Attachment 1

Date: March 4, 1999

(FACE SHEET) **ETHICAL REVIEW COMMITTEE, ICDDR,B.**

Principal Investigator: Dr. M. John Albert

Application No. 99-01

Title of Study: Studies on virulence of Vibrio cholerae 0139 Bengal

Trainee Investigator (if any): _____

Supporting Agency (if Non-ICDDR,B) SIDA/SAREC

Project Status: _____

[✓] New Study

[ ] Continuation with change

[ ] No change (do not fill out rest of the form)

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

1. Source of Population:
   - (a) Ill subjects — Yes No
   - (b) Non-ill subjects — (Yes) No
   - (c) Minor or persons under guardianship — Yes No

2. Does the Study Involve:
   - (a) Physical risk to the subjects — (Yes) (No)
   - (b) Social risk — Yes (No)
   - (c) Psychological risks to subjects — Yes (No)
   - (d) Discomfort to subjects — (Yes)
   - (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 NA
   - (c) Physical risk — (Yes) No
   - (d) Sensitive questions — Yes No NA
   - (e) Benefits to be derived — (Yes) No
   - (f) Right to refuse to participate or to withdraw from study — (Yes) No
   - (g) Confidential handling of data — Yes No NA
   - (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

March 4, 1999 _____ Trainee

ABSTRACT SUMMARY FOR ETHICAL REVIEW COMMITTEE

V. cholerae O139 is a recently discovered second aetiologic agent of epidemic cholera. Although there are striking similarities between *V. cholerae* O139 and the El Tor biotype of *V. cholerae* O1 (the traditional serogroup that causes cholera), the former differs from the latter in the possession of a polysaccharide capsule (CPS). By analogy with other encapsulated bacteria, the CPS of *V. cholerae* O139 will have important biological effects including increased virulence of the organism. The properties that will be studied are: serum resistance, invasion of epithelial cells, and proinflammatory cytokine production. CPS will also be assessed as a protective antigen. Recent isolates of *V. cholerae* O139 are CAMP haemolysin negative compared to earlier isolates which were positive. Comparison of clinical data of patients suggested that those infected with CAMP haemolysin positive isolates had a more severe disease compared to those infected with CAMP haemolysin negative isolates. Cloning of CAMP haemolysin gene, construction of a CAMP haemolysin negative mutant and studies with purified CAMP haemolysin will clarify the role of CAMP haemolysin in virulence. The data that will be generated by this proposal will lead to a better understanding of the virulence of *V cholerae* O139, and contribute to vaccine development.

1. To conduct one aspect of the study, production of inflammatory cytokines, peripheral blood from healthy volunteers is required.

2. Adult volunteer of either sex to be recruited from the laboratory (about 6 in total) will be required to donate 5 ml of venous blood. There will be momentary pain associated with drawing of blood. There are no alternatives other than drawing blood.

3. A trained phlebotomist will draw the blood.

4. The source of the blood sample will be kept anonymous.

5. Signed consent will be obtained.

6. There is no interview involved.

7. There is no direct benefit to the volunteer, but the society in general may benefit.

8. The study requires no records except a one time donation of 5 ml of blood.

INTERNATIONAL CENTRE FOR DIARRHOEAL DISEASE RESEARCH, BANGLADESH (ICDDR,B)
LABORATORY SCIENCES DIVISION

Informed Consent Form for Volunteers

Protocol entitled, "Studies on virulence of *Vibrio cholerae* O139 Bengal".

Vibrio cholerae O139 Bengal is a recently discovered, second aetiologic agent of cholera. It has some differences with the traditional, aetiologic agent of cholera, *Vibrio cholerae* O1. The most important difference is that it possesses an outer coat which is called capsule. Because of this capsule, we believe that the organism can cause a more severe disease. To investigate this possibility, we need to study the interaction between the organism and white blood cells from healthy people. On interaction, the white blood cells may release some chemicals known as cytokines which is an indication of increased virulence of the organism.

Therefore, we ask you to participate in the study by a one time donation of 5 ml (approx. one teaspoon) of venous blood. The blood will be drawn from a vein in your arm. There will be momentary pain associated with the drawing of blood. Should any complications arise, we will provide you appropriate treatment.

We will answer any questions you may have regarding the study. There is no direct benefit for you, but the society may benefit from this study.

The confidentiality of records will be maintained. You can also refuse to participate in the study.

If we find that the capsule may contribute to severity of disease, this knowledge can be used for treatment and prevention of cholera.

