International Centre for Diarrhoeal Disease Research, Bangladesh
CENTRE FOR HEALTH AND POPULATION RESEARCH
Mail : ICDDR,B, GPO Box 128, Dhaka-1000, Bangladesh
Phone: 880-2-8811751-60, Telex : 642486 ICDD BJ
Fax : 880-2-8823116, 8812530, 8811568, 8826050, 9885657, 8811686, 8812529
Cable : Cholera Dhaka

ESD
2003

Memorandum

8 December 2003

To : Dr. Firdausi Qadri
Dr. S. M. Faruque
PI of research protocol # 2003-041
Laboratory Sciences Division

From: Professor AKM Nurul Anwar
Chairman
Ethical Review Committee (ERC)

Sub : Approval of research protocol # 2003-041

Thank you for your research protocol # 2003-041 titled "The development of a *Vibrio cholerae* protein array and its application to the study of human immune responses". I have the pleasure to inform you that the ERC in its meeting held on 3rd December 2003 approved the protocol.

You shall conduct the study in accordance with the ERC-approved protocol; and shall be responsible for protecting the rights and welfare of the subjects and compliance with the applicable provisions of ERC Guidelines.

You shall also submit report(s) as required under ERC Guidelines. Relevant excerpt of ERC Guidelines and '*Annual/Completion Report for Research Protocol involving Human Subjects*' are attached for your information and guidance

Thank you and I wish you success in running the above-mentioned study.

cc: Associate Director
Laboratory Sciences Division

Attachment I

Date: 19/11/03

(FACE SHEET)

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Principal Investigator: Tinlawsi Qadri
S. M. Faruque

Application No.

Title of Study: "Development of V. cholerae protein assay and its application to the study of human immune response"

Protocol No: 2003-041

Trainee Investigator (if any): _____

Supporting Agency (if Non-ICDDR,B) NIH

Project Status To be started
RRC approved - 19th Nov, 2003

[✓] 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	No
(e)	Benefits to be derived	Yes	No
(f)	Right to refuse to participate or to withdraw from study	Yes	No
(g)	Confidential handling of data	Yes	No
(h)	Compensation &/or treatment where there are risks or privacy is involved in any particular procedure	Yes	No

5. Will Signed Consent Form be Required: *

(a)	From subjects	Yes	(No)
(b)	From 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.

Principal Investigator

Trainee

* Samples already collected under "protocol entitled" immune responses to V. cholerae in Bangladesh (No. 99006) to be used for the study. On consent form to protocol 99006, permission has been obtained to study immune response using the serum specimens. Stools from patients will be used for the study

Abstract summary

The protocol has two goals. The first goal of this project is to construct a *V. cholerae* "FLEX" gene repository in conjunction with the Harvard Institute of Proteomics (HIP). The *V. cholerae* repository will contain selected genes encoding predicted virulence factors, surface proteins, and *in vivo* expressed antigens discovered by a combination of technologies. This FLEXgene repository will then be used to perfect highly parallel recombinant protein expression methods, such that the expressed, full-length proteins have affinity tags to allow attachment to a microarray or "protein chip".

The second goal is to use the protein microarrays to analyze immune responses to the selected proteins. We will do this by comparing acute and convalescent sera from individual cholera patients in Bangladesh already enrolled in our ongoing project on the immune response studies in cholera (protocol no. 99006). These samples will be tested from randomly selected cholera patients (first 25 patients in the collection) from whom paired serum samples at the acute and convalescent phases are already available in our specimen bank. The microarrays will also be used to determine the responses in sera of rabbits immunized with *V.cholerae* O1. For this purpose stool extracts containing *V.cholerae* O1 and that cultured in the laboratory will be used. We will attempt to proteins on the microarray that react with sera from rabbits immunized with *V. cholerae* recovered directly from stool but not after *in vitro* growth, suggesting those proteins are expressed during *in vivo* infection. These experiments should identify potentially protective protein immune responses that have been simply missed by other methods of analysis. Such a result could contribute significantly to the development of - factors needed for the development of a acellular cholera vaccine based on these immunogenic, *in vivo*-induced proteins.

Clarification of to other points (as required in the attached list) are as follows:

1. Serum samples collected from cholera patients, adults and children will be studied. No consent will need to be taken since we will be testing samples for immune responses in patients and the intention is the same as that of the protocol under which permission for testing samples had been obtained. Cholera stools from patients which have been collected and formalin inactivated and stored at -70°C will also be used for the study

2. No risk is involved and no new samples will be collected.

3. No procedure will be involved which may be of any potential risk.

4. The information regarding the patient that is available from the study is kept confidential in a locked cabinet.

5. No consent form will be needed for the present protocol.

6. NA

7. The study will give useful information on the immune response to *V. cholerae*. The study will help better understanding of the proteins that may be important for virulence during infection and hence in the development of protective immune response.

8. Serum collected from cholera patients in an ongoing study will be used for the study. Formalin inactivated stool samples collected from cholera patients will be used for immunization of the rabbits. A provisional permission from AEEC has been obtained from the committee for the animal experiments.

International Centre for Diarrhoeal Disease Research, Bangladesh
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Cable : Cholera Dhaka

Memorandum

17 November 2003

To : Dr. Firdausi Qadri
Dr. S M Faruque
PIs of research protocol # 2003-041
Laboratory Sciences Division

From: David A Sack, MD
Chairman
Research Review Committee (RRC)
(Signed in his absence by
Professor Barkat-e-Khuda, Acting Director)

Sub : Approval of research protocol # 2003-041

Thank you for your memo dated 16th November 2003 with the modified version of your research protocol # 2003-041 titled: "The development of a *Vibrio cholerae* protein array and its application to the study of human immune responses", which the RRC considered in its meeting held on 13th November 2003. The issues raised by the RRC on your research protocol have been addressed in the modified version of the protocol to the satisfaction of the Committee. Accordingly, this research protocol is approved to proceed subject to the approval of the ERC/AEEC as may be necessary.

Terms of approval

The research protocol is approved as submitted for 2-year period from the date of starting the activities of the protocol. You should therefore notify the Committee Coordination Secretariat of the start date of the protocol.

This approval is only valid whilst you hold a position at the ICDDR,B; and in the event of your departure from the Centre, a new Principal Investigator will be designated for the research protocol.

This approval shall remain valid for starting the protocol for a period up to 2 years from the date of the approval of the ERC/AEEC, after two years, you shall have to seek approval (revalidation) of the RRC/ERC before starting the protocol. The RRC/ERC approval shall automatically deemed to be revoked after three years if the protocol is not started.

You should notify the RRC and the ERC immediately of any serious or unexpected adverse effects on participants or unforeseen events that might affect continued acceptability of the protocol.

Any changes to the research protocol require the submission and approval of an amendment/addendum. Substantial variations may require a new protocol.

Continued approval of this protocol is dependent on your periodically updating the Centre's database for the protocol to show the progress; and a final report/completion report should be submitted at the conclusion of the protocol.

You shall submit a report for time extension of the protocol (in form) if you are unable to complete the protocol within the time mentioned in the protocol.

The RRC should be notified if the project is discontinued before the expected date of completion. The report form is available at the Committee Coordination Secretariat and on the Centre's intranet.

You are responsible for systematic storage and retention of the original data pertaining to the research protocol; and the ownership of data after certain period shall be determined as per Centre's rules and regulations.

I wish you all the success in conducting the research protocol.

Cc: Associate Director
Laboratory Sciences Division

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

RESEARCH PROTOCOL
Protocol No. 2003-041

FOR OFFICE USE ONLY		
RRC Approval:	☒	☒Yes / ☐ Date14th Nov 2003
ERC Approval:	☐Yes / ☐No	Date:
AEEC Approval:	☐Yes / ☐No	Date:

Project Title: The development of a *Vibrio cholerae* protein array and its application to the study of human immune responses

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

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

Key words: *V. cholerae*, proteomics, gene expression, human immune response

Relevance of the protocol: The completion of the *Vibrio cholerae* genome sequence has provided the opportunity to apply highly parallel methods to the analysis of gene expression and human immune responses to *V. cholerae*. The initial goal of this project is to construct a *V. cholerae* "FLEX" gene repository in conjunction with the Harvard Institute of Proteomics (HIP). The *V. cholerae* repository will contain selected genes encoding predicted virulence factors, surface proteins, and *in vivo* expressed antigens discovered by a combination of technologies. This FLEXgene repository will then be used to perfect highly parallel recombinant protein expression methods, such that the expressed, full-length proteins have affinity tags to allow attachment to a microarray or "protein chip". Finally, we will use these protein chips to analyze human immune responses to the selected proteins, comparing acute and convalescent sera from individual cholera patients in Bangladesh enrolled in our ongoing Cholera Immune Response Study (CIRS). All of the work in the second year of the proposal will be conducted in Bangladesh, resulting in significant technology transfer and enhanced research capacity at the International Centre for Diarrhoeal Disease Research.

Programmes

- ☐ Child Health Programme
- ☐ Nutrition Programme
- ☒ Programme on Infectious Diseases & Vaccine Science
- ☐ Poverty and Health Programme
- ☐ Health and Family Planning Systems Programme
- ☐ Population Programme
- ☐ Reproductive Health Programme
- ☐ HIV/AIDS Programme

Principal Investigator: Dr. Firdausi Qadri/ Dr. S.M. Faruque **Division:** LSD **Phone:** 8811751-60 Extn, 2431,2410

Address: Mohakhali Dhaka, 1212, Dhaka, Bangladesh
Email: fqadri@icddrb.org,faruque@icddrb.org

Co-Principal Investigator(s): Dr. Stephen Calderwood, Dr. Regina LaRocque Dr. Leonardo Brizuela
Co-Investigator(s): Dr. Edward Ryan, Dr. Jason Harris, Dr. G.B.Nair

Revised on:15th April 2003

Student Investigator/Intern:

Collaborating Institute(s): Division of Infectious Diseases, Massachusetts General Hospital, Boston, Massachusetts Harvard Institute of Proteomics, Harvard Medical School, Boston, Massachusetts

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

Gender
- ☐ Male
- ☐ Females

Age
- ☐ 0 – 5 years
- ☐ 5 – 9 years
- ☐ 10 – 19 years
- ☐ 20 – 64 years
- ☐ 65 +

- ☐ Pregnant Women
- ☐ Fetuses
- ☐ Prisoners
- ☐ Destitutes
- ☐ Service providers
- ☐ Cognitively Impaired
- ☐ CSW
- ☐ Others (sera collected from patients in ongoing study, protocol- 99006 to be used)
- ☒ Animal

Project / study Site *(Check all the apply)*:
- ☐ Dhaka Hospital
- ☐ Matlab Hospital
- ☐ Matlab DSS area
- ☐ Matlab non-DSS area
- ☐ Mirzapur
- ☐ Dhaka Community
- ☐ Chakaria
- ☐ Abhoynagar
- ☐ Mirsarai
- ☐ Patyia
- ☐ Other areas in Bangladesh _____
- ☐ Outside Bangladesh
  name of country: _____
- ☐ Multi centre trial
  (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 (cohort or follow-up)
- ☐ Record Review
- ☐ Prophylactic trial
- ☐ Surveillance / monitoring
- ☐ Others: genetics and immunological 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: ☐

Acute and convalescent serum sample from 25 patients from ongoing study (protocol no. 99006 to be used)

Sub-group _____ ☐ ☐

Determination of Risk: Does the Research Involve *(Check all that apply)*:
- ☐ Human exposure to radioactive agents?
- ☐ Fetal tissue or abortus?
- ☐ Investigational new device?
  (specify_____)
- ☐ Human exposure to infectious agents?
- ☐ Investigational new drug
- ☐ Existing data available via public archives/source
- ☐ Pathological or diagnostic clinical specimen only

☒ Existing data available from investigator **Protocol 99006** ☐ 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
☐ only part of the diagnostic test NA

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: NIH

Yes/No

☐ ☒ Is the proposal being submitted for funding ?

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

(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

(Day, Month, Year - DD/MM/YY)

Beginning date___ASAP
End date___Two years from starting date

Cost Required for the Budget Period ($)

a. Ist Year *2nd Year* *3rd Year* *Other years*

US$ 6,480 *164,751*

b. Direct Cost :US $;163,352, *Total Cost* :US $171,231

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.

G·B·Nair
Name of the Associate Director

T. Sadhu
IN G·B·Nair
Signature

5/11/03
Date of Approval

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 PI Shiv Kapoor T. [illegible]

Date: 5/11/03

Name of Contact Person (if applicable)

Table of Contents

☐ Check here if appendix is included

PROJECT SUMMARY: Describe in concise terms, the hypothesis, objectives, and the relevant background of the project. Describe concisely the experimental design and research methods for achieving the objectives. This description will serve as a succinct and precise and accurate description of the proposed research is required. This summary must be understandable and interpretable when removed from the main application. (TYPE TEXT WITHIN THE SPACE PROVIDED).

