription factors will be used to map the critical cis control elements. It is likely that these efforts will give information about important signal pathways. This model system can be utilized in connection with butyrate stimulation or pathogen effects on the expression, thereby extracting information on host pathogen interactions. It is of importance to understand these basic regulatory mechanisms to develop alternative methods in order to tackle infectious pathogens, when bacterial strains resistant to classic antibiotics are an increasing problem.

Research Design and Methods

Describe in detail the methods and procedures that will be used to accomplish the objectives and specific aims of the project. Discuss the alternative methods that are available and justify the use of the method proposed in the study. Justify the scientific validity of the methodological approach (biomedical, social, or environmental) as an investigation tool to achieve the specific aims. Discuss the limitations and difficulties of the proposed procedures and sufficiently justify the use of them. Discuss the ethical issues related to biomedical and social research for employing special procedures, such as invasive procedures in sick children, use of isotopes or any other hazardous materials, or social questionnaires relating to individual privacy. Point out safety procedures to be observed for protection of individuals during any situations or materials that may be injurious to human health. The methodology section should be sufficiently descriptive to allow the reviewers to make valid and unambiguous assessment of the project. **(DO NOT EXCEED TEN PAGES, USE CONTINUATION SHEETS).**

I. STUDY SUBJECTS:

Patients attending the Clinical Research and Service Center (CRSC) of ICDDR,B will be screened for participation in the study. Adult *Shigella* infected patients from a study being conducted in Kamlapur may also be recruited in order to speed up the process of recruitment of increased number of patients.

Inclusion criteria

Among adult male patients (18-45 years of age), all presumptive cases of *Shigella* infection with a history of 0-3 days of diarrhea will be initially selected. Stool samples will be plated on MacConkey and SS (*Shigella-Salmonella*) agar plates and after overnight incubation of plates, serological confirmation of suspected *Shigella* colonies will be done by slide agglutination on the subsequent day. Only those patients who will have confirmed *Shigella spp* (n=19) in stool will be finally enrolled in the study (admission day). Stool samples will also be examined by direct microscopy for the presence of cyst and vegetative forms of intestinal parasites and ova of helminths and cultured for *Salmonella*, *Shigella* and *Aeromonas* species, *Vibrio cholerae* 01 and 0139 and *Campylobactor jejuni*. In addition, antimicrobial sensitivity tests will be performed. All patients will receive pivmecillinam immediately after admission as empiric therapy and drugs will be changed when necessary after obtaining the antimicrobial sensitivity pattern.

Exclusion criteria

Patients who report to having received antimicrobial treatment before attending the ICDDR,B hospital will not be included in the study. Patients presenting with clinical symptoms of other concomitant infections (chronic or otherwise) will not be included in the study. Patients initially

enrolled after preliminary serological confirmation will be dropped from the study if found to have other confounding infections (such as chronic respiratory infections, other concomitant gastrointestinal infections). However, necessary medications will be provided to the patients as per rules of CRSC.

All patients with diarrhea will be treated according to the current therapy protocols at CRSC, ICDDR,B for their respective diseases. Patients will be requested to stay in the hospital to facilitate disease monitoring and sampling. Peripheral blood, stool and rectal biopsies will be obtained from these patients accordingly as shown in the tables I & II (page 25) and flow chart I (page 26). Age matched healthy adult controls (n=19), without having any history of fever or intestinal infections within the past 3 months will be enrolled in the study. Subjects undertaking concomitant antimicrobial therapy due to chronic infections/diseases will not be included in the study.

Collection of Samples (Flow sheet I)

On admission day (patients will be enrolled after serological confirmation by slide agglutination test on the subsequent day i.e. day-1), 10 ml of blood and stool specimens will be collected from each patient. Stool specimens will be collected every day starting from the day of admission till 5 days after admission and on days-11 and 30 after admission as described. Venous blood and biopsy samples will be collected as shown in table I. From healthy subjects, all three specimens will be collected at a single time-point (Table II).

Blood: Peripheral blood mononuclear cells (PBMC) will be separated from blood upon ficoll-hypaque centrifugation at 300xg for 30 minutes. After washing in Hanks Balanced Saline Solution (HBSS, Gibco Laboratories) cells will be counted and suspended in RPMI 1640 medium supplemented with 20% fetal calf serum, 2% L-Glutamine and penicillin-streptomycin solution (1%) (Gibco). The PBMC will then be activated with anti-$CD3^+$-anti-$CD28^+$ and further cultured with either PMA plus ionomycin or purified antigens (Ipa A, Ipa B, lipoproteins (LP) from *Shigella*) for 3 days. Brefeldin (Sigma) will be added to the cells to inhibit further release of IFN-γ into the supernatant and induction and/or inhibition of IFN-γ at the single cell level will be assessed by flow cytometry and in supernatant by ELISA (developed in the lab, KI).

Granulocytes will be separated from the precipitating pellet-fraction of the Ficoll-paque density gradient centrifugation by the use of 3% Dextran sedimentation. Stimulation of granulocytes with the purified antigens (as above) will be carried out and the production of LL-37 peptide will be determined by quantitative ELISA and quantitative mRNA estimation by Real-time PCR [29]. Sodium Butyrate (1 nM/ml) will be added to the stimulated cells and incubated for 24 and 48 hours and then LL-37 will be measured as above.

Rectal biopsy: Repeated rectal biopsy samples will be obtained from patients during the acute (day-1) and the convalescent stages (day-11 & day-30) of the disease (tables I). Rectal biopsy specimen at a single time point will also be obtained from healthy controls. The samples will be used to assess the production of antibacterial peptides, beta-defensins (HBD-1) and LL-37 by immunohistochemistry as well as by Real-time PCR.

Immunohistochemistry and histology

The rectal biopsies will be fixed in 10% buffered formalin, processed, embedded in paraffin, cut in sequential 3 μm sections and then will be used either for hematoxylin and eosin staining for histological grading or for immunohistochemical staining. Staining for LL-37 and HBD-1 will be done as previously described with slight modification [24]. Sections will be deparaffinized and

microwave treatment will be given to the sections in citrate buffer (pH 5.3) followed by washing and blocking of intrinsic peroxidase activity. Sections will be incubated with anti-human LL-37 (rabbit polyclonal, 1/16000 dilutions) containing 1% normal goat serum overnight in room temperature followed by washing and incubation with goat-anti-rabbit antibodies for 60 min. After washing and incubation with avidin-biotin horseradish peroxidase complex (ABC-HRP Kit, DAKO) antibodies for 30 min in RT, the slides will be washed, substrate (DAB) will be added to develop color. Histological grading will be performed to determine the severity of inflammation in the rectum according to the standard procedure [2].

Real-Time PCR

Total RNA from rectal biopsy samples from each patient will be extracted using standard procedures, and will be reverse transcribed into cDNA following manufacturer's instructions (Life Technologies) using an oligo(dT) primer. A total of 10–50 ng retrotranscribed RNA will be used per PCR reaction. The primers and TaqMan probes for RT-PCR will be designed using the Primer Express software (Applied Biosystems, Foster City, CA). The TaqMan probes will be labeled with 6-carboxy fluorescein (Applied Biosystems). For LL-37, 5′-GAAGACCC-AAAGGAATGGCC and 5′-TCAGAGCCCAGA-AGCCTGAG primers will be used in combination with the 5'-TGT GGG CGG CAC ACC CTT TC-3' TaqMan probe. Three sets of CAP18 specific primers (GeneBank accession No. Z38026) will be used: 5′-GAATTCCGGCCATGAAGACCC, 5′-CAGAGACCCAGAAGCCTGAGC, 5′-GACTCT-GTCCTGGGTACAAGATTC. Primers and probes will be used at a final concentration of 300 nM and 200 nM, respectively. GAPDH amplifications will be conducted with the Pre-Developed TaqMan Assay Reagent specific for human GAPDH gene expression quantification (Applied Biosystems), according to manufacturer's indications. All PCR reactions will be set in triplicates using the TaqMan Universal PCR Master Mix (Applied Biosystems). Amplifications, detection, and analysis will be performed in an ABI PRISM 7700 system (Applied Biosystems). The difference between the LL-37 and HBD-1 content of patients at the acute stage with that in convalescence will be statistically analyzed with the Mann-Whitney test. *Gel Analysis of PCR Reactions:* For comparison and estimation of PCR efficacy, all samples amplified in this study will be analyzed by standard agarose gel electrophoresis.