Principal Investigators
Dr. Firdausi Qadri, Dr. S.M. Faruque,

Project Name
The development of a *Vibrio cholerae* protein array and its application to the study of human immune responses

Total Budget **US $ 171,231** Beginning Date ASAP Ending Date 2 years from starting date

The completion of the *Vibrio cholerae* genome sequence has provided the opportunity to apply highly parallel methods to the analysis of gene expression and human immune responses to *V. cholerae*. The initial goal of this project is to construct a *V. cholerae* "FLEX" gene repository similar to those already being constructed by the HIP for the yeast, human, and *Pseudomonas aeruginosa* genomes. The *V. cholerae* repository will contain selected genes encoding predicted virulence factors, surface proteins, and *in vivo* expressed antigens discovered by a combination of technologies. This FLEXgene repository will then be used to perfect highly parallel recombinant protein expression methods, such that the expressed, full length proteins have affinity tags to enable attachment to a microarray or "protein chip". Finally, we will use these protein chips to analyze human immune responses to the selected proteins, comparing acute and convalescent sera from individual cholerae patients in Bangladesh enrolled in the CIRS. All of the work in the second year of the proposal will be conducted in Bangladesh, resulting in significant technology transfer and enhanced research capacity at the International Centre for Diarrhoeal Disease Research in Bangladesh.

There are three **SPECIFIC AIMS** in the present proposal. In **Specific Aim #1**, we will construct a *V. cholerae* FLEXgene repository of approximately 140 clones of key genes. Each clone in the repository will consist of an independent and unique bacterial open reading frame (from the initiation codon to the last codon) cloned into a "master vector" and sequence confirmed. In **Specific Aim #2**, we will develop a *V. cholerae* FLEXprotein repository of a subset of these selected genes. Each ORF will be moved by directional recombinational cloning into a variety of expression vectors, introducing amino- and/or carboxy-terminal affinity tags to the encoded proteins as needed. We will utilize two different approaches to express and purify as many of the ORFs in the FLEXgene repository as possible: (a) introducing the expression plasmids into *Escherichia coli* to express individual proteins. Because of the ease of cloning from the master vector to a variety of different expression vectors by recombinational cloning, we can investigate several different expression vectors to determine an optimal system(s) for each protein; (b) we will also investigate the use of a novel approach being developed at HIP, termed Nucleic Acid-Programmable Protein Array (NAPPA). In this approach, genes encoding ORFs are moved into a protein expression plasmid, these plasmids themselves are spotted on a microarray, and *in vitro* transcription/translation is carried out *in situ*. The expressed protein has an appropriate tag at one end, facilitating capture of the translated product directly at that spot on an appropriately coated microarray. In **Specific Aim #3**, we will investigate use of the *V. cholerae* FLEXprotein microarray to screen for immune responses against these key proteins. In the first approach, we will compare recognition of proteins on the microarray by convalescent versus acute sera from patients infected with *V. cholerae* enrolled in the CIRS. Detection of antibody bound to the protein on the microarray is done by adding a second, fluorescently labeled, anti-human IgG antibody. In the second approach, rice-water stool from patients with acute cholera is directly mixed with formalin at the bedside, to preserve proteins expressed *in vivo* by *V. cholerae*. For comparison, *V. cholerae* is grown *in vitro* and also treated with formalin. Formalin-fixed organisms from both samples are recovered, and proteins recovered from lysates. Rabbits are immunized with these preparations, and the sera from the rabbits is used to probe the protein microarray. We wish to identify proteins on the microarray that react with sera from rabbits immunized with *V. cholerae* recovered directly from stool but not after *in vitro* growth, suggesting those proteins are expressed during *in vivo* infection. These experiments should identify potentially protective protein immune responses that have been simply missed by other methods of analysis. Such a result could contribute significantly to the development of an acellular cholera vaccine based on these immunogenic, *in vivo*-induced proteins.

9/9/2

KEY PERSONNEL (List names of all investigators including PI and their respective specialties)

Name		Professional Discipline/ Specialty	Role in the Project
1.	Firdausi Qadri	Immunology Section, LSD, ICDDR,B	Co-PI (LSD)
2	S.M.Faruque	Molecular Genetics Section, LSD, ICDDR,B	Co-PI (LSD)
3.	G.B. Nair	ICDDR,B	Coinvestigator
4.	Stephen B. Calderwood	Clinical Medicine, Immunology, Microbiology Massachusetts General Hospital, Boston, MA, USA	Co-PI (MGH)
5.	Edward T. Ryan	Clinical Medicine, Immunology, Microbiology: Massachusetts General Hospital, Boston, MA, USA	Co-invest (MGH)
6.	Regina C. LaRocque	Clinical Medicine, Immunology, Microbiology: Massachusetts General Hospital, Boston, MA, USA	Research Fellow
7.	Leonardo Brizuela	Biologist, Harvard Institute of Proteomics	Co-invest (HIP)
8.	Jason B. Harris	Clinical Medicine, Immunology, Microbiology: Massachusetts General Hospital, Boston, MA, USA	Research Fellow
9.	Regina C. LaRocque	Clinical Medicine, Immunology, Microbiology: Massachusetts General Hospital, Boston, MA, USA	Research Fellow
10.	John J. Mekalanos	Microbiology Harvard Medical School, Boston, MA, USA	Collaborator
11.	Joshua LaBaer	Harvard Institute of Proteomics	Collaborator

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.

Hypothesis **:**

A protein microarray representing key open-reading frames of *V. cholerae* can be developed and used as a high-throughput tool to assess human immune responses during cholera infection in the endemic setting of Bangladesh.

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).

In **Specific Aim #1**, we will construct a *V. cholerae* FLEXgene repository of ca. 140 clones of key genes. Each clone in the repository will consist of an independent and unique bacterial open reading frame (from the initiation codon to the last codon) cloned into a "master vector" and sequence confirmed.

In **Specific Aim #2**, we will develop a *V. cholerae* FLEXprotein repository of a subset of these selected genes.

In **Specific Aim #3**, we will investigate use of the *V. cholerae* FLEXprotein microarray to screen for immune responses against these key proteins.

Background of the Project including Preliminary Observations

Describe the relevant background of the proposed study. Discuss the previous related works on the subject by citing specific reference++++--999*s. Describe logically how the present hypothesis is supported by the relevant background observations including any preliminary results that may be available. Critically analyze available knowledge in the field of the proposed study and discuss the questions and gaps in the knowledge that need to be fulfilled to achieve the proposed goals. Provide scientific validity of the hypothesis on the basis of background information. If there is no sufficient information on the subject, indicate the need to develop new knowledge. Also include the **significance and rationale** of the proposed work by specifically discussing how these accomplishments will bring benefit to human health in relation to biomedical, social, and environmental perspectives. (DO NOT EXCEED 5 PAGES, USE CONTINUATION SHEETS).

1. Pathogenesis of *V. cholerae* infection. *V. cholerae* is a gram-negative bacillus that causes a severe, dehydrating diarrhea in humans (Kaper 1995). There are an estimated 5 to 7 million cases worldwide of cholera each year, with more than 100,000 deaths. Cholera is endemic in a number of countries worldwide. In such countries, for example in Bangladesh and India, cholera cases occur year-round, although the majority of cases occur during distinct cholera seasons (Glass 1982).

V. cholerae can be differentiated by the lipopolysaccharide (LPS) component of the outer membrane. Strains of *V. cholerae* that produce cholera belong to serogroup O1 or O139. *V. cholerae* O1 is divided into two biotypes, classical and El Tor, and the O1 serogroup can be differentiated into two major serotypes, Inaba and Ogawa (Kaper 1995). Serotype differences are due to variation in presence of three O antigen factors, A, B and C (Stroeher 1992). In 1992, *V. cholerae* O139 also emerged as a cause of cholera in South and Southeast Asia (Ramamurthy 1993). Several lines of evidence suggest that this new serogroup is derived from El Tor biotype of *V. cholerae* O1, by substitution of genes encoding the O139 O antigen and acquisition of the ability to produce a capsule (Bik 1996; Calia 1994; Comstock 1996; Comstock 1995).

The major virulence factor for all toxigenic strains of *V. cholerae* is cholera toxin, which consists of a single, enzymatically active A subunit associated with five B subunits (Gill 1976). During assembly of cholera holotoxin, A and B subunits transiently enter the periplasmic space, and assemble before being secreted across the outer membrane by a dedicated type II secretion system (Hirst 1987; Sandkvist 1993). The pentamer of B subunits binds to its receptor on the eukaryotic cell, the ganglioside GM_1, and the A subunit is translocated, where it activates adenylate cyclase, elevates cAMP, and causes chloride secretion through the apical CFTR transmembrane channel, causing secretory diarrhea (Cassel 1978; Gabriel 1994; Gill 1975; Gill 1978; Mekalanos 1979).

A second major virulence factor of *V. cholerae* is the toxin-coregulated pilus (TCP), whose expression is regulated in classical strains of *V. cholerae* by the same environmental growth conditions as is cholera toxin (Taylor 1987). TCP is essential for colonization and virulence in both animal models and human volunteer studies (Taylor 1987; Herrington 1988). The major structural protein of TCP is TcpA a 20.5 kDa structural protein homologous to the type IV pili of *Neisseria gonorrhoeae, Pseudomonas aeruginosa, Moraxella bovis,* and *Bacteroides nodosus* (Shaw 1990).

In addition to TCP, the *V. cholerae* genome encodes two other type IV pili, the mannose-sensitive hemagglutinin (MSHA) and PilA (Heidelberg 2000) MSHA is expressed by *V. cholerae* O1 El Tor strains but is rarely expressed by classical strains (Jonson 1991a). Recent studies have shown that mutation of *mshA* or pre-treatment with antibody to MshA have no effect on the ability of El Tor *V. cholerae* to colonize mice and a deletion of *mshA* in *V. cholerae* showed no defect in colonization of human volunteers (Tacket 1998). More recently, Fullner *et al.* (1999) described a four-gene cluster, *PilABCD*, encoding a third type IV pilus in *V. cholerae*. Deletion of *PilA* had no effect on colonization in infant mice. PilD had previously been shown to be the prepilin peptidase for a number of type IV pilus-related proteins, including four proteins in the Eps cluster (EpsGHIJ), four proteins in the MSHA cluster (MshABCD), and one type IV pilus-like gene in the TCP cluster, TcpB (Marsh 1998). However, no other work has been done previously on the PilA pilus of *V. cholerae*.

In addition, a number of other gene products are associated with *V. cholerae* adherence and/or colonization, including those encoded by the *acf* cluster (accessory colonization factor) (Peterson 1988; Parsot 1991), a hemagglutinin not inhibited by mannose, fucose and other sugars (MFRHA) (Franzon 1993; Franzon 1986), and the outer membrane protein IrgA (iron regulated protein A) (Goldberg 1990). TCP, however, is the only *V. cholerae* colonization factor whose importance in human disease has been demonstrated (Kaper 1995; Herrington 1988).

2. Immune responses to *V. cholerae* infection. Infection with *V. cholerae* induces protective immunity against subsequent cholera (Cash 1974a; Cash 1974b; Levine 1979). In Bangladesh and cholera caused by classical

strains severe enough to require medical attention at a health care facility has been shown to decrease the risk of subsequent disease by approximately 90% (Glass 1985a). Studies from Bangladesh have also shown a higher degree of protection from subsequent disease after infection with *V. cholerae* classical strains than El Tor strains (Glass 1985a; Clemens 1991). Similarly, volunteer studies in non-endemic settings have shown that infection with classical biotype *V. cholerae* O1 provides 100% protection from disease due to classical biotypes, while El Tor infection provides 90% protection from disease due to El Tor biotypes. Such protection has been shown to last at least three years in volunteer studies (Levine 1981). In these studies, infections with Ogawa or Inaba serotypes protected from re-infection with both serotypes, although cross challenge was more likely to be associated with excretion of *V. cholerae* in stool, and prolonged stool excretion was more likely to be associated with clinical diarrhea at rechallenge (Losonsky 1996).

Systemic immune responses after infection with *V. cholerae* have been studied in depth, though many details of protective mucosal immune responses after cholera have not yet been elucidated (Kaper 1995; Levine 1992; Svennerholm 1994). Both systemic and mucosal antibacterial and antitoxin immune responses are measurable after cholera. Antibacterial immune responses appear to be the more important of the two since protection from clinical disease is associated with the inability to culture *V. cholerae* from stool of volunteers after re-challenge, and since previous disease due to cholera toxin-producing *V. cholerae* O1 does not protect from subsequent disease caused by cholera toxin-producing *V. cholerae* O139, and since nontoxigenic vaccine strains of *V. cholerae* induce immunity equivalent to that conferred by toxigenic strains (Levine 1988).

The best characterized of the antibacterial immune responses induced by *V. cholerae* is the vibriocidal antibody. The vibriocidal antibody assay is a bactericidal assay requiring the presence of complement-fixing antibody bound specifically to vibrios; this serum antibody response increases after clinical cholera. The vibriocidal response is comprised of both IgM and IgG antibodies and subsequent infection/rechallenge may lead to maturation of the vibriocidal response from IgM to IgG (Losonsky 1996). In non-endemic settings and in volunteer studies, a marked increase in both vibriocidal and antitoxic antibody titers occur in infected individuals after clinical disease (Cash 1974b; Levine 1981; Snyder 1981, Clements 1982); vibriocidal antibody levels return to baseline within six months of primary disease; and antitoxin levels decline over 1-2 years. Epidemiological studies in Bangladesh have shown that vibriocidal antibody levels increase with age. In this endemic setting, vibriocidal antibodies are detectable in approximately 40-80% of individuals by the age of 10-15 years (Glass 1985a; Mosley 1968a; Mosley 1968b; Mosley 1969a). Studies in Bangladesh have also demonstrated that an inverse relationship exists between vibriocidal antibody titers and the susceptibility of individuals to intestinal colonization with *V. cholerae* and symptomatic cholera (Glass 1985a; Mosley 1968a; Mosley 1968b; Mosley 1969a). In such seroepidemiological studies, every two-fold rise in the vibriocidal geometric mean titer is associated with an approximately 50% decrease in the attack rate of cholera (Glass 1982; Glass 1985a; Mosley 1969a; Mosley 1969b; Mosley 1970). No such association is found for any other systemic immune response yet examined.

It is currently unclear why the vibriocidal response is the only recognized predictor of protection from cholera, and why no such association exists for serum antitoxin responses (Benenson 1968; Glass 1983). Since *V. cholerae* is a non-invasive organism, and there is no disruption of the intestinal epithelium during cholera, a serum complement-fixing antibody response would be predicted to have minimal activity during mucosal infection with *V. cholerae*. Non-complement binding secretory IgA (sIgA) is the primary antibody in the intestinal lumen and would be predicted to be the primary active antibody during cholera. One possibility is that vibriocidal antibody is a surrogate marker for intestinal sIgA responses that are the primary mediators of immunity to cholera (Kaper 1995).

The primary component of the vibriocidal antibody response is most often thought to be directed against LPS (Kaper 1995). Some researchers, however, feel that a majority of vibriocidal antibody activity may actually be directed against *V. cholerae* membrane proteins (Richardson 1989; Sears 1984; Attridge 1983; Neoh 1970). Studies vary, but it appears that a large component of the vibriocidal antibody response can be absorbed with *V. cholerae* LPS (Holmgren 1977). Short term protection against *V. cholerae* can also be induced with parenteral administration of purified LPS (Mosley 1970). Despite such findings, studies in Bangladesh have failed to show a relationship between levels of serum anti-LPS IgG and protection from cholera (Glass 1985a).

Other immune responses which have been studies in V. cholerae infection include those directed against its type IV pili, including MSHA and and TCP. In Bangladesh, approximately 50% of patients with *V. cholerae* O1 or O139 infection develop serum IgG and approximately 65% develop serum IgA antibody to MSHA (Qadri 1997b). Twenty of 28 (86%) of these Bangladeshi patients with *V. cholerae* O1 and 11 of 17 (65%) patients with *V. cholerae* O139 showed increases in IgA and IgM antibody-secreting cells specific for this antigen, and approximately 70% of infected individuals developed anti-MSHA antibodies in stool (Qadri 1997b). Overall, approximately 90% of individuals in Bangladesh develop anti-MSHA responses after cholera (Qadri 1997b). Anti-MSHA immune responses have not been measured in volunteer studies, but in Peru, where cholera has only

recently been reintroduced, anti-MSHA response rates after cholera appear to be approximately 60% (Svennerholm 1994).

Intestinal colonization by *V. cholerae* is a prerequisite for the development of immune responses during cholera, and TCP has been shown to be required for intestinal colonization by *V. cholerae* (Herrington 1988). In volunteer studies in North America, anti-TCP responses were not found in convalescent sera of individuals after challenge with *V. cholerae*; however, low level anti-TCP immune responses were present in convalescent sera from 3 of 6 individuals with cholera in an endemic area, Indonesia (Hall 1991). It is not clear why antibody responses to a key colonization antigen, TCP, are not more prominent in normal volunteers but perhaps immune responses to this pilus require repeated intestinal exposures to *V. cholerae* (see Preliminary Studies below for our recent data). No studies have yet been published on the role of the PilA pilus in human infection, nor on whether humans develop an immune response to this protein following natural infection or challenge of volunteers (see Preliminary Studies below for our recent data).

Cholera toxin (CT) is a potent immunogen and a very potent immunoadjuvant (Kaper 1995; Lycke 1986). The majority of the anti-toxin immune response is directed against the B subunit (CtxB), while the immunoadjuvant properties of holotoxin are associated with the enyzmatically active A subunit (Lycke 1997; Lycke 1992). Serum anti-CT and anti-CtxB responses increase markedly after cholera; however, antitoxin responses have not been shown to protect from intestinal colonization or from clinical disease (Glass 1985a). Seroepidemiological studies in Bangladesh have not shown an increase in the prevalence or titers of anti-CT serum antibody with age, in contrast to what is seen with vibriocidal antibodies (Benenson 1968). Serum anti-cholera toxin immune responses have, however, been shown to be sensitive markers for recent exposure to CT-producing *V. cholerae* (Glass 1985a).

To summarize our understanding of the immune response to cholera, despite four decades of characterizing immune responses following *V. cholerae* infection and challenge studies in humans, there is still no satisfactory immune response documented that has sufficient sensitivity and specificity to adequately predict protection from intestinal colonization or clinical illness following exposure. The serum vibriocidal antibody response is the best predictor currently available, but the components of *V. cholerae* against which the vibriocidal antibody response are directed have not been fully defined. Definition of the antigens mediating protective immunity to *V. cholerae* would allow identification of individuals in a population susceptible to disease on exposure, provide an important immune response to measure in vaccine trials, and potentially allow design of a vaccine containing specific protective antigens.

3. Study of gene expression in *V. cholerae in vivo*, in animal models. A number of techniques have been described to identify gene expression in bacteria *in vivo*, in animal models. The first of these techniques was *in vivo* expression technology (IVET), a technique that generated fusions between bacterial promotor fragments and two genes, one essential for *in vivo* growth and the other providing an indicator activity for *in vitro* expression. A modification of IVET was subsequently developed by Camilli *et al* (Camilli 1994; Camilli 1995), in which the promotor fragments were fused to a gene encoding transposase, and cloned in a *V. cholerae* strain that contained a tetracycline resistance cassette elsewhere in the chromosome, flanked by two *res* sites, such that activation of the promotor induced expression of transposase and resulted in excision of the tetracycline resistance cassette. Strains containing *in vivo*-activated promotors could be screened for as tetracycline-resistant clones following *in vitro* growth (promotor OFF), but tetracycline-sensitive clones (promotor ON) following infant mouse infection. This procedure, recombinase IVET (RIVET) identified a number of genes in *V. cholerae* specifically induced during infection of infant mice, including a methyl-accepting chemotaxis protein and VieSAB, encoding an *in vivo*-expressed sensor kinase and two distinct response regulatory proteins (Camilli 1995; Lee 1998). RIVET has subsequently been used to study the spatiotemporal expression of selected *V. cholerae* genes (*tcpA* and *ctxAB*) in infant mice (Lee 1999), as well as in a mutagenesis screen to identify *V. cholerae* genes required for expression of *ctxAB* and *toxT in vivo* (Lee 2001). This latter screen identified several chemotaxis genes, a methyl-accepting chemotaxis protein, *capK* (involved in biofilm-associated exopolysaccharide synthesis), *rtxB* (encoding the transport gene for the RTX toxin of El Tor *V. cholerae*; Fullner 1999b), *vieS*, *mfrha* (the mannose/fucose-resistant hemagglutinin), and other genes, as important regulators of *in vivo* colonization in mice.

Signature-tagged mutagenesis (STM) is another procedure designed to identify genes uniquely expressed *in vivo*. This procedure utilizes specific sequence tags to individually mark each of a set of mutants. Such a set is then used to infect an animal model and following *in vivo* growth, the pooled organisms recovered are compared to the input pool for the absence of sequence-tagged mutants; these mutants lack the ability to grow *in vivo* and are therefore absent in the output pool (Hensel 1995). Application of STM to *V. cholerae* has suggested that a number of genes are necessary for growth in infant mice, including genes in the TCP cluster, as well as *pta*, *ptlA*, and genes involved in purine biosynthesis (Chiang 1998). However, since humans are the only known

natural hosts for *V. cholerae* infection, determination of genes expressed during infection in animal models may not identify genes uniquely required for human infection.

4. Gene microarray analysis on human samples. Recently, our group has collaborated with the Camilli and Schoolnik labs to apply gene microarray technology to directly assay the expression of genes in *V. cholerae* recovered from stool of human patients with cholera, using as a comparison sample, genes expressed *in vitro,* to ascertain genes specifically up-regulated during human infection (Merrell 2002). Our results with this approach suggest that *V. cholerae* recovered from human stool show *down*-regulated expression of the known *V. cholerae* virulence factors, such as cholera toxin, TCP, and others, as well as genes involved in chemotaxis and methyl-accepting chemotaxis proteins. In contrast, there is *up*-regulation of genes required for biosynthesis of amino acids, iron uptake, and formation of a periplasmic nitrate reductase complex. These results, which provide new insights into the state of *V. cholerae* excreted in the stool (and potentially hyperinfectious for the next host), also suggest that *V. cholerae* recovered from stool may express different genes than those at the site of primary infection in the upper small intestine. This suggests that while gene microarray experiments may continue to yield insights on genes in *V. cholerae* expressed *in vivo*, microarray experiments on organisms recovered from stool may not provide information about the key antigens expressed during human infection of the upper gastrointestinal tract.

5. Application of proteomics to *in vivo* gene expression by bacteria. Over the last few years, genome sequencing projects have generated a vast amount of information and revolutionized biological research. DNA microarray-based studies of large numbers of genes is just one example of methods used for high-throughput manipulation and analysis of gene expression. Nevertheless, in spite of the spectacular advances made in this area, the genome projects will not truly benefit human health without detailed biochemical analyses of protein function, which are required understanding of how they can be targeted for therapeutic intervention, and to obtain information about the human immune responses to individual bacterial proteins.

One critical deficiency in genomic studies is that the bulk of sequence information has yet to be converted into forms that can be used easily in high-throughput experiments. For instance, genomic sequence information has primarily been used to generate small oligonucleotides for DNA microarrays and transcriptional profiling experiments, while most functional assays are still being performed with proteins expressed from genomic or cDNA libraries. These libraries almost invariably exist as pools of clones, and moreover suffer from problems of incomplete gene representation and the presence of non-coding regions that may affect gene expression. The traditional libraries are consequently incompatible with high-throughput experimentation, and cannot take advantage of genomic sequence information.

Two different applications of proteomics are being developed to complement genomic studies. In the first, proteins expressed during *in vivo* infection are identified using mass spectroscopy and other techniques, yielding information similar to that revealed by gene microarray experimentation. While useful results come from such proteomic experiments, they suffer the same concerns as application of gene microarray to specific human samples, i.e., that the organisms recovered for example in stool may not be representative of organisms at a different infectious site.

The second application of proteomics is at a much earlier stage of development and seeks to construct a protein expression library of each gene in the genome of the organism of interest. To this end, the Harvard Institute of Proteomics (HIP; http://www.hip.harvard.edu) is combining DNA sequence data with novel recombinational cloning techniques to create a new type of gene library. These libraries, referred to as FLEX (Full-Length Expression-ready) repositories, consist of individual DNA clones that each encode a different full-length open reading frame (ORF). A FLEXgene repository when complete, therefore, would contain all the genes of a particular organism as a set of fully arrayed, sequence-verified, and annotated (in a relational database) full-length DNA clones (Brizuela 2001). The clone of each ORF can be easily moved into a protein expression vector, and these used to create a protein microarray or FLEXprotein repository (see below). All of the FLEX repositories being constructed at HIP are freely available to all scientists (academic, governmental, or commercial), in the hope that they will eventually provide a universal research standard. Access to the

information in the FLEX repositories will allow the development of novel experimental technologies, such as assaying for novel functional activities or examining the properties of panels of purified proteins.

6. Preliminary Studies.

a. IVIAT. *In vivo*-induced antigen technology (IVIAT) is a novel method that circumvents the limitations of use of animal models and microarray experiments on organisms in stool, allowing the direct identification of proteins expressed at sufficient levels during human infection to be immunogenic (Handfield 2000). In this procedure, sera from patients convalescing from the infection in question are pooled and extensively absorbed against the *in vitro*-grown cognate pathogen; this procedure removes antibodies recognizing antigens expressed during *in vitro* growth. The absorbed convalescent sera is then used to probe a library of genomic fragments of the pathogen of interest, expressed from an inducible promoter in *E. coli*, using a Western blot approach. Clones in the expression library reacting with the absorbed, convalescent sera encode proteins expressed during human infection, but not expressed during *in vitro* growth.