Rabbit model A nonsurgical rabbit model of shigellosis will be developed according to the established method [30]. In brief, New Zealand White rabbits (n=72), each weighing 2 to 2.5 kg will be housed in an isolated room. Rabbits will be selected based on a number of tests including physical examination, complete blood count, rectal swab culture and fecal examination for parasites. Only healthy, *coccidia*-free rabbits with culture free of enteric pathogens will be used for the study. These rabbits will be kept on pre-inoculation fasting for 36 hours and will be provided with a single dose of tetracycline oral suspension in water. Pathogenic, wild type *Shigella flexneri* strain will be used that will be pre-confirmed biochemically and serologically by slide agglutination test. Antibioitic susceptibility will also be checked by standard disk diffusion process. Bacterial inoculum will be prepared as done previously [30]. The bacterial suspension in PBS (pH 7.4) will be adjusted to 10^{10} CFU/ml by spectrophotometric method at 600 nm. Each rabbit will be given 1 ml of this suspension in 14 ml of sterile brain heart infusion (BHI) broth. On the day of inoculum, rabbits will be anesthetized with ketamine (35 mg/kg) and xylazine (5 mg/kg) intramuscularly. Each rabbit will also receive 75 mg of cimetidine hydrochloride intravenously via the marginal car vein to inhibit gastric secretion. After 15 minutes, 15 ml of 5% sodium bicarbonate solution will be administered orally with a plastic feeding tube. A second dose of 5% sodium bicarbonate will be administered 15 min later, followed by 15 ml of the BHI bacterial suspension (as described above). After 20 min of oral inoculation, 2 ml of tincture of opium will be inoculated intraperitonially to reduce intestinal motility. Within twenty-four hours after inoculation, rabbits are expected to

develop symptoms of shigellosis. Rectal swabs will be cultured on MacConkey and TSA plates. A set of 36 rabbits will be treated with 40 mM sodium n-butyrate (pH 7.2) in Tris buffer. Another set of 36 rabbits will not be given any treatment (flow chart-rabbit model, pg 27). The treatment will be given orally by orogastric plastic feeding tube every 24 hours after inoculation of the bacteria (flow chart). Rabbits will be killed by an overdose of intravenous sodium Pentothal for collection of intestinal tissue samples at specified time intervals (flow chart-rabbit model, pg 27).

Intestinal tissue samples from rabbits will be analyzed to study the change in expression and production of antibacterial peptide CAP18 during acute infection. Anti-CAP18 antibody will be used to study expression in the intestine and the gene encoding CAP18 will be studied by Real time PCR specially developed for studying rabbit peptides. Since the antibacterial and anti-inflammatory effect of sodium butyrate has been shown already in the rabbit model of shigellosis [25], the effect of butyrate on the production of CAP18 in rabbits will be studied [31].

Sample size

Based on a previous study that showed that 90% healthy individuals show a positive response for LL-37 and 30% patients show a positive response in the acute stage [24]. Therefore, we require a sample size of 15 in each group (with 95% confidence limit and a power of 90%). With 20% loss to follow-up the required sample size in each group will be 19. In the rabbit model of shigellosis [25], it has been shown that the effect of sodium butyrate on the severity of inflammation as assessed by histology using a scoring system, the mean score ± SD in untreated group was 8.2 ± 2.9 and in the butyrate treated group was 5.1 ± 1.0 (with 95% confidence limit and a power of 90%). The required sample size in each group is 11. With 10% loss to adverse complications, the required sample size will be 12 in each group.

Data Analysis

For normally distributed data, we will use appropriate parametric tests (eg. t test) to compare between groups. In case the data is skewed, we will use nonparametric tests. Then, statistical analysis will be done using Wilcoxon signed-rank test in comparing differences in the immunological parameters in different days within the same group of patients and Mann Whitney U test in comparing patients versus healthy controls. Similarly, Wilcoxon signed-rank test will be used in comparing *Shigella* infected rabbits at the different time points and Mann Whitney U test in comparing butyrate treated rabbits with untreated rabbits. Statistical calculations will be performed using the JMP software (SAS Institute Inc., Carey, NC, USA) program.

Collaborative Arrangements

The proposed study is based on the collaboration between Professor Jan Andersson, Head of the Division of Infectious Medicine, Huddinge University Hospital, Karolinska Institutet, Sweden and the PI at ICDDR,B.

Training of a Ph.D student will be carried out for 3 years (the agreement period) and based on the achievement and performance of the student, he/she will be continued in the next 3 years' project to complete the study program.

Studies using immunohistochemical techniques, histology, and enzyme-linked immunosorbant assay will be carried out at ICDDR,B since the techniques have been standardized and the equipment needed for carrying out the experiments are available at ICDDR,B. Study involving the rabbit model of shigellosis will be done in ICDDR,B. The synthetic peptide LL-37 as well as the specific antibodies have been developed in the Department of Medical Biochemistry and Biophysics (MBB), Karolinska Institutet (KI) and will be applied for immunohistochemical staining in ICDDR,B. However, for quantitative estimation of tissue content of the antibacterial peptides by Real time PCR, the method is being established and standardized at the MBB, Microbiology and Tumorbiology Center (MTC). The facilities are available at the Department of Medical Biochemistry and Biophysics as well as at the Division of Infectious Diseases, Huddinge University Hospital at the Karolinska Institutet. For this work, rectal biopsies as well as tissue materials from the Rabbit shigellosis model will be carried in dry ice to KI and the relevant part of the work will have to be carried out by the principal investigator at KI and later the technology and the experimental know how will be transferred to ICDDR,B, Dhaka.

Ethical Assurance for Protection of Human Rights

The ethical implications are outlined below.

1. For inclusion of patients and healthy subjects (controls), informed consents will be required according to the guide lines of the local ethical committee at ICDDR,B. (Consent forms of the previous study is attached for review).

2. Patients will receive clinical care and therapy free of charge. The study will not in any way interfere with the management and treatment of the patients. Patients may discontinue their participation in the study at any time point. This decision will not have any influence on the clinical management or therapy of the patients.

3. The proposed study involves repeated sampling of blood, stool and rectal mucosa from patients. In case of healthy subjects, sampling will be done once. None of the procedures are harmful and none will result in permanent physical damage or injury. All samples from controls will be collected at one time point only, to monitor the constancy of the research parameters in the healthy adult subjects. Approximately 10 ml of venous blood (from median cubital vein) will be taken from adults (3 times, total volume of blood, 30 ml. There may be a momentary pain and a very small chance of bruising at the site of insertion of the needles. To minimize the chance of infection, aseptic precautions will be taken and disposable, sterile syringes and needles will be used for drawing blood.

4. Before obtaining rectal biopsy specimen, the bleeding parameters (prothrombin time and platelet counts) will be checked. If these are found normal, then there is no risk involved as only a few millimeters of the superficial mucosa will be taken. With a flexible sigmoidoscope seven tiny pieces of biopsies (about the size of mustard seeds, 2 mm across) will be obtained from the rectosigmoid area of adults at three time points. This instrument has a tube which will be passed through anus up to 10-12 cm and with a biopsy needle, pinches of rectal biopsies (about the size of mustard seeds, 2 mm across) will be obtained. All sampling procedures will be performed by trained and well-experienced clinician in the procedures concerned. This procedure have been performed previously at ICDDR,B in conjunction with studies of immune responses in shigellosis. No serious side effects have been associated with sampling in previous studies. Bleeding, though seldom

and usually minor, may occur from the site of biopsy and there is a very rare possibility of perforation during endoscopy, since the procedure will be performed by a trained and well-experienced clinician, an investigator of this study. To prevent such complications, every possible precaution will be taken, and the patient and controls will be kept under observation for 3-4 hours after the sigmoidoscopic examination for any untoward effect if it were to occur. Prompt and necessary treatment will be provided. Appropriate measures will be taken to avoid introduction of any infection during the procedure.