We have used the IVIAT procedure of Handfield *et al.* (Handfield 2000) to screen for genes in *V. cholerae* expressed during human infection (unpublished data). For these studies, we pooled sera from ten patients convalescing from cholera, extensively absorbed this pooled sera against an *in vitro*-grown El Tor *V. cholerae* O1 strain isolated from one of the ten patients, and then used the sera to probe an expression library of genomic fragments from El Tor *V. cholerae* O1 strain N16961 (Heidelberg 2000). Using this approach, we identified more than thirty positive clones. Included in these positive clones are genes previously detected by a variety of screens in infant mice using IVET, RIVET, and STM (Camilli 1995; Chiang 1998; Lee 2001), including TcpA, three methyl-accepting chemotaxis proteins, and CapK. This is the first documentation of a strong human immune response to TcpA following El Tor *V. cholerae* O1 infection, with particular note that the gene encoding *tcpA* was hit 8 different times in the screen. We do not yet know whether TcpA reactivity was found at high levels in a subset of patients out of the ten whose sera was pooled, or if most or all of the patients are reactive.

The IVIAT approach also identified reactivity of convalescent sera with clones not previously detected in screens using infant mice, such as clones encoding TcpF; MshA; LuxP (a periplasmic protein that is a key component in one of the three quorum-sensing systems in *V. cholerae* recently described; Miller 2002), and four hypothetical ORFs. Of particular interest, all four of the hypothetical ORFs identified are encoded on chromosome II of *V. cholerae*, the smaller chromosome (Trucksis 1998), which has many fewer genes required for growth in standard laboratory media but that contains many of the genes necessary for adaptation of *V. cholerae* to grow in unique environments (Heidelberg 2000). The fact that all four hypothetical ORFs were from chromosome II raises the possibility that these four ORFs may encode functions specifically required for growth in the human intestine.

Most interestingly, the gene sequence identified most frequently in the IVIAT screen was that encoding PilA, the structural subunit of the third type IV pilus in *V. cholerae* (17 hits in the screen). In addition, IVIAT identified antibody in convalescent sera to PilQ, a protein that acts as an outer membrane secretin for type IV pili in other bacterial species. This is the first suggestion that PilA and its assembly apparatus may be expressed during human infection and may be involved in colonization of the human gastrointestinal tract. The fact that convalescent sera from cholera patients specifically recognizes PilA and PilQ suggests that the genes for these proteins may be uniquely expressed during human infection, and may play a role in *V. cholerae* pathogenesis not suspected by previous *in vitro* or animal model experiments.

These results suggest that IVIAT provides a technology to directly detect genes expressed during human infection that are not detected by current methods using animal models. One limitation of our IVIAT screen so far is that the screen is clearly not yet saturated. For example, we did not yet identify any clones encoding the genes for cholera toxin, which must be expressed during human infection and which we know is immunogenic. The opportunity to screen a FLEXprotein microarray of key proteins from *V. cholerae*, utilizing convalescent human sera, would be a natural complement to our ongoing IVIAT studies.

b. FLEXgene and FLEXprotein arrays. Recently, HIP has taken on the task of constructing a FLEX repository for all of the genes in the *Pseudomonas aeruginosa* genome. In a matter of several months, 5,070 genes encoding ORFs from this organism have been successfully cloned into a FLEXgene repository. We therefore believe that construction of the *V. cholerae* FLEX repository of 140 ORFS proposed below can be accomplished relatively quickly and easily.

Most recently, investigators at the HIP (collaborators on the present application) have described expression of individual proteins in bacteria from a human FLEXgene repository, and high-throughput

purification of these expressed proteins under a variety of conditions (Braun 2002). They have begun working on an improved expression system, called Nucleic Acid-Programmable Protein Array (NAPPA), which is described in the Research Design and Methods below and is a very novel means of creating a protein microarray at high density, sufficient for measuring immune responses to a large number of proteins simultaneously.

In conclusion, we believe that the study of *V. cholerae* biology and pathogenesis, and the development of effective vaccines against *V. cholerae*, depend on the identification of protective immune responses to key proteins of the organism expressed during human infection. Constructing a *V. cholerae* FLEX library of these key proteins will allow us to fully exploit the recently obtained genomic information of this pathogen (Heidelberg 2000) in designing and performing experiments to characterize gene expression *in vivo* and human immune responses to *in vivo* induced proteins. The development of a *V. cholerae* FLEX library of key proteins on a microarray will also allow us to transfer and establish key technology for analysis of immune responses to *V. cholerae* infection in the endemic area of Bangladesh. We propose to focus initially on a subset of approximately 140 key proteins in *V. cholerae*, both to establish and test the methodology in a manner that provides the highest likelihood of success, as well as because the limited funds available in the current supplement do not yet allow a genome-wide approach.

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).

1. Overview.

In the first year of the project, we propose to select ca. 140 key open reading frames from the genomic sequence of El Tor *V. cholerae* O1 (Heidelberg 2000), PCR amplify each of these ORFs (without 5' and 3' regions) into a "master vector", flanked by sites that facilitate directional recombinational cloning into a variety of expression vectors. Each of these ORFs will be sequence confirmed, to insure that our *V. cholerae* FLEXgene repository of key genes is correct in sequence. We will then utilize two different approaches to expressing proteins from this FLEXgene repository, one which involves moving the gene for each ORF into a protein expression vector, expressing individual proteins in *E. coli*, and spotting the individually expressed proteins onto a protein microarray; and the other, a novel approach termed NAPPA, in which the genes encoding the ORFs are moved into a protein expression plasmid. These plasmids are spotted themselves on a microarray and *in vitro* transcription/translation occurs *in situ*, incorporating an appropriate tag on the expressed protein, therefore allowing capture of the translated protein directly at that location on an appropriately coated microarray.

In year 1, the construction and sequencing of the FLEXgene repository of the ca. 140 ORFs of *V. cholerae*, and construction of the FLEXprotein microarray will be done through a subcontract with the Harvard Institute of Proteomics. Testing of the protein microarrays for specific reactivity with sera from patients convalescing from cholera will be done at the Massachusetts General Hospital. In year 2, we will take approximately 100 of the spotted protein microarrays to the International Centre for Diarrhoeal Disease Research (ICIDR,B) in Bangladesh, for two sets of studies: one will compare acute and convalescent sera from patients with cholera for immune responses to the proteins on the microarray; and the second will utilize immunization of rabbits with formalin-fixed *V. cholerae* recovered directly from human stool, to ascertain proteins expressed in *V. cholerae* during human infection. All of the experiments in year 2 will be conducted at the ICIDR,B, and the post-doctoral fellow on the project, Dr. Regina LaRocque, will work full-time in Bangladesh that second year, to transfer the protein microarray technology and use of the fluorescent plate reader to the Centre. Note that the success of the proposed project does not depend on successful protein expression from every gene in the FLEXgene library, just a sufficient number to assess the feasibility of using protein microarrays to assess human immune responses in a parallel and more comprehensive manner.

2. Selection of the open reading frames of *V. cholerae* to be included in the FLEXgene library.

We will initially amplify by PCR and clone into a master vector, the following set of ORFs; this number is greater than 100, because we anticipate that a number of genes in the FLEXgene repository may not express proteins sufficiently to be used in a protein microarray initially; the goal is to have a subset of approximately 100 proteins on the microarray. The genes we will select are those thought to be involved in colonization by *V. cholerae*, production of toxic activity, regulation of virulence gene expression, chemotaxis and motility, quorum sensing, key outer membrane proteins, proteins involved in the type II secretion system of *V. cholerae*, and genes detected by previous *in vivo* screens, including those in our IVIAT experiments. Our initial gene set, therefore, will include: the genes in the TCP island, in which there are a total of 29 ORFs, including *tcpA-T* (excluding *tcpG*); *toxT*; the genes encoding an accessory colonization factor, *acfA-D*; *aldA; tagA,D* and *E*; and six additional ORFs (Karaolis 2001); the genes in the MSHA gene cluster, of which there are 16 ORFs, *mshA-Q* (Marsh 1999); the *pilA-D* genes (PilD has previously been shown to be an important pre-pilin peptidase for processing of several key proteins in *V. cholerae* (including PilA) (Fullner 1999; Marsh 1998); *pilQ* (found in our IVIAT screen); the genes encoding *ctxAB*, as well as the eight other genes that are part of the cholera toxin genetic element, *zot*, *cep*, *ace*, *orfU*, *rstABCR* (Waldor 1996); the four genes in the RTX toxin gene cluster, *rtxA-D* (Lin 1999); the gene *hlyA* encoding a secreted hemolysin (Menzl 1996); genes regulating virulence, including *toxRS*, the hemagglutinin-protease, *aphA* and *aphB* (Kovacikova 1999), and the gene encoding an alternative sigma factor *rpoS* (Yildiz 1998); 18 genes involved in chemotaxis, organized in three clusters on the genome (Heidelberg 2000; Gosink 2002); 17 genes encoding methyl-accepting chemotaxis proteins (Merrell 2002); 5 flagellin genes, *flaA-E*, (Klose 1998); 8 genes involved in three quorum sensing systems of *V. cholerae*, including *luxL-U* exclusive of *luxR* and *luxT* (Miller 2002); the 12 genes encoding the general secretory (*eps*) pathway in *V. cholerae* necessary for secretion of cholera toxin (Sandkvist 1997; Scott 2001); 2 key outer membrane proteins, *ompU* and *ompT* (Provenzano 2001); and genes expressed in *in vivo* model systems, including *vieSAB* (Lee 1998); *capK* (IVIAT results; Hang et al. 2003); *cadA*, involved in the acid tolerance response of *V. cholerae* (Merrell 1999); and the four hypothetical ORFs discovered in our IVIAT screen.

3. Construction of a *V. cholerae* FLEXgene repository of approximately 140 clones.

The success of any genomics initiative relies, in part, on the use of a high throughput strategy to clone large number of genes and to manipulate them (create and sub-clone alternative forms). The advent of recombinational cloning greatly facilitates this task by enabling the rapid transfer of any defined ORF between a variety of appropriate destination vectors required for heterologous expression (Walhout, 2000). All clones defining the targeted 140 full length genes to be used in our studies will consist of sequence-verified, full length ORFs in recombination-based vectors (Creator system). These constructs will be generated by low cycle PCR-amplification from *V. cholerae* genomic DNA. Preliminary optimization of PCR conditions will be carried out and then high throughput PCR and cloning of the genes will follow. The clones in the *V. cholerae* FLEX library will be individually constructed by PCR amplification from a genomic library provided by John Mekalanos' laboratory. In short, oligonucleotides 40-50 bases in length, containing ORF- specific sequences and vector-specific sequences, will be used to amplify the genes. The PCR reaction products will be size verified and PCR products will then be captured into the pDNR-DUAL (Clontech) master vector to allow recombination-based cloning.

All the steps involved in the procedure described above, as well as most of the rest of the cloning and sequencing steps below, have been fully automated and optimized during the development of the human and yeast projects at the HIP. All the reactions are performed in 96 well plates. The Institute has fully operative workstation automation (by use of TECAN Genesis 150 liquid handling robots) with capacity to assemble 800 clones per week (up to the DNA preparation step).

The next step in the construction of the repository is the introduction of the clones into *E. coli* by a high throughput transformation procedure, colony picking and DNA preparation. We believe that for the FLEXgene repository to reach its full potential, every single clone has to be sequence verified and correspond to the wild-type form of the gene in question. Given the mutation rate documented during pilot experiments using *P. aeruginosa* genomic DNA (1 mutation/1200 bases), and to maximize the chance that the wild type form of the gene is obtained at the end of the process, four colonies for each transformation are selected for DNA preparation and two for sequencing. HIP uses an ABI3700 DNA analysis system to achieve high standards of sequence quality.

We will make use of the Oracle-based database originally built to support the generation of the human, yeast and *Pseudomonas* gene repositories. The database was originally used to manage processes associated with a) sequence acquisition, b) oligo design, c) clone tracking (to relate the cloning history and container positions of clones representing genes) and d) sequence analysis (clone verification). We will now extend its applications so

it can collate, manage and analyze all the *V. cholerae* gene, protein expression and serum reactivity data generated in this project. The HIP intends to immediately devote a portion of its capacity to building the *V. cholerae* FLEXgene repository, and we expect completion of this step within the first 3-6 months of obtaining support for the project.