5. Permission for carrying out research in animals will be obtained from the animal experimental ethical committee (AEEC).

Risk Analysis

A delay in recruiting patients and healthy controls can delay the completion of the project in the stipulated time period and the implementation of the project will be difficult.

Dissemination and Use of Findings

Describe explicitly the plans for disseminating the accomplished results. Describe what type of publication is anticipated: working papers, internal (institutional) publication, international publications, international conferences and agencies, workshops etc. Mention if the project is linked to the Government of Bangladesh through a training program.

Research findings will be published in international journals to make the results available to all researchers in the relevant fields and will be presented in international conferences. Results obtained from this study may help in better understanding the role of protective immune mechanisms in shigellosis and the immunopathogenic processes involved in the development of disease. In the wake of alarmingly increasing rate of antimicrobial resistant strains, it is of utmost importance to understand the basic regulatory mechanisms to develop alternative methods that may be cheap and effective complementary therapy to antibiotics for tackling the pathogens and treatment of patients. Thus the study may provide information on better therapeutic interventions as well as alternate methods for treatment of shigellosis and better formulation of protective and efficacious vaccine against all *Shigella* infections.

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DETAILED BUDGET

Project Title: Innate and Adaptive Immune Response in Shigella Infection.
Name of PIs: (1) Rubhana Raqib, Laboratory Sciences Division, International Centre for Diarrhoeal Diseases Research, Bangladesh
(2) Jan Andersson, Division of Infectious Diseases, Huddinge University Hospital, Karolinska Institutet.

Funding Source: SIDA/SAREC Starting Date: January, 2002 Closing Date: December, 2004
Total Amount: 273,698 US$ (2,824,563 SEK)

BUDGET FOR ICDDR,B Yearly Budget, 1 US$=10.32 SEK

	Salary Support				**1st Year**		**2nd Year**		**3rd Year**		**Total in 3 yrs**	
	Personnel	Position	Effort%	Salary	**US$**	SEK	**US$**	SEK	**US$**	SEK	**US$**	SEK
1	Rubhana Raqib	NOB	20%	11556	2,311	23,850	2,427	25,042	2,548	26,294	7,285	75,186
2	PK Bardhan	Sr. Medi.Ofl	5	16572	828	8,545	869	8,972	0	0	1,697	17,517
3	Hamidur Rahman	ARB,in-chrg	5	12984	649	6,698	681	7,033	0	0	1,330	13,730
4	Research Officer	GS-5	100	4000	4,000	41,280	4,200	43,344	4,410	45,511	12,610	130,135
5	Rsrch Asst/phD Student	GS-4	100	3060	3,060	31,579	3,213	33,158	3,374	34,816	9,647	99,553
6	Lab Attendent	GS-1	100	1908	1,908	19,691	2,003	20,675	2,104	21,709	6,015	62,074
7	Research Physician		100	2,400	2,400	24,768	2,520	26,006	2,646	27,307	7,566	78,081
	Sub Total				**15,156**	156,410	**15,914**	164,230	**15,081**	155,637	**46,151**	476,277
	Travel											
8	Local Travel				400	4,128	300	3,096	200	2,064	900	9,288
9	International Travel				3,000	30,960	3,000	30,960	3,000	30,960	9,000	92,880
	Sub Total				**3,400**	35,088	**3,300**	34,056	**3,200**	33,024	**9,900**	102,168
	Supplies and Materials											
11	Immunological Assays:											
	Antibodies, conjugates, recombinant protein				3,000	30,960	3,000	30,960	2,500	25,800	8,500	87,720
	Tissue culture, reagents for rabbit models				2,500	25,800	2,500	25,800	2,000	20,640	7,000	72,240
12	Laboratory Supplies:											
	Plasticware, glassware, office supplies				1,500	15,480	1,000	10,320	1,000	10,320	3,500	36,120
	Chemicals and media				1,000	10,320	1,000	10,320	500	5,160	2,500	25,800
	Sub Total				**8,000**	82,560	**7,500**	77,400	**6,000**	61,920	**21,500**	221,880

	1st Year		2nd Year		3rd Year		Total in 3 yrs	
	US$	SEK	**US$**	SEK	**US$**	SEK	**US$**	SEK
13 **Interdepartmental Services**								
Pathological Tests	1,000	10,320	1,000	10,320	600	6,192	2,600	26,832
Microbiological tests	1,000	10,320	1,000	10,320	600	6,192	2,600	26,832
Biochemistry Tests	900	9,288	600	6,192	600	6,192	2,100	21,672
Patient Study								
Study ward costs (30x5x3x25$)	3,750	38,700	3,000	30,960	1,500	15,480	8,250	85,140
Saline, drugs, food costs etc	700	7,224	700	7,224	500	5,160	1,900	19,608
Endoscopy charges (20 $x3x30 plus 30x20$)	1,000	10,320	1,000	10,320	400	4,128	2,400	24,768
Traveller's clinic and utility	900	9,288	900	9,288	300	3,096	2,100	21,672
Wage loss for follow up & controls	1,200	12,384	1,200	12,384	600	6,192	3,000	30,960
Rabbit model, cell lines and animal house related cost	600	6,192	1,200	12,384	300	3,096	2,100	21,672
Sub Total	**11,050**	114,036	**10,600**	109,392	**5,400**	55,728	**27,050**	279,156
14 **Other Contractual Services**								
Subsistence, Rent, Communications, Electricity, Utility	1,000	10,320	1,000	10,320	1,000	10,320	3,000	30,960
Repair, Maintanence, Freight charges, liq Nitorgen, dr	1,000	10,320	600	6,192	600	6,192	2,200	22,704
Printing and Publication	400	4,128	400	4,128	1,000	10,320	1,800	18,576
Staff development, training, workshop, unforseen	200	2,064	200	2,064	200	2,064	600	6,192
Sub Total	**2,600**	26,832	**2,200**	22,704	**2,800**	28,896	**7,600**	78,432
15 Capital Equipment	**2,500**	25,800	**2,000**	20,640	**0**	0	**4,500**	46,440
Costs at ICDDR,B	**42,706**	440,726	**41,514**	428,422	**32,481**	335,205	**116,701**	1,204,353
16 Overhead 25%	10,677	110,181	10,378	107,106	8,120	83,801	29,175	301,088
Total Costs at ICDDR,B	**53,383**	550,907	**51,892**	535,528	**40,601**	419,006	**145,876**	**1,505,442**

Swedish Project Leader: Jan Andersson, Head, Division of Infectious Diseases, Karolinska Institutet, Huddinge University Hospital, Stockholm, Sweden

BUDGET FOR KAROLINSKA INSTITUTET

Yearly Budget, 1 US$=10.32 SEK

			1st Year		2nd Year		3rd Year		Total in 3 yrs	
1 Salary Support	Effort%	Salary	**US$**	SEK	**US$**	SEK	**US$**	SEK	**US$**	
Anette Hofmann	50		14,560	145,600	14,560	145,600	14,560	145,600	43,680	
Laboratory Attendent	15		4,560	45,600	4,560	45,600	4,560	45,600	13,680	
Animal Care taker	15		0	0	0	0	4,600	46,500	9,200	
Sub Total			19,120	191,200	19,120	191,200	23,720	237,700	66,560	
Overhead 18%			3,442	34,416	3,442	34,416	4,270	42,786	11,981	
Sub Total			**22,562**	**225,616**	**22,562**	**225,616**	**27,990**	**280,486**	**78,541**	
2 International Travel										
Visit to ICDDR,B			1,500	15,000	1,500	15,000	1,500	15,000	4,500	
Sub Total			**1,500**	15,000	**1,500**	15,000	**1,500**	15,000	**4,500**	
3 Supplies and Materials										
DNA-polymerase + nucleotide supply			4,000	40,000	4,000	40,000	2,000	20,000	6,000	
Animal supply, Rabbits			3,000	30,000	3,000	30,000	3,000	30,000	9,000	
Cell lines, tissue culture media, purification of protein			1,200	12,000	1,000	10,000	900	9,000	3,100	
Biochemical analysis and toxicity assays			2,000	20,000	1,500	15,000	1,000	10,000	4,500	
Plastic disposals, glass ware, photo material,			1,500	15,000	1,500	15,000	1,000	10,000	4,000	
Sub Total			11,700	117,000	11,000	110,000	7,900	79,000	30,600	
Overhead 18%			2,106	21,060	2,700	27,000	2,412	24,120	7,218	
Sub Total			**13,806**	138,060	**13,700**	137,000	**10,312**	103,120	**37,818**	
4 Applying inst. Administered by Cooperating inst.										
Purchase and shipment of reagents, cell cultures, biological material to ICDDR,B			3,500	36,120	3,500	36,120	3,500	36,120	10,500	108,360
Overhead 18%			630	6,502	630	6,502	630	6,502	1,890	19,505
Sub Total			**4,130**	**42,622**	**4,130**	**42,622**	**4,130**	**42,622**	**12,390**	127,865
TOTAL COST AT KAROLINSKA INSTITUTET			**41,998**	419,976	**41,892**	418,916	**43,932**	439,316	**127,821**	1,319,111

	1st Year		2nd Year		3rd Year			
	US$	SEK	**US$**	SEK	**US$**	SEK	**US$**	**SEK**
Yearly Costs at ICDDR,B	53,383	550,913	51,892	535,525	40,601	419,002	145,876	1,505,440
Yearly Costs at KI	41,998	433,419	41,892	432,325	43,932	453,378	127,822	1,319,123
SUB-TOTAL	**95,381**	984,332	**93,784**	967,851	**84,533**	872,381	**273,698**	2,824,563

GRAND TOTAL FOR THREE YEARS **273,698 US$**
2,824,563 SEK

Budget Justifications

Please provide one page statement justifying the budgeted amount for each major item. Justify use of man power, major equipment, and laboratory services.

1. Full salaries of the research officer, research assistant, lab attendant, 50% of study physician, 30% salary of the PI (ICDDR,B) and 5% salary of the co-investigators is requested.
2. Charges for patients include costs for study ward, out-patient ward, medicines, hospital supplies and hospital meals during their stay at the study ward.
3. Costs for paying patients and controls for follow-up and wage loss and meals.
4. Interdepartmental charges include costs of microbiological, pathological, histopathological and biochemical tests.
5. Costs for supplies and reagents required for various immunological and microbiological assays, disposable plasticware, glassware.
6. Expenses for publication has been requested.
7. International travel is required per year for carrying out work at KI in Sweden, for analysis and group discussions of results and writing up of manuscripts at KI and at ICDDR,B.
8. Local travel is required for visiting patients / household contacts to remind and encourage them to complete their follow-up visits.
9. Costs for spare parts such as disposable knives, knife holder, etc are requested.

Sequence of tasks within time frame:

	Year 1	Year 2	Year 3
Initial set up	———		
Patient enrolment with follow-up	———	———	———
Laboratory assays		———	———
Data analysis and writing up		———	———

Itemized specific tasks for each listed investigator:

1. Rubhana Raqib (PI)
   Will standardize methods and supervise work in the lab at ICDDR,B. Coordinate specimen collection from patients. Carry out quantification of mRNA of antimicrobial peptide-related work at KI. Data compilation and analysis. Writing up of results obtained.
2. Jan Andersson (PI)
   Scientific and academic feedback. Technical support for immune response related work.
3. Birgitta Agerberth
   Scientific and academic feedback. Establishment and standardization of some methods and relevant technical support.
4. Gudmundur H. Gudmundsson
   Scientific and academic feedback. Coordination in obtaining purified proteins, antibodies, biopsy materials etc from relevant laboratories and relevant technical support.
5. Firdausi Qadri
   Supervise work in the lab, scientific and academic feedback.
6. PK Bardhan
   Sigmoidoscopy and patient related work, scientific and academic feedback.
7. Hamidur Rahman
   Perform animal model studies, specimen collection from rabbit model.
8. Medical officer
   Patient enrolment and clinical management, obtaining history, determining age, gender, nutritional status and to obtain blood specimens.
9. Research Officers
   Carry out experiments specific for the protocol involving immunological and microbiological techniques.
10. Lab Attendent/field workers
    Motivate patients and healthy controls for enrolment. Sample collection from study population and processing for storage. Labeling and storing. Visiting patients at their respective homes to encourage patients to come to the Center for follow-up visits.

APPENDIX-I

Table I. Collection of samples from *Shigella*-infected patients (n=19)*

Days (D) from enrollment	Samples collected from each patients		
	Blood	Stool	Rectal biopsy
D-1	+	+	+
D-2, 3, 4	–	+	–
D-5	+	+	–
D-11	+	+	+
D-30	+	+	+

Patients* (18-45 yrs old) infected with *Shigella* attending the outpatient at the Clinical research and service Center of ICDDR,B will be initially screened and later selected for the study. Blood (10 ml) and rectal biopsy samples (8 pieces) will be collected thrice from the patients.

Table II. Collection of samples from Healthy controls (n=19)†

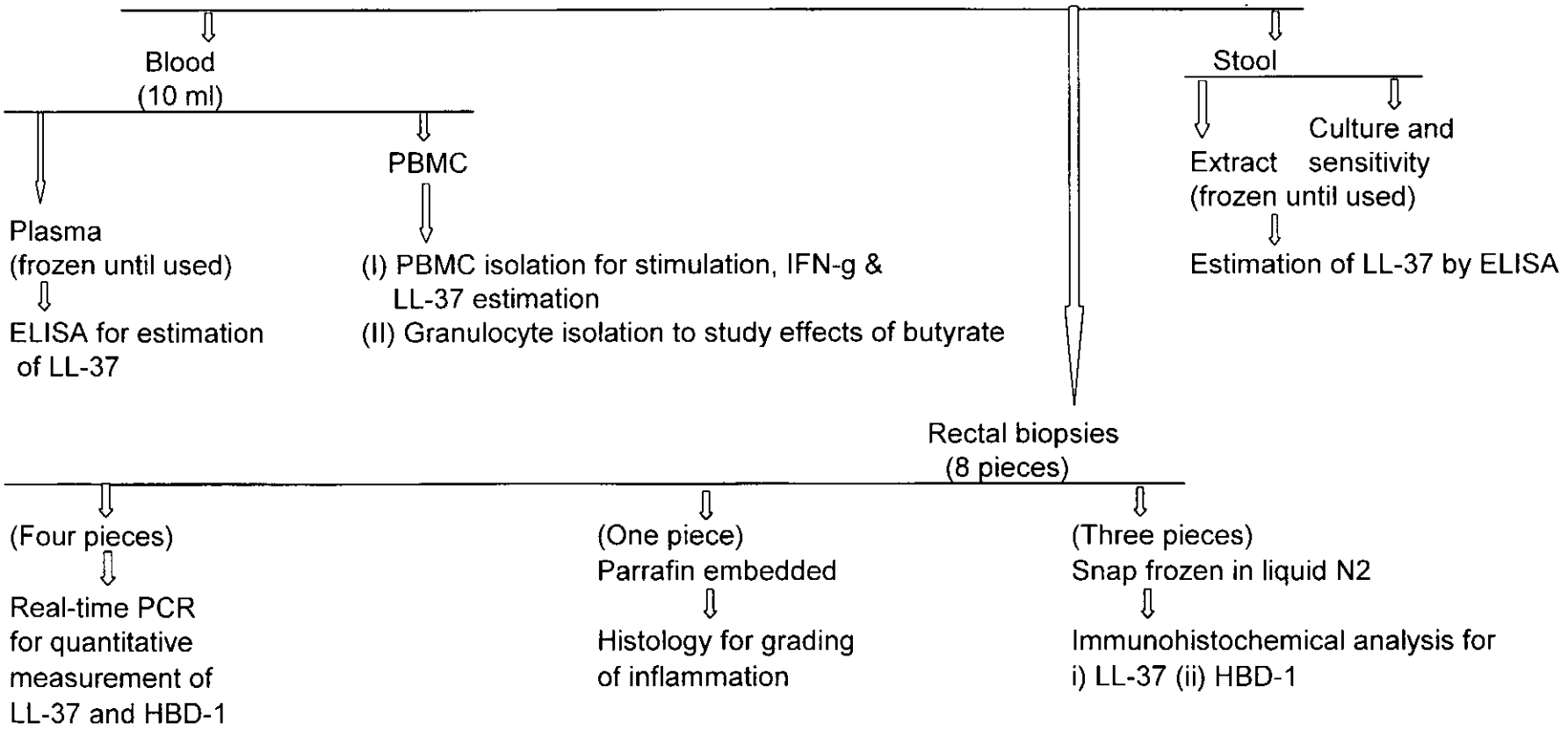
	Samples collected from each individual		
	Blood	Stool	Rectal biopsy
Day of Enrollment only	+	+	+