4. Development of a *V. cholerae* FLEXprotein repository.

Each ORF will be subsequently moved into expression vectors to produce amino- and/or carboxy-terminal fusions of the encoded proteins, as needed. Traditional approaches for making expression constructs are laborious and require individualized cloning strategies that may have to be altered for every different assay. This process is obviously not compatible with high-throughput manipulation of genes. Recombinational cloning is a novel technique that exploits the ability of certain enzymes to cleave DNA at specific sequences and then rejoin the ends with other matching sequences, and in precise reading frame, during a single concerted reaction. The BD Creator System allows efficient transfer of genes of interest into a variety of vectors using one easy step. The BD Creator technology utilizes *in vitro* recombination between two vectors. This recombination takes place at specific loci called *loxP* sites, mediated by Cre Recombinase. Cre-*loxP* recombination leads to directional transfer of a DNA fragment from a single donor vector into any of the variety of protein expression/acceptor vectors, bypassing traditional sub-cloning steps.To facilitate purification, immobilization/capture and detection of the expressed proteins, they will be expressed with a tag. Investigators at HIP have chosen five tags to be tested and for which they will be generating the proper vectors. Three of these tags, which are relatively short, are the FLAG-tag, the His-tag and the biotinylation signaling peptide tag. The advantage of using short tags is that the molecular weights of expressed proteins will not increase significantly. The other two tags currently used at HIP are glutathione-S-transferase (GST) and the maltose-binding protein (MBP). In addition to facilitating affinity purification and detection, these two domains are able to solubilize and/or to increase protein expression. All of these protein tags will be engineered with a protease site permitting removal of the tag after purification of the refolded fusion protein.

a) **Use of expression plasmids in bacteria to express individual proteins for use on the microarray.** Purification tags are widely used to produce proteins of high purity following expression in bacteria. Because purification tags can be genetically attached to the protein, they are suited to high throughput operations such as envisioned here. Investigators at HIP have developed efficient protocols for high-throughput (96-well format) protein expression and affinity purification from bacteral cultures (Braun, 2002). Each of the expression systems used at HIP has advantages and disadvantages for different proteins. Because of the ease of the recombinational cloning system and moving cloned ORFs from the master vector to a variety of different expression vectors, we can utilize several different expression vectors with a subset of the genes cloned in the FLEXgene repository, to determine an optimal system(s) for expressing *V. cholerae* proteins in an *E. coli* host, and capturing these on an array. For example, solutions containing His_6- or GST-tagged proteins can be robotically spotted onto derivatized glass slides or high-density microtiter plates, coated with zinc or anti-GST antibody, respectively. The HIP has developed systems for expressing proteins in this manner at concentrations of approximately 1 μg/ml, sufficient for manufacturing approximately 200 spotted microarrays.

b) **Investigation of the use of a novel approach, termed Nucleic Acid-Programmable Protein Array (NAPPA), to produce proteins in a cell-free system from plasmid DNA spotted on a microarray, with immobilization of the resulting proteins *in situ*.** It would certainly be advantageous to construct a protein array without the need to individually prepare each protein. Investigators at HIP are developing a novel, high-throughput method for protein expression and purification termed Nucleic Acid-Programmable Protein Array (NAPPA). This approach replaces the complex process of spotting purified proteins with the simple process of spotting plasmid DNA. Proteins are expressed from the individually spotted plasmid DNAs by *in vitro* transcription/translation and immobilized *in situ*, minimizing direct manipulation of the protein (Figure 1). They are developing and refining high-throughput techniques for the automation of protein expression and purification using cell free expression systems (Kigawa *et al.*, 1999; Madin *et al.*, 2000). They will carry out a systematic and simultaneous comparison of the rabbit reticulocyte, bacterial and wheat germ systems for transcription/translation, as well as of different tagging and purification protocols, using subsets of the genes. In the development and systematic automation of the cell-free synthesis for this approach, we will benefit from the collaboration of HIP with Prof. S. Yokoyama's group in Tokyo, who have developed coupled transcription/translation in cell free systems for the generation of isotope-

labeled proteins on the scale needed for structural studies (Kigawa 1999). As described above, the recombinational cloning system allows ready movement of the DNA encoding each ORF from the *V. cholerae* FLEXgene library into the necessary expression vectors to facilitate easy comparison of multiple systems for cell-free expression and attachment of various tags to the expressed protein for capture. In preliminary experiments with the NAPPA approach, described more thoroughly below, investigators at HIP have been able to obtain expression for a number of proteins at concentrations of 5.6 ng protein/μl plasmid DNA spot; this amount of protein is more than sufficient for use in the present proposal. We will use the protein expression and arraying technology currently being developed at the HIP as an alternate approach to produce the microarrays necessary for testing immune responses to proteins in the *V. cholerae* FLEX repository.

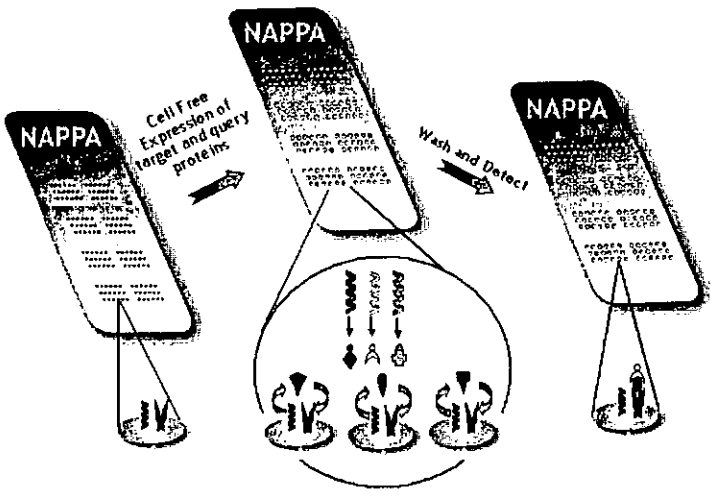

Figure 1. Schematic representation of screening protein-protein interactions with NAPPA. (A) On each spot, a target plasmid and an affinity capture molecule are linked to the prepared glass slide. (B) The slide is bathed with the cell-free *in vitro* transcription/translation mix, containing one or more query plasmids expressing different affinity tags. (C) The target proteins are expressed and immobilized on the spots, as are query proteins if they bind to that target. (D) The wells are then washed to remove unbound protein. (E) Target-query complexes are detected by fluorescence.

Investigators at HIP have been able to reliably express and specifically immobilize multiple *in vitro* synthesized proteins on to a microarray plate coated with GST antibody and demonstrate that protein-specific antibodies detect just their cognate corresponding protein, and not other proteins on the array. Investigators at HIP have also adapted NAPPA to a microarray format on a glass slide. First, anti-GST antibody and plasmid DNA were linked to aminosilane treated glass slides. The slide was then bathed in reticulocyte lysate to express target proteins. The target proteins were expressed with a C terminal GST fusion and as such, were immobilized by the GST-specific antibody on the slide. Specific detection of bound protein was done with a monoclonal antibody to the GST tag.

This suggests that current (and future) applications of NAPPA may be used as an alternate approach in developing a *V. cholerae* FLEXprotein microarray for the approximately 100 key proteins envisioned in this proposal. The NAPPA approach has the advantage of utilizing *in vitro* transcription/translation, which avoids many of the problems of expressing membrane or secreted proteins in intact *E. coli*.

Note that it is not necessary for all 140 genes in the *V. cholerae* FLEXgene repository to be successfully expressed utilizing either expression of individual proteins in *E. coli* or the NAPPA approach. We only seek to express a sufficient number of proteins by one or both methods to allow assessment of the feasibility of using a protein microarray to assess human immune responses following *V. cholerae* infection in a more parallel and comprehensive manner than current approaches.

5. Use of the *V. cholerae* FLEXprotein microarray for screening for immune responses against these proteins.

a. Use with human sera from patients convalescing from *V. cholerae* infection in Bangladesh.

Serum as well as other clinical specimens (stool, saliva) are being collected from cholera patients from the ongoing parent NIH/ICIDR project over the last 3 years (Protocol 99006). As part of this project, we have already obtained serum samples on presentation and at convalescence, from patients, children and adults diagnosed with cholera at the Centre and entered into the study (275 have been studied so far).These samples are being stored in an organized manner and can be retrieved from storage as and when necessary. As part of this project, cholera patients as well as their household contacts are being studied. Specimens including blood are collected from the patients at the acute stage of infection, that is as soon as the diagnosis for *V. cholerae* O1/O139 has been made. This is usually within the second day of hospitalization and considered as study 'day 2' post-onset of infection. The cholera patients present to the ICDDR,B hospital with 24 hours of onset of illness (Qadri et al. 1997). These blood samples are used for separation of mononuclear cells and for serum or plasma. The patients and other study subjects are also followed up at early (day 7) and late convalescence (day 21) stages. These samples are being used for carrying out immunological assays including the vibriocidal , ALS, ELISPOT assays and for LPS, CT, MSHA specific ELISAs (Qadri et al. 2003,Saha et al submitted).

We have also obtained permission for carrying out studies using genomics and proteomics in this study (Addendum to protocol 99006, appendix I). For this proposal, we will utilize acute and convalescent serum samples from a subset of approximately 25 randomly selected patients with *V. cholerae* O1 cholera to determine human immune responses to the *V. cholerae* proteins represented on the microarray.

Sera obtained from patients is frozen at -70°C until used. We will test randomly selected serum samples collected from the first 25 V. cholerae O1 infected patients in whom follow-up has been collected upto day 21 of the study. In pilot experiments, we will examine various dilutions of convalescent serum (1:50, 1:100, 1:500, 1:1000, 1:2000, and 1:5000) to determine an optimal dilution of serum to be used with the protein microarray. After binding of the serum dilutions to the protein microarray and washing, we will then add a second anti-human IgG antibody, linked to a fluorescent dye that can be detected by the fluorescent plate reader described in the equipment requested. Once we have determined the concentration of first antibody that appears optimal, we will also investigate various dilutions of the second antibody to optimize detection. We desire a dilution of each of these two reagents, such that the fluorescent signal from a particular spot on the protein microarray is roughly proportional to the amount of antibody to that protein in the serum sample. Ideally (and if possible), we would like to determine dilutions of the first and second antibodies that produce results in a linear response range.

Once these dilutions are determined, then we will compare the magnitude of antibody responses in a subset of 25 patients to each of the proteins on the microarray, comparing responses in convalescent serum with that same patient's responses during the acute phase, to ensure that the antibody responses detected in convalescence result from the acute infection. The goal is to find several protein antigens on the microarray that are specifically recognized following cholera infection. Based on our IVIAT results in the preliminary studies,

we anticipate that these responses will be seen at least to TcpA, MshA, PilA, PilQ, several chemotaxis genes, and the four hypothetical ORFs. Of interest will be whether responses to each of these proteins are shared broadly across patients or are found only a smaller subset of patients. Ideally, we would like to identify proteins to which the majority of patients with acute *V. cholerae* infection develop a robust immune response, as these will suggest proteins specifically expressed *in vivo* during human infection and potentially useful as vaccine antigens.

b. Immunization of rabbits with formalin-fixed *V. cholerae* recovered from human stool, to determine proteins expressed during human infection as compared to proteins expressed during *in vitro* growth.

In these experiments, strongly dark field positive rice-water stool from patients with acute cholera will be directly mixed with formalin at the bedside, to preserve proteins expressed *in vivo* by *V. cholerae* in the stool, as well as to inactivate the organisms; a separate aliquot of the rice-water stool will be put on ice without formalin, serially diluted, and plated to determine the colony-forming units per milliliter of liquid stool. For comparison with organisms recovered from *in vivo* infection, the same strain of El Tor *V. cholerae* will be grown *in vitro* in L broth, and handled in a similar manner. The formalin-fixed organisms from both samples will be recovered by centrifugation and washing in phosphate-buffered saline. Note that a large proportion of organisms in the dark field positive rice-water stool from patients with cholera are *V. cholerae* (Qadri et al.. Faruque et al. observations over the last 10 years) suggesting that analysis of organisms recovered directly from stool, without culture purification, is reasonable to compare with that of organisms recovered in pure culture from broth.

We will then utilize both the formalin-killed whole organisms, as well as proteins recovered from lysates of the killed organisms (to reduce the LPS content of the preparations), to immunize rabbits. The immunogens will be administered by subcutaneous injections to rabbits, with boosting at 2, 4, and perhaps 6 weeks. Following immunization and euthanasia, rabbits will be exsanguinated to recover post-immune sera. This will be compared to pre-immune sera from the same rabbit.

Sera from the rabbits will be used to develop the protein microarrays, in this case utilizing fluorescently-labeled anti-rabbit IgG as the second antibody, to determine proteins present on the microarray that react with sera from rabbits immunized with *V. cholerae* recovered directly from stool but not from *V. cholerae* following *in vitro* growth. The purpose of these experiments is to identify proteins in the microarray specifically expressed during *in vivo* human infection.

We know from our previous studies using a DNA microarray directly on organisms recovered from stool that the transcripts for several genes involved in virulence are no longer expressed in organisms in stool, even though the transit time in acute cholera from the upper gastrointestinal tract to rice-water stool is probably only a matter of 15-30 minutes. Because of the very short half-lives of bacterial mRNAs (generally 1-5 minutes), this time lag may be sufficient for virulence genes to turn off and mRNA may, therefore, no longer be detectable in stool organisms. For this reason, we believe that examining organisms in stool for proteins expressed during infection may be more useful than examining RNA transcripts, because proteins made in the upper gastrointestinal tract will most likely still be present on organisms excreted in the rice-water stool due to the much longer half-life of proteins in bacteria after synthesis.