†Age and socioeconomic status matched healthy controls will be enrolled.

Flow Chart: ADULTS (AGE18-45 YRS)

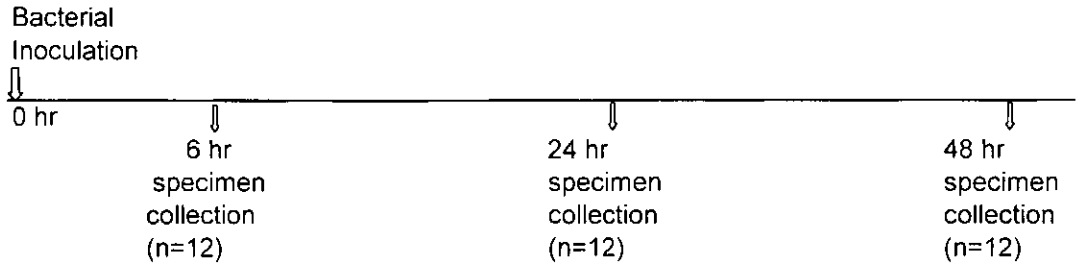
Sample collection and sequence of work

Patients infected with *Shigella* : Venous blood (from median cubital vein, 3 times), stool (7 times) and rectal biopsy (by flexible sigmoidoscopy, 3 times)
Healthy controls: Blood, stool and biopsy will be collected only once

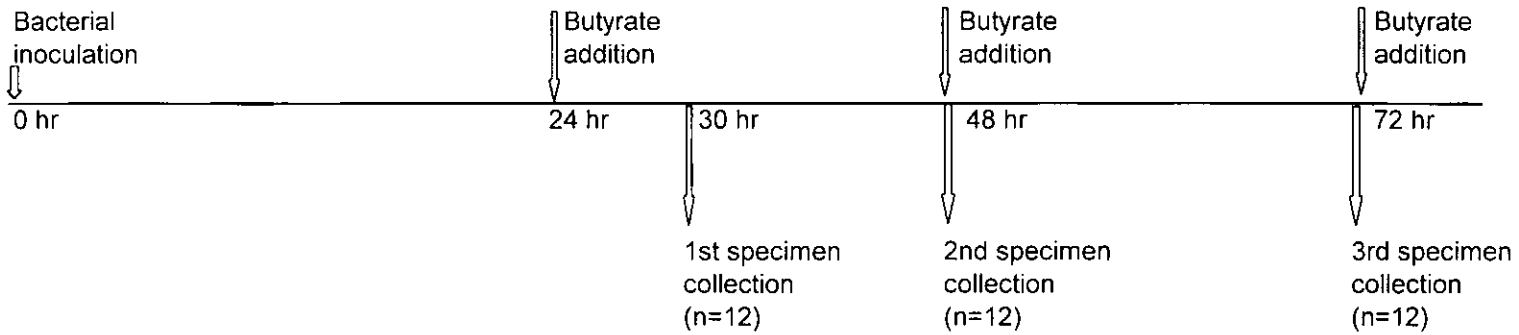


Flow Chart- Rabbit model for shigellosis
Schedule of specimen collection with and without butyrate treatment

Without butyrate treatment (n=36)



With butyrate treatment (n=36)



Note: Total number of rabbits used for inoculation with *Shigella* bacteria will be n=72. At each time point there will be 24 rabbits, 12 of which will receive butyrate treatment and 12 will not receive this treatment. Specimen will be used for studying quantitative PCR and immunostaining.

APPENDIX V

Protocol No: 2001-026

Protocol Title: Innate and Adaptive Immune Responses in *Shigella* Infection.

Principal Investigator: Rubhana Raqib

Organization: International Centre for Diarrhoeal Disease Research, Bangladesh

CONSENT FORM FOR ADULT PATIENTS WITH SHIGELLA INFECTION

You have bloody diarrhoea which is caused by a pathogen called *Shigella*. Very little is known about the immunopathogenic mechanisms of this disease. 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 provide information on better therapeutic interventions and management of this disease. Since you have dysentery, we request you to help us by participating in this study. Your participation in this study may help to save lives in future due to this bloody-diarrhea.

During the study period, you will be examined thoroughly and you will receive necessary treatment of this hospital. For this study, you will be required to stay in the hospital for 4 days and will be discharged on the 5th day. You will be requested to come for follow-up visits on days 11 and 30 after admission. About 10 ml of blood (two tea-spoon full) will be collected from a vein on your forearm (median cubital vein) on the day of admission and 5, 11 and 30 days after that (4 times). Other than momentary pain and a very small chance of bruising at the site of insertion of the needles, drawing blood will not cause any harm to you. To minimize the chance of infection, we will take aseptic precautions and use disposable, sterile syringes and needles for drawing blood. Stool samples will also be collected from you on these above-mentioned days.

You will be examined by a narrow, flexible tube-like instrument called flexible sigmoidoscope which will be passed through your anus (10-12 cm from anus) to examine the rectosigmoid area of the large intestine. A lubricating jelly (bacteriostatic) will be applied locally at the anal orifice so that you will only feel a slight discomfort during this examination. Eight tiny pieces of biopsies (about the size of a mustard seed, 2 mm across) will be obtained from your rectum. Repeated biopsy samples will be obtained from you three times, on the day of admission, 11 and 30 days later. Bleeding, though seldom and usually minor, may occur from the site of biopsy and there is a rare possibility of perforation during endoscopy. The chance of rectal perforation is very small as this procedure will be performed by a trained and well-experienced clinician, an investigator of this study. To prevent such complications, every possible precaution will be taken, and you will be kept under observation for 3-4 hours after the sigmoidoscopic examination for any untoward effect if it were to occur. Necessary treatment will be provided. Appropriate measures will be taken to avoid introduction of any infection during the procedure.

All information/data of this study will be kept confidential and will be provided to you upon your request. We will keep all the information and results of laboratory tests performed on you under lock and key and only the investigators of this study would have access to the information. In case of future use of information in the form of Abstract or publication, the anonymity of participant will be strictly maintained.