Facilities Available

Describe the availability of physical facilities at the place where the study will be carried out. For clinical and laboratory-based studies, indicate the provision of hospital and other types of patient's care facilities and adequate laboratory support. Point out the laboratory facilities and major equipment that will be required for the study. For field studies, describe the field area including its size, population, and means of communications. **(TYPE WITHIN THE PROVIDED SPACE).**

Laboratory: Most Facilities in the Immunology and Molecular Genetics laboratory, Animal Resources Branch and at the LSD are adequate for the study. The fluorescent scanner needed for the project will be procured from funds for the study.

Describe plans for data analysis. Indicate whether data will be analyzed by the investigators themselves or by other professionals. Specify what statistical software packages will be used and if the study is blinded, when the code will be opened. For clinical trials, indicate if interim data analysis will be required to monitor further progress of the study. **(TYPE WITHIN THE PROVIDED SPACE).**

Equipment needed- An applied Precision arrayWOrk"e" microarray scanner, with accompanying computer and analysis software will be purchased for ICDDR,B from the project funds. This scanner handles standard 1" by 3" microscope
Slides, has a scanning resolution of 10 microns (sufficient for the protein microarrays that will be developed for the proposal) and has the capability of reading both Cy3 and Cy5 labels, and has a cassette for loading multiple slides. This piece of equipment would allow the investigators at the ICDDR,B to stain protein microarrays made at the Harvard Medical School.
Analysis of data: Accompanying computer and analysis software with arrayWOrk"e" will be used for analysis of data obtained from the proteomic studies.

Ethical Assurance for Protection of Human Rights

Describe in the space provided the justifications for conducting this research in human subjects. If the study needs observations on sick individuals, provide sufficient reasons for using them. Indicate how subject's rights are protected and if there is any benefit or risk to each subject of the study.

Human Subjects

Access to serum samples from 25 patients infected with *V. cholerae* is available from project, already approved and ongoing at the ICDDR,B, supported by an NIH-funded grant under the International Collaborations in Infectious Disease Research Program (project 99006). The patient samples used in year 2 in determining human immune responses to the proteins on the microarray will be taken from samples already approved and being collected as part of the ICIDR project; no additional human studies are proposed in this study, apart from using these samples already being collected and stored.

In protocol no. 99006 and addendum to it we have obtained permission for the use of the specimens for immunological and molecular biological assays which include gene and proteomic analyses. Addendum and approval in Appendix 1.

Use of Animals

Describe in the space provided the type and species of animal that will be used in the study. Justify with reasons the use of particular animal species in the experiment and the compliance of the animal ethical guidelines for conducting the proposed procedures.

1. Description of proposed use of rabbits.

No animal studies are proposed in year 1 of the project. Animal studies in year 2 of the project will take place in ICDDR,B. In brief, rice-water stool from patients with acute cholera will be directly mixed with formalin, to preserve proteins expressed *in vivo* by *V. cholerae* in the stool, as well as to inactivate the organisms; a separate aliquot of the rice-water stool will be put on ice without formalin, serially diluted, and plated to determine the colony forming units per milliliter of liquid stool. The formalin-fixed organisms will be isolated by centrifugation of stool and washing in phosphate-buffered saline. Note that >99% of organisms in rice-water stool of patients with cholera are *V. cholerae*.

We will investigate the use of both killed whole organisms as an immunogen, as well as recovering proteins from a lysate of the organisms, to reduce the LPS content of the preparation. The immunogen will be administered by subcutaneous injections on the back of adult New Zealand white rabbits, <1.0 ml per site, using a needle of 22 gauge or smaller, to raise antibodies to antigens expressed by *V. cholerae in vivo*. Two and four weeks later, the rabbits will be boosted with the same immunogen. In some experiments, rabbits may receive a booster injection at six weeks as well. Sera (1-5 ml) will be collected from the central ear artery prior to immunization and 9-11 days after the last boost, and the sera will be used to assay immune responses to proteins on the microarray; results will be compared to those using the pre-immune sera from the same rabbit. Following euthanasia, rabbits will be exsanguinated via cardiac puncture.

2. Justification of the use of animals.

Cholera infects more than seven million people in the world annually, with a sizable number of deaths, particularly in children. The long-range goal of this project is to characterize specific virulence genes in *V. cholerae* expressed *in vivo*, and to use knowledge of these genes to facilitate *V. cholerae* vaccine development for prevention of human infection.

3. Procedures for reducing discomfort, distress and pain of the animals.

All animals will be given appropriate sedation and/or anesthesia as needed. Every effort will be made to keep the animals as comfortable as possible during the experiments.

4. Procedures for euthanasia.

Rabbits will be sacrificed by receiving intramuscular ketamine (50 ml/kg) followed by an overdose of intravenous pentobarbital (100 mg/kg). This method is consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

5. Permission from AEEC, ICDDR,B- Permission will be obtained from the committee for the animal studies

Literature Cited

Identify all cited references to published literature in the text by number in parentheses. List all cited references sequentially as they appear in the text. For unpublished references, provide complete information in the text and do not include them in the list of Literature Cited. There is no page limit for this section, however exercise judgment in assessing the "standard" length.

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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 programme.

The results of this work will be disseminated through publication in international peer-reviewed journals, as well as through international scientific presentations.

Collaborative Arrangements

Describe briefly if this study involves any scientific, administrative, fiscal, or programmatic arrangements with other national or international organizations or individuals. Indicate the nature and extent of collaboration and include a letter of agreement between the applicant or his/her organization and the collaborating organization. **(DO NOT EXCEED ONE PAGE)**

This proposal involves collaboration between Massachusetts General Hospital and the Harvard Institute of Proteomics at Harvard Medical School, during which the FLEXgene and FLEXprotein microarrays will be developed and the International Centre for Diarrhoeal Diseases Research in Dhaka, Bangladesh, where the microarrays will be used, a fluorescent plate reader purchased, and experiments done to determine immune responses to proteins on the microarray, as well as utilizing the protein microarrays to screen for antigens expressed in *V. cholerae in vivo* during human infection.

BIOGRAPHICAL SKETCH

POSITION TITLE

Senior Scientist and Head, Immunology Unit, ICDDR,B

)AUSI QADRI

)CATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include doctoral training.)

INSTITUTION AND LOCATION	DEGREE (if applicable)	YEAR(s)	FIELD OF STUDY
:rsity of Dhaka, Bangladesh	BS	1975	Science
:rsity of Dhaka, Bangladesh	Masters	1977	Biochemistry
pool University, UK	Ph.D.	1980	Biochemistry
)R,B, Dhaka, Bangladesh	Post-doctoral fellowship	1986-1988	Immunology

A. Selected peer-reviewed publications (in chronological order). (selected 80 peer reviewed publications).

1. **Firdausi Qadri**, Edward T. Ryan , A.S.G. Faruque, Firoz Ahmed, Ashraful Islam Khan, Md. Monirul Islam, Syed Mohd. Akramuzzaman, D. A. Sack and Stephen B. Calderwood Antigen specific IgA antibodies secreted from circulating B cells are an effective marker for recent local immune responses in patients with cholera: comparison with antibody secreting cell responses and other immunological markers. Infection and Immunity; 2003, 71, 4808-4814

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5. Merrell SD, Butler SM, **Qadri F**, Dolgananov NA, Alam A, Cohen MB, Calderwood SB, Schoolnik GK, Camilli A. Host-induced epidemic spread of the cholera bacterium. Nature, 2002, 417,642-645.

6. **Qadri F**, Raqib R, Ahmed F, Rahman M T, Wenneras C, Das SK, Alam NH, Mathan MM, Svennerholm AM. Increases in inflammatory mediators in children and adults infected with *V. cholerae* O1 and O139. Clin Diagn Lab Immunol. 2002, 9, 221-229.

7. **Qadri F**, Asaduzzaman M, Wenneras C, Mohi G, Albert MJ, Abdus Salam M, Sack RB, Jertborn M,R McGhee JR, Sack DA, Holmgren J. Enterotoxin –specific immunoglobulin E responses in humans after infection or vaccination with diarrhea-causing enteropathogens. Infect. Immun. 2000, 68, 6077-81.

8..Raqib R, Mia SM, **Qadri F**, Alam TI, Alam NH, Chowdhury AK, Mathan MM, Andersson J. Innate immune responses in children and adults with shigellosis. Infect. Immun. 2000, 68, 3620-3629.

9. **Qadri F**, Giron JA, Helander A, Begum YA, Asaduzzaman M, Xicohtencatl-Cortes, Negrete E, Albert MJ. Human antibody response to longus type IV pilus and study of its prevalence among enterotoxigenic *Escherichia coli* in Bangladesh using monoclonal antibodies. Journal of Infectious Diseases. 2000, 181, 2071-2074.

10. **Qadri F**, Wenneras C, Bardhan PK, Albert MJ, Sack RB, Svennerholm A-M. Safety and immunogenicity of an oral, inactivated enterotoxigenic *Escherichia coli* plus cholera toxin B subunit vaccine in Bangladeshi adults and children Vaccine. 2000, 18, 2704-2712

11. **Qadri F**. Swadesh Kumar Das, A.S.G. Faruque, George J. Fuchs, M. John Albert, R. Bradley Sack and Ann-Mari Svennerholm. Prevalence of toxin types and colonization factors in enterotoxigenic *Escherichia coli* isolated during a two year period from diarrheal patients in Bangladesh J. Clin Micr. 38, 2000, 27-31.

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URRICULUM VITAE OF APPLICANT

Family name: FARUQUE — Date of birth: Nov 11, 1956

First name(s): SHAH M. — Nationality: Bangladeshi

Education (subjects, university or school, degree, year)

h.D., Molecular Biology, University of Reading, Reading, England, 1988

1.Sc., Biochemistry, University of Dhaka, Dhaka, Bangladesh, 1979

.Sc. (Honours) Biochemistry, University of Dhaka, Dhaka, Bangladesh, 1978

Present and most recent positions held (type of position, institution/authority, dates)

989-Present: Scientist, International Center for Diarrhoeal Disease Research, Bangladesh.

988-1989 : Assistant Professor, Department of Biochemistry, University of Dhaka.

984-1988 : Lecturer, Department of Biochemistry, University of Dhaka.

). Recent publications: list only the five most important and relevant publications over the last five years (papers in press or submitted for publication are also acceptable).
Please give full bibliographic references: author(s), title, journal, volume, page numbers, year

ECENT PUBLICATIONS

1. **Faruque SM**, Mekalanos JJ. Pathogenicity islands and phages in Vibrio cholerae evolution. Trends. Microbiology. 2 11:505-510.

2. **Faruque SM,** Chowdhury N, Kamruzzaman M, Ahmad QS, Faruque AS, Salam MA, Ramamurthy T, Nair GB, Wein A, Sack DA. Reemergence of epidemic Vibrio cholerae O139, Bangladesh. Emerg Infect Dis. 2003; 9:1116-1122.

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BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
Stephen B. Calderwood	Associate Professor of Medicine (Microbiology & Molecular Genetics)

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing. Include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Harvard University, Cambridge MA	A.B.	1971	Biology
Harvard Medical School, Boston MA	M.D.	1975	Medicine

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. DO NOT EXCEED TWO PAGES.