There will be no direct benefit to you as a result of participation in this study. However, the results of this study will help us to understand better about this disease and will thus benefit the society. We will compensate for any wage loss and travel costs that you may incur for each follow-up visit.

It is your decision to participate in this study. Even after initial participation in the study, you have the right to withdraw yourself at any time at your will. If you are found to have concurring infections besides shigellosis after enrollment in the study and would require to be excluded from the study, or if you do not agree to participate or want to withdraw from the study, you will receive the standard treatment of this hospital. If you agree to 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 patient	Date
Signature of the investigator	Date
Signature of a witness	Date

APPENDIX V

Protocol No: 2001-026

Protocol Title: Innate and Adaptive Immune Responses in *Shigella* Infection.

Principal Investigator: Rubhana Raqib

Organization: International Centre for Diarrhoeal Disease Research, Bangladesh

CONSENT FORM FOR ADULT PATIENTS WITH SHIGELLA INFECTION

You have bloody diarrhoea which is caused by a pathogen called *Shigella*. Very little is known about the immunopathogenic mechanisms of this disease. 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 provide information on better therapeutic interventions and management of this disease. Since you have dysentery, we request you to help us by participating in this study. Your participation in this study may help to save lives in future due to this bloody-diarrhea.

During the study period, you will be examined thoroughly and you will receive necessary treatment of this hospital. For this study, you will be required to stay in the hospital for 4 days and will be discharged on the 5th day. You will be requested to come for follow-up visits on days 11 and 30 after admission. About 10 ml of blood (two tea-spoon full) will be collected from a vein on your forearm (median cubital vein) on the day of admission and 5, 11 and 30 days after that (4 times). Other than momentary pain and a very small chance of bruising at the site of insertion of the needles, drawing blood will not cause any harm to you. To minimize the chance of infection, we will take aseptic precautions and use disposable, sterile syringes and needles for drawing blood. Stool samples will also be collected from you on these above-mentioned days.

You will be examined by a narrow, flexible tube-like instrument called flexible sigmoidoscope which will be passed through your anus (10-12 cm from anus) to examine the rectosigmoid area of the large intestine. A lubricating jelly (bacteriostatic) will be applied locally at the anal orifice so that you will only feel a slight discomfort during this examination. The bleeding parameters (prothrombin time and platelet counts) will be checked. If these are found normal, then eight tiny pieces of biopsies (about the size of a mustard seed, 2 mm across) will be obtained from your rectum. Repeated biopsy samples will be obtained from you three times, on the day of admission, 11 and 30 days later. Bleeding, though seldom and usually minor, may occur from the site of biopsy and there is a rare possibility of perforation during endoscopy. The chance of rectal perforation is very small as this procedure will be performed by a trained and well-experienced clinician, an investigator of this study. To prevent such complications, every possible precaution will be taken, and you will be kept under observation for 3-4 hours after the sigmoidoscopic

examination for any untoward effect if it were to occur. Necessary treatment will be provided. Appropriate measures will be taken to avoid introduction of any infection during the procedure.

All information/data of this study will be kept confidential and will be provided to you upon your request. We will keep all the information and results of laboratory tests performed on you under lock and key and only the investigators of this study would have access to the information. In case of future use of information in the form of Abstract or publication, the anonymity of participant will be strictly maintained.

There will be no direct benefit to you as a result of participation in this study. However, the results of this study will help us to understand better about this disease and will thus benefit the society. We will compensate for any wage loss and travel costs that you may incur for each follow-up visit.

It is your decision to participate in this study. Even after initial participation in the study, you have the right to withdraw yourself at any time at your will. If you are found to have concurring infections besides shigellosis after enrollment in the study and would require to be excluded from the study, or if you do not agree to participate or want to withdraw from the study, you will receive the standard treatment of this hospital. If you agree to 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 patient | Date

_____ _____

Signature of the investigator | Date

_____ _____

Signature of a witness | Date

Protocol No: 2001-026

Protocol Title: Innate and Adaptive Immune Responses in *Shigella* Infection.

Principal Investigator: Rubhana Raqib

Organization: International Centre for Diarrhoeal Disease Research, Bangladesh

CONSENT FORM FOR HEALTHY ADULT CONTROL

Bloody diarrhea due to pathogen "*Shigella*" may cause severe complications. Very little is known about the immunopathogenic mechanisms of this disease. In order to understand more about this illness and development of immune responses of the host against it, we are conducting a study. Results obtained from this study may help to understand protective immunity and provide information on better therapeutic interventions and management of this disease. Healthy subjects are needed to be examined in order to compare the findings in health to that in *Shigella* infection. For this purpose we request you to participate in this study. Your participation in this study may help to save many lives in future due to this bloody-diarrhea.

For this purpose, you will be examined by a qualified physician for a routine check up. For our study, we will collect single samples of blood, stool, urine and rectal biopsies from you. Approximately 10 ml (two tea-spoon full) of venous blood will be taken from a vein on your forearm (median cubital vein). Other than momentary pain and a very small chance of bruising at the site of insertion of the needles, drawing blood will not cause any harm to you. To minimize the chance of infection, we will take aseptic precautions and use disposable, sterile syringes and needles for drawing blood.

You will be examined by a narrow, flexible tube-like instrument called flexible sigmoidoscope which will be introduced into the rectum (10-12 cm from anus) to examine the rectosigmoid area of the large intestine. A lubricating jelly (bacteriostatic) will be applied locally at the anal orifice so that you will feel only little discomfort during this examination. The bleeding parameters (prothrombin time and platelet counts) will be checked. If these are found normal, then eight tiny pieces of biopsies (about the size of mustard seed, 2 mm across) will be obtained from your rectum. Bleeding, though seldom and usually minor, may occur from the site of biopsy and there is a rare possibility of perforation during endoscopy. The chance of rectal perforation is very small, as this procedure will be performed by a trained and well-experienced clinician, one of the investigators of this study. To prevent such complications, every possible precaution will be taken, and you will be kept under observation for 3-4 hours after the sigmoidoscopic examination for any untoward effect if it were to occur. Necessary treatment will be provided. Appropriate measures will be taken to avoid introduction of any infection during the procedure.

All information/data of this study will be kept confidential and will be provided to you upon your request. We will keep all the information and results of laboratory tests performed on you under lock and key and only the investigators of this study would have access to the information. In case of future use of information in the form of publication, the anonymity of participant will be strictly maintained.

This procedure will not benefit you in any way but it will help us to better understand the normal immune status of an adult living in areas endemic for diarrheal diseases and will help others in future. It is your decision to participate in this study. We will compensate for any wage loss and travel costs that you may incur while participating in this study. If you agree to 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 control Date

_____ _____

Signature of the investigator Date

_____ _____

Signature of the witness Date

প্রটোকল নং: ২০০১-০২৬
প্রটোকল শিরনাম: ইনেট এন্ড এডাপটিভ ইমিউন রেসপন্স ইন শিগেলা ইনফেকসান ।
(Innate and Adaptive Immune Responses in *Shigella* Infection.)
প্রধান গবেষক: রুবহানা রকীব
প্রতিষ্ঠান: আন্তর্জাতিক উদারাময় গবেষনা কেন্দ্র, বাংলাদেশ মহাখালী, ঢাকা-১২১২

সম্মতিপত্র "শিগেলা" (প্রাপ্ত বয়স্ক)