Research and Professional Experience

1975-78	Medical Residency, Massachusetts General Hospital, Boston, MA
1978-80	Clinical and Research Fellow in Infectious Diseases, Massachusetts General Hospital, Boston, MA
1981	Chief Resident in Medicine, Massachusetts General Hospital, Boston, MA
1981-84	Instructor in Medicine, Harvard Medical School, Boston, MA
1984-88	Assistant Professor of Medicine, Harvard Medical School, Boston, MA
1985-88	Upjohn Scholar, Department of Microbiology and Molecular Genetics (Laboratory of Dr. John Mekalanos), Harvard Medical School, Boston, MA
1988-91	Assistant Professor of Medicine (Microbiology and Molecular Genetics), Harvard Medical School, Boston, MA
1991-	Associate Professor of Medicine (Microbiology and Molecular Genetics), Harvard Medical School, Boston, MA
1992-	Editorial Board, *Infection and Immunity*
1994-98	Bacteriology and Mycology-1 Study Section, National Institutes of Health, Bethesda, MD

1995- Physician and Chief, Division of Infectious Diseases, Massachusetts General Hospital, Boston, MA
1996-98 Chair, Bacteriology and Mycology-1 Study Section, National Institutes of Health, Bethesda, MD

Awards and Honors
1971 Summa cum laude
1971 Phi Beta Kappa
1975 Alpha Omega Alpha
1989 Fellow, American College of Physicians
1990 Fellow, Infectious Diseases Society of America

Selected Original Publications (out of a total of 60):

1. Calderwood SB, Auclair F, Donohue-Rolfe A, Keusch GT, Mekalanos JJ. Nucleotide sequence of the Shiga-like toxin genes of *Escherichia coli*. Proc Natl Acad Sci USA 1987; 84:4364-4368.
2. Calderwood SB, Mekalanos JJ. Iron regulation of Shiga-like toxin expression in *Escherichia coli* is mediated by the *fur* locus. J Bacteriol 1987; 169:4759-4764.
3. Hovde CJ, Calderwood SB, Mekalanos JJ, Collier RJ. Evidence that glutamic acid 167 is an active-site residue of Shiga-like toxin I. Proc Natl Acad Sci USA 1988; 85:2568-2572.
4. Goldberg MB, DiRita VJ, Calderwood SB. Identification of an iron-regulated virulence determinant in *Vibrio cholerae*, using Tn*phoA* mutagenesis. Infect Immun 1990; 58:55-60
5. Goldberg MB, Boyko SA, Calderwood SB. Transcriptional regulation by iron of a *Vibrio cholerae* virulence gene and homology of the gene to the *Escherichia coli* Fur system. J Bacteriol 1990; 172:6863-6870.
6. Goldberg MB, Boyko SA, Calderwood SB. Positive transcriptional regulation of an iron-regulated virulence gene in *Vibrio cholerae*. Proc Natl Acad Sci USA 1991; 88:1125-1129.
7. Litwin CM, Boyko SA, Calderwood SB. Cloning, sequencing and transcriptional regulation of the *Vibrio cholerae fur* gene. J Bacteriol 1992; 174:1897-1903.
8. Butterton JR, Stoebner JA, Payne SM, Calderwood SB. Cloning, sequencing, and transcriptional regulation of *viuA*, the gene encoding the ferric vibriobactin receptor of *Vibrio cholerae*. J Bacteriol 1992; 174:3729-3738.
9. Goldberg MB, Boyko SA, Butterton JR, Stoebner JA, Payne SM, Calderwood SB. Characterization of a *Vibrio cholerae* virulence factor homologous to the family of TonB-dependent proteins. Mol Microbiol 1992; 6:2407-2418.
10. Litwin CM, Calderwood SB. Analysis of the complexity of regulation by Fur in *Vibrio cholerae*. J Bacteriol 1994; 176:240-248.
11. Lam MS, Litwin CM, Carroll PA, Calderwood SB. *Vibrio cholerae fur* mutations associated with loss of repressor activity: Implications for the structural-functional relationships of Fur. J Bacteriol 1994; 176:5108-5115.
12. Butterton JR, Calderwood SB. Identification, cloning and sequencing of a gene required for ferric vibriobactin utilization by *Vibrio cholerae*. J Bacteriol 1994; 176:5631-5638.
13. Butterton JR, Beattie DT, Gardel CG, Carroll PA, Hyman T, Killeen KP, Mekalanos JJ, Calderwood SB. Heterologous antigen expression in *Vibrio cholerae* vector strains. Infect Immun 1995; 63:2689-2696.
14. Pozsgay V, Trinh L, Shiloach J, Robbins JB, Donohue-Rolfe A, Calderwood SB. Purification of subunit B of Shiga toxin using a synthetic trisaccharide-based affinity matrix. Bioconjugate Chemistry 1996; 7:45-55.
15. Tashima KT, Carroll PA, Rogers MB, Calderwood SB. Relative importance of three iron-regulated outer membrane proteins for *in vivo* growth of *Vibrio cholerae*. Infect Immun 1996; 64:1756-1761.
16. Butterton JR, Shahin RA, Calderwood SB. Development of a germ-free mouse model of *Vibrio cholerae* infection. Infect Immun 1996; 64:4373-4377.
17. Calderwood SB, Baker MA, Carroll PA, Michel JA, Arbeit RD, Ausubel FM. Use of cleaved amplified polymorphic sequences to distinguish strains of *Staphylococcus epidermidis*. J Clin Microbiol 1996; 34:2860-2865.
18. Carroll PA, Zhao G, Boyko SA, Winkler ME, Calderwood SB. Identification, sequencing, and enzymatic activity of the erythrose 4-phosphate dehydrogenase gene of *Vibrio cholerae*. J Bacteriol 1997; 179:293-296.
19. Watnick PI, Eto T, Takahashi H, Calderwood SB. Purification of *Vibrio cholerae* Fur and estimation of its intracellular concentration by antibody sandwich enzyme-linked immunosorbent assay. J Bacteriol 1997; 179:243-247.
20. Butterton JR, Ryan ET, Acheson DWK, Calderwood SB. Co-expression of the B subunit of Shiga toxin 1 and EaeA from enterohemorrhagic *Escherichia coli* in *Vibrio cholerae* vaccine strains. Infect Immun 1997; 65:2127-2135.

21.Ryan ET, Butterton JR, Smith RN, Carroll PA, Crean TI, Calderwood SB. Protective immunity against *Clostridium difficile* toxin A induced by oral immunization with a live, attenuated *Vibrio cholerae* vector strain. Infect Immun 1997; 65:2941-2949.
22. Ryan ET, Butterton JR, Zhang T, Baker MA, Stanley SL, Jr., Calderwood SB. Oral immunization with attenuated vaccine strains of *Vibrio cholerae* expressing a dodecapeptide repeat of the serine-rich *Entamoeba histolytica* protein fused to the cholera toxin B subunit induces systemic and mucosal antiamebic and anti-*V. cholerae* antibody responses in mice. Infect Immun 1997;65:3118-3125.
23. Carroll PA, Tashima KT, Rogers MB, DiRita VJ, Calderwood SB. Phase variation in *tcpH* modulates expression of the ToxR regulon in *Vibrio cholerae*. Mol Microbiol 1997; 25:1099-1111.
24. Rahme LG, Tan M-W, Le L, Wong SM, Tompkins RG, Calderwood SB, Ausubel FM. Use of model plant hosts to identify *Pseudomonas aeruginosa* virulence factors. Proc Natl Acad Sci USA 1997; 94:13245-13250.
25. John MDV, Hibberd PL, Karchmer AW, Sleeper LA, Calderwood SB. *Staphylococcus aureus* prosthetic valve endocarditis: Optimal management and risk factors for death. Clin Infect Dis 1998;26:1302-1309.
26. Calia KE, Waldor MK, Calderwood SB. Use of representational difference analysis to identify genomic differences between pathogenic strains of *Vibrio cholerae*. Infect Immun 1998; 66:849-852.
27. Watnick PI, Butterton JR, Calderwood SB. The interaction of the *Vibrio cholerae* transcription factors, Fur and IrgB, with the overlapping promoters of two virulence genes, *irgA* and *irgB*. Gene 1998;209:65-70.

28. Hang L, John M, Asaduzzaman M, Bridges EA. Vanderspurt C, Kirn TJ, Taylor RK, Hillman, JD, Handfield,M, Ryan E.T and Stephen B. Calderwood 2003, PNAS, 100(14): 8508-13

29. Firdausi Qadri, Edward T. Ryan , A.S.G. Faruque, Firoz Ahmed, Ashraful Islam Khan, Md. Monirul Islam, Syed Mohd. Akramuzzaman, D. A. Sack and Stephen B. Calderwood Antigen specific IgA antibodies secreted from circulating B cells are an effective marker for recent local immune responses in patients with cholera: comparison with antibody secreting cell responses and other immunological markers. Infection and Immunity; 2003, 71, 4808-4814

NAME	POSITION TITLE
Edward T. Ryan	Instructor in Medicine

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing. Include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Princeton University, NJ	A.B.	1984	Biochemical Sciences
Harvard University, MA	M.D.	1988	Medicine

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. DO NOT EXCEED TWO PAGES.

Research and Professional Experience

1988-91 Intern, Junior Resident and Senior Resident, Internal Medicine, Massachusetts General Hospital, Boston, MA
1991-92 Clinical Fellow, Infectious Disease Unit, Massachusetts General Hospital, Boston, MA
1992 Physician, Department of Internal Medicine, FHP-Asia; Guam Memorial Hospital, Guam
1993 Chief Resident in Medicine, Massachusetts General Hospital, Boston, MA
1993-1994 Instructor in Medicine, Harvard Medical School, Boston, MA
1994 Dalton Traveling Fellow, London School of Hygiene and Tropical Medicine, London, UK
1994-97 Clinical and Research Fellow, Infectious Disease Unit, Massachusetts General Hospital, Boston, MA
1996-97 Fellow in Human Rights & Medicine; Center for the Study of Society & Medicine, College of Physicians & Surgeons, Columbia University, New York, NY
1996-97 Warren-Whitman-Richardson Traveling Fellow, Harvard Medical School, Boston, MA
1997 Visiting Scientist/International Fellow, International Centre for Diarrhoeal Disease Research (ICDDR,B), Dhaka, Bangladesh
1997- Assistant in Medicine, Massachusetts General Hospital, Boston, MA
1998- Director, Travel Advice & Immunization Center, Massachusetts General Hospital, Boston, MA
1998- Director, Tropical Medicine Clinic, Massachusetts General Hospital, Boston, MA

Awards and Honors

1984 *summa cum Laude* in Biochemical Sciences, Princeton University
1984 Phi Beta Kappa, Princeton University
1984 Ralph G. Treen Memorial Scholarship for Achievement in Biological Sciences, PrincetonUniversity
1988 *Cum Laude et Thesi Propria,* Harvard University
1994 Diploma in Tropical Medicine and Hygiene, Royal College of Physicians of London
1994 Duncan Gold Medal, First Prize, London School of Hygiene and Tropical Medicine
1994 Edward H. Kass Award for Clinical Excellence, Massachusetts Infectious Diseases Society
1996 Young Investigator Award, Honorable Mention, American Society of Tropical Medicine & Hygiene

Bibliography

Articles

1. Marti GE, Ryan ET., Papadopoulos NM, Filling-Katz M, Barton N, Fleischer TA, Rick M, Gralnick HR. Polyclonal B-cell lymphocytosis and hypergammaglobulinemia in patients with Gaucher's disease. Am J Hematol 1988:29:188-194
2. Ryan ET. Surgical nosocomial infections. Res S Phys 1991;2:11-15.
3. Ryan ET, Ecker JL, Christakis NA, Folkman J. Hirschspring's disease: associated abnormalities and demography. J Ped Surg 1992;27:76-81.
4. Ryan ET, Pak PH, DeSanctis RW. Myocardial infarction mimicked by acute cholecystitis. Ann Intern Med 1992;116:218-220.
5. Young JA, Ryan ET. Parasitic infections of the anterior segment. International Ophthal. Clinics 1996;36:49-71.

6. Butterton JR, Ryan ET, Shahin RA, Calderwood SB. Development of a germfree mouse model of *Vibrio cholerae* infection. Infect. Immun. 1996;64:4373-4377.
7. Butterton JR, Ryan ET, Acheson DWK, Calderwood SB. Coexpression of the B subunit of Shiga toxin 1 and EaeA from enterohemorrhagic *Escherichia coli* in *Vibrio cholerae* vaccine strains. Infect. Immun. 1997;65:2127-2135.
8. Ryan ET, Butterton JR, Smith RN, Carroll PA, Crean TI, Calderwood SB. Protective immunity against *Clostridium difficile* toxin A induced by oral immunization with a live, attenuated *Vibrio cholerae* vector strain. Infect. Immun. 1997;65:2941-2949.
9. Ryan ET, Butterton JR, Zhang T, Baker MA, Stanley Jr SL, Calderwood SB. Oral immunization with attenuated vaccine strains of *Vibrio cholerae* expressing a dodecapeptide repeat of the serine rich *Entamoeba histolytica* protein (SREHP-12) fused to the cholera toxin B subunit induces sytemic and mucosal antiamebic and anti-*V. cholerae* antibody responses in mice. Infect. Immun. 1997:65:2941-2949.
10. Hang L, John M, Asaduzzaman M, Bridges EA. Vanderspurt C, Kirn TJ, Taylor RK, Hillman, JD, Handfield,M, Ryan E.T and Stephen B. Calderwood 2003, PNAS, 100(14): 8508-13
11. Firdausi Qadri, Edward T. Ryan , A.S.G. Faruque, Firoz Ahmed, Ashraful Islam Khan, Md. Monirul Islam, Syed Mohd. Akramuzzaman, D. A. Sack and Stephen B. Calderwood Antigen specific IgA antibodies secreted from circulating B cells are an effective marker for recent local immune responses in patients with cholera: comparison with antibody secreting cell responses and other immunological markers. Infection and Immunity; 2003, 71, 4808-4814

Texts

1. Hibberd PL, Ryan ET, Baker AS. *Pneumocystic carinii,* parasitic infection, and the eye. In: Albert DM, Jakobiec FA, eds. Principles and Practice of Ophthalmology. Philadelphia: WB Saunders, 1994:3065-3077.
2. Ryan, ET, Baker AS. Systemic bacterial infections and the eye. In: Albert DM, Jakobiec FA, eds. Principles and Practice of Ophthalmology. Philadelphia: WB Saunders, 1994:3006-3010.
3. Ryan ET, Swartz MN. Dermatological Infections. In: Roitt IM, Delves PJ, eds. Encyclopedia of Immunology, Second Edition. London. Academic Press Ltd., 1998:750-754.
4. Ryan ET, Maguire JH. Parasitic infections and the eye. In: Albert DM, Jakobiec FA, eds. Principles and Practice of Ophthalmology. Philadelphia: WB Saunders, (in press).
5. Ryan ET. Tropical infectious diseases: ocular manifestations. In: Guerrant RL, Krogstad DJ, Maguire JH, Walker DH, Weller PF, eds. Tropical Infectious Diseases: Principles, Pathogens and Practice. New York: Churchill Livingstone, (in press). (textbook & pocket manual).