আপনি শিগেলা নামক এক প্রকার জীবানুর আক্রমনে সৃষ্ট রক্ত আমাশয়ে ভুগছেন যা কখনো কখনো আপনার শরীরে মারাত্মক জটিলতা সৃষ্টি করতে পারে। এই রোগে শরীরে কিভাবে জটিলতা সৃষ্টি হয় তা এখন পর্যন্ত সম্পুর্ণরূপে জানা যায়নি। এই রোগটি সম্পর্কে ভালভাবে জানার জন্য এবং এর বিরুদ্ধে প্রতিরোধ ক্ষমতা বাড়ানোর জন্য আমরা একটি গবেষনা করছি। এই গবেষনা হতে প্রাপ্ত ফলাফল ভবিষ্যতে এ রোগ প্রতিরোধের উপায় সম্পর্কে ভালভাবে জানতে এবং উন্নত চিকিৎসার মাধ্যমে এ রোগ প্রশমনে আমাদের সাহায্য করবে। যেহেতু আপনি রক্ত আমাশয়ে ভুগছেন, এই গবেষনায় অংশ গ্রহন করার জন্য আমরা আপনাকে অনুরোধ করছি; আপনার অংশ গ্রহন ভবিষ্যতে রক্ত আমাশয়ের প্রকোপ হতে অনেক মূল্যবান জীবন রক্ষা করতে পারে।

গবেষনা চলাকালীন সময় আপনার সম্পুর্ণ শারীরিক পরীক্ষা করা হবে এবং আপনাকে হাসপাতালের প্রয়োজনীয় চিকিৎসাসেবা দেওয়া হবে। এই পবেষনার জন্য আপনাকে ৪ দিন হাসপাতালে ভর্তি থাকতে হবে এবং পঞ্চম দিনে আপনি হাসপাতাল ত্যাগ করতে পারবেন। তবে গবেষনায় অন্তর্ভূক্তির ১১ দিন এবং ৩০ দিন পরে আপনাকে পুনরায় হাসপাতালে আসতে আমরা অনুরোধ করব। ভর্তির দিন এবং ভর্তির ৫,১১,এবং ৩০ দিন পরে (৪ বার) আপনার হাতের শিরা থেকে ১০ মিঃ লিঃ (দুই চা চামচ) মত রক্ত নেওয়া হবে। যে স্থানে সুঁই প্রবেশ করানো হবে সে স্থানে ক্ষনস্থায়ি ব্যাথা এবং ন্যূনতম ক্ষেত্রে কাল দাগ পড়া ছাড়া রক্ত নেওয়ার সময় আপনার অন্য কোন সমস্যা হবেনা। যে কোন প্রকার সংক্রমন কমাতে আমরা সম্পুর্ণ জীবানুমুক্ত পরিবেশে রক্ত সংগ্রহ করব এবং রক্ত সংগ্রহের জন্য জীবানুমুক্ত সিরিঞ্জ এবং সুঁই (একাবর ব্যবহার করে ফেলে দেওয়া হয় এমন) ব্যবহার করব। উপরি উল্লিখিত দিনগুলোতে আপনার মল এবং মূত্রও আমরা সংগ্রহ করব। আপনার মলদ্বারে একটি নমনীয় নলের মত যন্ত্র (flexible sigmoidoscope) ঢুকিয়ে (মলদ্বার থেকে ১০-১২ সেঃমিঃ অভ্যন্তরে) বৃহদন্তের রেকটোসিগময়েড এলাকা পরীক্ষা করা হবে। এই প্রক্রিয়ায় আপনার মলদ্বারে এক প্রকার পিচ্ছিল জেলী (ব্যাকটেরিয়ার প্রতিরোধক) লাগান হবে যাতে আপনার মলদ্বার পিচ্ছিল হয় এবং পরীক্ষার সময় আপনি কোন অসুবিধা বোধ না করেন। ভর্তির দিন এবং ভর্তির ১১ দিন এবং ৩০ দিন পর মোট তিনবার আপনার মলাশয় থেকে ৮ টুকরা ঝিল্লি (সরিষার বীজ আকৃতির, ২ মিঃমিঃ) সংগ্রহ করা হবে। ঝিল্লি সংগ্রহ করার আগে রক্তপাতের নিয়ামকসমূহ (প্রথ্রম্বিন জমাট বাধার সময় এবং অনুচক্রিকার সংখ্যা) পরীক্ষা করা হবে । এই পক্রিয়ায় খুব কম ক্ষেত্রে মলদ্বারে সামান্য রক্তপাত এবং ক্ষত সৃষ্টি হতে পারে। মলাশয়ে ক্ষত হওয়ার সম্ভাবনা খুবই কম থাকবে কারন এই প্রক্রিয়া পরিচালিত হবে একজন প্রশিক্ষিত এবং অভিজ্ঞ চিকিৎসক দ্বারা যিনি এই পবেষনার একজন অনুসন্ধানকারী। যে কোন ধরনের জটিলতা রোধে সম্ভাব্য সবধরনের সতর্কতা অবলম্বন করা হবে এবং পরীক্ষার পর ৩-৪ ঘন্টা আপনাকে পর্যবেক্ষন করা হবে যাতে যে কোন ধরনের প্রতিকুল অবস্থা সৃষ্টি হলে সাথে সাথে ব্যবস্থা নেওয়া যায়। এই প্রক্রিয়ার সময় যে কোন ধরনের সংক্রমন রোধে যথাযথ ব্যবস্থা গ্রহন করা হবে এবং প্রয়োজনীয় চিকিৎসা সরবরাহ করা হবে ।

এই গবেষনার সমস্ত তথ্য / উপাত্ত গোপন রাখা হবে, তবে আপনি জানতে আগ্রহী হলে আপনার অনুরোধে আপনাকে সরবরাহ করা হবে । গবেষনাগারে করা রোগীর বিভিন্ন পরীক্ষার তথ্য এবং ফলাফল তালাবদ্ধ করে রাখা হবে এবং শুধুমাত্র এই গবেষনার অনুসন্ধানকারীরা এবং প্রয়োজনে আইনপ্রয়োগকারী সংস্থা এই সমস্ত তথ্য জানবেন। ভবিষ্যতে গবেষনা প্রকাশনার জন্যে যদি এই সমস্ত তথ্য ব্যবহার করা হয় সেক্ষেত্রে অংশগ্রহনকারীর নাম গোপন রাখা হবে ।

এই গবেষনায় আপনি সরাসরি লাভবান হবেন না; কিন্তু গবেষনায় প্রাপ্ত ফলাফল এই রোগ সম্পর্কে ভালভাবে জানতে আমাদের সাহায্য করবে এবং ফলশ্রুতিতে পুরো সমাজ উপকৃত হবে। ছুটির পর প্রতিবার হাসপাতালে আসার জন্য আপনাকে আপনার ঐ দিনের মজুরি এবং যাতায়াত খরচ আমরা দিয়ে দেব।

আপনি এই গবেষনায় অংশ গ্রহন করবেন কিনা তা আপনার একান্ত ব্যক্তিগত সিদ্ধান্ত । এমনকি গবেষনায় অংশ গ্রহনরে পর যে কোন সময় নিজেকে প্রত্যাহার করে নেওয়ার অধিকার আপনার থাকবে। গবেষনায় অংশ গ্রহনরে পর যদি দেখা যায় যে আপনার অন্য কোন অসুখ আছে তখন আপনাকে এই গবেষনা থেকে প্রত্যাহার হবে; অথবা আপনি গবেষনায় অংশগ্রহন করতে না চাইলে অথবা অংশগ্রহনের পর নিজেকে প্রত্যাহার করে নিলেও এই হাসপাতালের সকল চিকিৎসাসেবা যথারীতি পাবেন। আপনি যদি স্বেচ্ছায় এই গবেষনায় অংশ গ্রহন করতে সম্মত হন তবে অনুগ্রহ করে নিচে আপনার সই বা বামহাতের বৃদ্ধাঙ্গুলির টিপসই দিন।

আপনার সহযোগীতার জন আপনাকে ধন্যবাদ।

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স্বাক্ষর/বাম হাতের বৃদ্ধাঙ্গুলির টিপ সই তারিখঃ

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অনুসন্ধানকারীর স্বাক্ষর তারিখঃ

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প্রত্যক্ষদর্শীর স্বাক্ষর তারিখঃ

প্রটোকল নং: ২০০১-০২৬
প্রটোকল শিরনাম: ইনেট এন্ড এডাপটিভ ইমিউন রেসপন্স ইন শিগেলা ইনফেকসান ।
(Innate and Adaptive Immune Responses in *Shigella* Infection.)
প্রধান গবেষক: রুবহানা রকীব
প্রতিষ্ঠান: আন্তর্জাতিক উদারাময় গবেষনা কেন্দ্র, বাংলাদেশ মহাখালী, ঢাকা-১২১২

সম্মতিপত্র "কন্ট্রোল" (প্রাপ্ত বয়স্ক)

"শিগেলা" নামক এক প্রকার জীবানুর আক্রমনে সৃষ্ট রক্ত আমাশয় মানব দেহে মারাত্মক জটিলতা সৃষ্টি করতে পারে। এই রোগে শরীরে কিভাবে জটিলতা সৃষ্টি হয় তা এখন পর্যন্ত সম্পুর্ণরূপে জানা যায়নি। এই রোগটি সম্পর্কে আরো ভালভাবে জানার জন্য এবং এর বিরুদ্ধে প্রতিরোধ ক্ষমতা বাড়ানোর জন্য আমরা একটি গবেষনা করছি। গবেষনার ফলাফল ভবিষ্যতে এ রোগ প্রতিরোধের উপায় সম্পর্কে ভালভাবে জানতে এবং উন্নত চিকিৎসার মাধ্যমে এ রোগ প্রশমনে আমাদের সাহায্য করবে। এই রোগে আক্রান্ত রোগীর অবস্থা সুস্থ ব্যক্তির সাথে তুলনা করার জন্য এই গবেষনায় সুস্থ ব্যক্তির অংশ গ্রহন বাঞ্ছনিয়। এই উদ্দেশ্যে, এই গবেষনায় অংশ গ্রহন করার জন্য আমরা আপনাকে অনুরোধ করছি; আপনার অংশগ্রহন ভবিষ্যতে রক্ত আমাশয়ের প্রকোপ হতে অনেক মূল্যবান জীবন রক্ষা করতে পারে।

এই গবেষনায় একজন দক্ষ চিকিৎসক আপনার প্রয়োজনীয় শারীরিক পরীক্ষা করবে। আমরা মাত্র একবার আপনার কাছ থকে রক্ত, মল এবং মলাশয়ের ঝিল্লি সংগ্রহ করব। আপনার হাতের শিরা থেকে ১০ মিঃলিঃ (দুই চা চামচ) মত রক্ত নেওয়া হবে। যে স্থানে সুঁই প্রবেশ করানো হবে সে স্থানে ক্ষনস্থায়ি ব্যাথা এবং ন্যূনতম ক্ষেত্রে কাল দাগ পড়া ছাড়া রক্ত নেওয়ার সময় আপনার অন্য কোন সমস্যা হবেনা। যে কোন প্রকার সংক্রমন কমাতে আমরা সম্পুর্ণ জীবানুমুক্ত পরিবেশে রক্ত সংগ্রহ করব এবং রক্ত সংগ্রহের জন্য জীবানুমুক্ত সিরিঞ্জ এবং সুঁই (একাবর ব্যবহার করে ফেলে দেওয়া হয় এমন) ব্যবহার করব। আপনার মলদ্বারে একটি নমনীয় নলের মত যন্ত্র (flexible sigmoidoscope) ঢুকিয়ে (মলদ্বার থেকে ১০-১২ সেঃমিঃ অভ্যন্তরে) বৃহদন্তের রেকটোসিগময়েড এলাকা পরীক্ষা করা হবে। এই প্রক্রিয়ায় আপনার মলদ্বারে এক প্রকার পিচ্ছিল জেলী (ব্যাকটেরিয়া প্রতিরোধক) লাগান হবে যাতে আপনার মলদ্বার পিচ্ছিল হয় এবং পরীক্ষার সময় আপনি কোন অসুবিধা বোধ না করেন। আপনার মলাশয় থেকে আট টুকরা ঝিল্লি (সরিষার বীজ আকৃতির, ২ মিঃমিঃ) সংগ্রহ করা হবে। ঝিল্লি সংগ্রহ করার আগে রক্তপাতের নিয়ামকসমূহ (প্রথ্রম্বিন জমাট বাধার সময় এবং অনুচক্রিকার সংখ্যা) পরীক্ষা করা হবে । এই প্রক্রিয়ায় খুবই কম ক্ষেত্রে মলদ্বারে সামান্য রক্তপাত এবং ক্ষত সৃষ্টি হতে পারে। মলাশয়ে ক্ষত হওয়ার সম্ভাবনা খুবই কম থাকবে কারন এই প্রক্রিয়াটি পরিচালিত হবে একজন প্রশিক্ষিত এবং অভিজ্ঞ চিকিৎসক দ্বারা যিনি এই গবেষনার একজন অনুসন্ধানকারী। উপরে উল্লিখিত জটিলতা রোধে সম্ভাব্য সব ধরনের সতর্কতা অবলম্বন করা হবে এবং পরীক্ষার পর ৩-৪ ঘন্টা আপনাকে পর্যবেক্ষনে রাখা হবে যাতে যে কোন ধরনের প্রতিকুল অবস্থা সৃষ্টি হলে

সাথে সাথে ব্যবস্থা নেওয়া যায়। এই প্রক্রিয়ায় যে কোন ধরনের সংক্রমন রোধে যথাযথ ব্যবস্থা গ্রহন করা হবে এবং প্রয়োজনীয় চিকিৎসা সরবরাহ করা হবে।

এই গবেষনার সমস্ত তথ্য / উপাত্ত গোপন রাখা হবে, তবে আপনার অনুরোধে আপনাকে সরবরাহ করা হবে । গবেষনাগারে করা অংশগ্রহনকারীর বিভিন্ন পরীক্ষার তথ্য এবং ফলাফল তালাবদ্ধ করে রাখা হবে এবং শুধুমাত্র এই গবেষনার অনুসন্ধানকারীরা এবং প্রয়োজনে আইনপ্রয়োগকারী সংস্থা এই সমস্ত তথ্য জানবেন। ভবিষ্যতে গবেষনা প্রকাশনার জন্যে যদি এই সমস্ত তথ্য ব্যবহার করা হয় সেক্ষেত্রে অংশগ্রহনকারীর নাম গোপন রাখা হবে ।

এই গবেষনায় আপনি সরাসরি লাভবান হবেন না, কিন্তু গবেষনায় প্রাপ্ত ফলাফল উদারাময় উপদ্রুত এলাকায় বসবাসরত প্রাপ্ত বয়স্ক ব্যক্তির স্বাভাবিক রোগ প্রতিরোধ ক্ষমতা সম্পর্কে জানতে আমাদের সাহায্য করবে এবং ভবিষ্যতে অন্যদের এ রোগের প্রকোপ থেকে রক্ষা পেতে সাহায্য করবে। এই গবেষনায় অংশ গ্রহন করা বা না করার সিদ্ধান্ত একান্ত ই আপনার। এই গবেষনায় অংশ গ্রহন করতে আসার জন্য আপনাকে আপনার ঐ দিনের মজুরি এবং যাতায়াত খরচ আমরা দিয়ে দেব। আপনি যদি স্বেচ্ছায় এই গবেষনায় অংশ গ্রহন করতে সম্মত হন তবে অনুগ্রহ করে নিচে আপনার সই বা বামহাতের বৃদ্ধাঙ্গুলির টিপসই দিন।

আপনার সহযোগিতার জন্য আপনাকে ধন্যবাদ।

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স্বাক্ষর/বাম হাতের বৃদ্ধাঙ্গুলির টিপ সই তারিখঃ

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অনুসন্ধানকারীর স্বাক্ষর তারিখঃ

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প্রত্যক্ষদর্শীর স্বাক্ষর তারিখঃ