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.

The budget includes costs for personnel, costs for microbiological and immunological reagents and supplies. Funds for a fluorescent plate reader will be purchased from the funds for carrying out the study with the protein microarrays at ICDDR,B

Other Support

Describe sources, amount, duration, and grant number of all other research funding currently granted to PI or under consideration. (DO NOT EXCEED ONE PAGE FOR EACH INVESTIGATOR)

Firdausi Qadri

1. Studies to evaluate vaccines against watery diarrhoea suitable for use in Bangladesh Funding Sida-SAREC (ending 2005)

2. Immune response to V. cholerae in Bangladesh (NIH/ICTDR, Ending 2005)

3. Phase I/II safety and immunogenicity studies of Peru 15, a live attenuated oral vaccine candidate for *Vibrio cholerae* O1 in Bangladeshi volunteers both adults and children (Funding Domi, Ending 2004).

Other support for S.M. Faruque

Phase I/II safety and immunogenicity studies of Peru 15, a live attenuated oral vaccine candidate for *Vibrio cholerae* O1 in Bangladeshi volunteers both adults and children (Funding Domi, Ending 2004)..

T Phages and genomic variation in V. cholerae evolution., Donor: NIH (Role in project: Co-PI)

Characterization of V. cholerae O1 and non-O1 in Bangladesh, Donor: Ellison Foundation (Role in project: Co-PI)

Check List

After completing the protocol, please check that the following selected items have been included.

1. Face Sheet Included [X]
2. Approval of the Division Director on Face Sheet [X]
3. Certification and Signature of PI on Face Sheet, #9 and #10 [x]
4. Table on Contents [X]
5. Project Summary [X]
6. Literature Cited [X]
7. Biography of Investigators [X]
8. Ethical Assurance [X]
9. Consent Forms [NA]
10. Detailed Budget [X]

International Centre for Diarrhoeal Disease Research, Bangladesh
CENTRE FOR HEALTH AND POPULATION RESEARCH
Mail : ICDDR,B, GPO Box 128, Dhaka-1000, Bangladesh
Phone: 880-2-8811751-60. Telex : 642486, ICDD BJ
Fax : 880-2-8823116, 8812530, 8811568, 8826050, 9885657, 8811686, 8812529
Cable : Cholera Dhaka

Date : 5/11/03

To : Prof. David A. Sack
Chairperson, RRC

From : P.I. Dr. Firdausi Qadri

Subject: Waiver of external review for NIH funded project

We would like to submit a protocol to RRC entitled "the development of a *Vibrio cholerae* protein array and its application to the study of human immune responses". The protocol has been funded by NIH (Grant No.3 U01 A1058935-04S1) with a subcontract to ICDDR,B.

This is a collaborative study between ICDDR,B and the Division of Infectious Diseases, Massachusetts General Hospital, Boston and the Harvard Institute of Proteomics, Harvard Medical School. Since the project underwent critical review by NIH, we would request you to kindly waive the requirement for the external review process from the Centre. We would like to submit the project for the November RRC meeting.

Thanking you

cc: Mr. Bejoy R. Saha, Manager TED

DETAILED BUDGET FOR INITIAL BUDGET PERIOD

DIRECT COSTS ONLY

FROM 05/01/03 THROUGH 04/30/04

PERSONNEL (Applicant organization only)

NAME	ROLE ON PROJECT	TYPE APPT. (months)	% EFFORT ON PROJ.	INST. BASE SALARY	DOLLAR AMOUNT REQUESTED (omit cents) SALARY REQUESTED	FRINGE BENEFITS	TOTAL
Stephen B. Calderwood	Principal Investigator	12	5		8,335.	2,259.	10,594.
Regina C. LaRocque	Post-Doc	12	85	50,000.	35,417.	9,598.	45,015.
SUBTOTALS →					43,752.	11,857.	55,609

CONSULTANT COSTS — 0

EQUIPMENT (Itemize) — 0

SUPPLIES (Itemize by category)

Molecular biology reagents $3,000
Immunology reagents $3,000

6,000.

TRAVEL — 2,000.

See Budget Justification

PATIENT CARE COSTS — INPATIENT 0.

OUTPATIENT 0.

ALTERATIONS AND RENOVATIONS (Itemize by category) — 0.

OTHER EXPENSES (Itemize by category)

See Budget justification

466.

SUBTOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD — 64,093

CONSORTIUM/CONTRACTUAL COSTS — DIRECT COST HMS $103,224; ICDDR $6,000 — 109,224.

FACILITIES AND ADMINISTRATION COSTS HMS $56,776; ICDDR $1,480 — 57,256.

TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD (Item 7a, Face Page) → $ 130,573

SBIR/STTR Only: FIXED FEE REQUESTED

Principal Investigator/Program Director (Last, First, Middle): Qadri Dr. Firdausi & Faruque Dr.S M

DETAILED BUDGET FOR INITIAL BUDGET PERIOD — For 2 years

DIRECT COSTS ONLY

PERSONNEL (APPLICANT ORGANIZATION ONLY)	DOLLAR AMOUNT REQUESTED							TOTAL
	1st Year	2nd Year						
NAME		ROLE ON PROJECT	TYPE APPT (months)	% EFFORT ON PROJECT	INST BASE SALARY	FRINGE BENEFITS	TOTALS	
Dr. Firdausi, Qadri		PI	12	20%	23,455	-	23,455	
Dr.S.M. Faruque		PI	12	20%	21,916	-	21,916	
Sr.Research Officer			12	92%	5,627	-	5,627	
Research Officer		GS-5	12	50%	2,382	-	2,382	
Office clerk		GS-3	12	50%	1,578	-	1,578	
Sub Total						-	54,958	54,958
CONSULTANT COSTS								
SUPPLIES (Itemize by category)								
Nunc plates							1,500	
Enzyme conjugates for ELISA assays							2,700	
Other immunochemicals, substrates buffers, cell separation media etc.							1,500	
Tissue culture plastic							800	
Tissue culture media							797	
Reagent for buffers							1,500	
Office supplies							1,200	
Laboratory supplies							4,000	
Molecular biology reagents							6,000	
Chemical and media							1,390	
Miscellaneous							702	
Sub Total							22,589	22,589
Travel								
International Travel	6,000						6,000	
Sub Total	6,000						6,000	12,000
Inter-Departmental								
Animal Resoures							3,000	
Maintenance							1,000	
Sub Total							4,000	4,000
Equipment (Microassary scanner)							65,000	65,000
PATIENT CARE COSTS								
CONSORTIUM/CONTRACTUAL COSTS							152,547	158,547
SUB TOTAL DIRECT COSTS	6,000						12,204	12,084
INDIRECT COSTS (8%)	480							
TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD	6,480						164,751	171,231

S. Main
04-11-2003
BP-MM-XXXY
Shamima Main
Controller, Budget & Costing

Signature

INTERNATIONAL CENTRE FOR DIARRHOEAL DISEASE RESEARCH, BANGLADESH
Mail : ICDDR,B, GPO Box 128, Dhaka-1000, Bangladesh
Phone : 871751-60, Telex : 675612 ICDD BJ
Fax : 880-2-883116, 886050, 871568, 871686, Cable : Cholera Dhaka

CENTRE
FOR HEALTH AND
POPULATION RESEARCH

Memorandum

13 May 2002

To : Dr. Firdausi Qadri
Laboratory Sciences Division

From: David A Sack, M D
Chairman, Research Review Committee (RRC)

Sub : Proposal for an addendum to protocol # 99-006

Thank you for your memo of 7 May 2002 proposing an addendum to protocol # 99-006 entitled "Immune response to *V. cholerae* in Bangladesh", which the RRC approved in its meeting held on 9th May 2002. You are advised to submit a modified version of your protocol incorporating all modifications/addenda approved by the Committee, for record and future reference.

Thank you.

Copy: Associate Director
Laboratory Sciences Division

International Centre for Diarrhoeal Disease Research, Bangladesh
CENTRE FOR HEALTH AND POPULATION RESEARCH
Mail : ICDDR,B, GPO Box 128, Dhaka-1000, Bangladesh
Phone : (880-2) 8826318 / 8810117, PABX: 8811751-60
Fax : 880-2-8823116, 8826050, 8811568, 8811686, Cable: Cholera Dhaka
Email: analam@icddrb.org or ted@icddrb.org or brsaha@icddrb.org

MEMORANDUM

3 June 2002

To : Dr. Firdausi Qadri
Laboratory Sciences Division

Dr. A. S. G. Faruque
Clinical Sciences Division

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

Sub : Protocol # 99-006

Thank you for your addendum protocol # 99-006 entitled "Immune response to *V. cholerae* in Bangladesh". I am pleased to inform you that the ERC in its meeting held on 29th May 2002has approved the addendum proposal.

Please submit a modified copy of the protocol incorporating all approved addendum, for the ERC file.

Thank you.

cc: i) Associate Director
Laboratory Sciences Division

ii) Acting Associate Director
Clinical Sciences Division

International Centre for Diarrhoeal Disease Research, Bangladesh.
CENTRE FOR HEALTH AND POPULATION RESEARCH
Mail : ICDDR, B, GPO Box 128, Dhaka-1000, Bangladesh
Phone: 880-2-8811751-60, Telex : 642486 ICDD BJ
Fax : 880-2-8823116, 8812530, 8811568, 8826050, 9885657, 8811685, 8812529
Cable : Cholera Dhaka

Addendum to P. Prot
99006
Permission approved

RRC/ERC

Date: 14/5/02

To: The Chairperson, ERC

From: Drs. A.S.G. Faruque and Firdausi Qadri, PIs of protocol No. 99006

Subject: Addendum to protocol (No. 99006) entitled "Immune response to *V. cholerae* in Bangladesh" (PI's Drs. A.S.G. Faruque and Firdausi Qadri).

We have been conducting a study on the immune response in cholera patients for which we are enrolling patients in different age groups. The study is ongoing and we are recruiting patients according to our study criteria. We are studying the systemic and mucosal immune responses in the patients. We have been studying antibody secreting cell responses (ASC) in patients as well as have permission to carry out antibody in lymphocyte supernantant (ALS) in 20 patients (approved addendum to protocol, June 2001).

We would now like permission for some minor additions:

1. To carry out the the ALS assay in 10 more patients, that is in total, study 30 patients for this response. This will enable us to analyse the results more effectively since the collection of samples was not uniform in the first few enrollees from the 20 patients in which we had permission to carry out the assay. For this purpose we will collect 30 ml of blood from patients on day 7 after onset of disease. As before, this will be carried out in adults, 18-45 years of age and with exclusion criteria as in the previous addendum.

2. We would also like to collect additional samples from the patients, apart from blood, saliva and feces that we now collect. This will include spontaneously occurring vomitus from patients in our study. This vomitus is otherwise discarded. The feces and now the vomitus will be used for microbiological, molecular biological and immunological assays. The vomitus will also be used for similar purposes.

3. We would now like to add some investigators as key collaborators in our study for the molecular biological, gene and proteomic analyses and will include:

a. Professor John J. Mekalanos, Chairman, Department of Microbiology, Harvard Medical School

b. Dr. Andrew T. Camilli, Department of Microbiology, Tufts University Medical School

c. Dr. S.M. Faruque, Head , Molecular Genetics Section, LSD, ICDDR,B

d. Dr. G.B. Nair, Associate Director, LSD

The revised protocol, the previous addendum to the protocol, copies of RRC/ERC approvals to the addendum, and the revised consent forms to include collection of vomitus is attached (both in English and Bangla) .

Thanking you

To,

2.12.2003

Chairman
ERC, ICDDR,B
Dhaka.

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

Ref.: Comments on the protocol entitled "Development of V. cholerae protein array and its application to the study of human immune response (Protocol# 2003-041)"

Under this protocol, a *V. cholerae* gene repository will be constructed. It will then be used to identify immunogenic protein/antigen(s) using protein micro array system. It is a very interesting approach to identify candidate protein molecule(s) capable of inducing immune response from a large number of proteins.

The serum samples will be taken from the stored samples collected under a previous protocol # 99-006. So, there is no need of any consent form for this protocol. In addition, the investigators will use rabbits for raising immune sera against *V. cholerae*. The protocol for using rabbits for such experiments is in conformity with standard procedures.

The protocol does not have any issues of ethical concern. Therefore, the protocol can be considered for ethical clearance