If you agree to participate in this study, please sign below:

_____

Signature of volunteer Date: _____

_____

Signature of witness Date: _____

_____

Signature of investigator Date: _____

আন্তর্জাতিক উদরাময় গবেষণা কেন্দ্র, বাংলাদেশ
।। ল্যাবরেটরী সায়েন্স ডিভিশন ।।

ভিবরিও কলেরী ০১৩৯ বেঙ্গল, সম্প্রতি আবিষ্কৃত দ্বিতীয় কলেরা সৃষ্টিকারী জীবানু। গতানুগতিক কলেরা সৃষ্টিকারী জীবানু ভিবরিও কলেরী ০১ এর সাথে ভিবরিও কলেরী ০১৩৯ এর কিছু পার্থক্য আছে যার মধ্যে বিশেষভাবে উল্লেখযোগ্য হচ্ছে 'বাইরের আবরন' যা ক্যাপসুল নামে পরিচিত। আমরা মনে করি, এই ক্যাপসুলের উপস্থিতির কারনে এর রোগ সৃষ্টির প্রবনতা তুলনামূলকভাবে বেশী। এই সম্ভাব্যতা যাচাইয়ের জন্য কলেরা জীবানু এবং সুস্থ মানুষের শ্বেত কনিকার পারস্পরিক সম্পর্কের উপর আমাদের গবেষণা প্রয়োজন। এই পারস্পরিক সম্পর্কের ভিত্তিতে শ্বেতকনিকা কিছু রাসায়নিক পদার্থ যা 'সাইটোকাইন' নামে পরিচিত নিঃসরন করে এবং এর মাধ্যমেই জীবানুর একাধিক রোগ সৃষ্টিকারী প্রবনতার দিক নির্দেশনা পাওয়া যায়।

সেইজন্য আমরা আপনাকে গবেষণায় অংশগ্রহন করে মাত্র একবার ৫ সি.সি. (এক চা চামচ) পরিমান রক্ত দেবার জন্য অনুরোধ করছি। এই রক্ত আপনার বাহুর শিরা থেকে সংগ্রহ করা হবে। রক্ত সংগ্রহের সময় অস্থায়ীভাবে খুবই সামান্য অস্বস্তি লাগতে পারে। আপনার কোন সমস্যা দেখা দিলে আমরা প্রয়োজনীয় চিকিৎসার ব্যবস্থা করব।

গবেষণা সম্পর্কে আপনার সকল প্রশ্নের উত্তর জানাতে আমরা সচেষ্ট থাকব। এই গবেষণায় আপনি সরাসরি লাভবান না হলেও সমাজ উপকৃত হবে। গবেষণার সকল তথ্য গোপন রাখা হবে। আপনি স্বেচ্ছায় গবেষণায় অংশগ্রহন করা থেকে বিরত থাকতে পারেন।

আপনি যদি এই গবেষণায় অংশগ্রহনে সম্মত হন, তবে নিচে স্বাক্ষর দিন।

____
রক্ত দাতার স্বাক্ষর — তারিখ:

____
সাক্ষীর স্বাক্ষর — তারিখ:

____
গবেষকের স্বাক্ষর — তারিখ:

Principal Investigator: Last, first, middle _Albert MJ

International Centre for Diarrhoeal Disease Research, Bangladesh

RESEARCH PROTOCOL

FOR OFFICE USE ONLY
Protocol No: Date:
RRC Approval: Yes/ No Date:
ERC Approval: Yes/No Date:

1. Title of Project (Do not exceed 60 characters including spaces and punctuations)
Studies on virulence of *Vibrio cholerae* O139 Bengal

2a. Name of the Principal Investigator(s) (Last, Middle, First).	2b. Position / Title	2c. Qualifications
M. John Albert and Andrej Weintraub	Research Microbiologist	Ph.D,MRCPath

3. Name of the Division/ Branch / Programme of ICDDR,B under which the study will be carried out.
Laboratory Sciences Division

4. Contact Address of the Principal Investigator
4a. Office Location:
ICDDR,B
GPO Box 128
Dhaka-1000, Bangladesh

4b. Fax No: 880 2 872529/883116
4c. E-mail: albert@icddrb.org
4d. Phone / Ext: 880 2 602440

5. Use of Human Subjects Yes [ ] No [x]

5a. Use of Live Animal Yes [x] No [ ]

5b. If Yes, Specify Animal Species
Rabbit, Mouse

6. Dates of Proposed Period of Support
(Day, Month, Year - DD/MM/YY)
1/1/99 – 31/12/2001

7. Cost Required for the Budget Period
7a. *1st Year* ($): 75,619 ***2nd Year* ($):** 75,627 ***5th Year*:** 75,616
7b. Direct Cost ($) 219,365 **Total Cost ($)** 226,862

8. 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.

Prof. V.I. Mathan — Name of the Division Director
Signature
14/10/98 — Date of Approval

9. 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.

10. Signature of PIs
Date: 14/10/98

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☐ 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 Investigator Albert MJ, , Weintraub A., Qadri F, Rahman M, M. Mathan

Project Name: Studies on virulence of *Vibrio cholera* O139 Bengal

Total Budget ($) 226.862 Beginning Date 1/1/99 Ending Date 31/12/2001

PROJECT SUMMARY

V. cholerae O139 is a recently discovered second aetiologic agent of epidemic cholera. Although there are striking similarities between *V. cholerae* O139 and the El Tor biotype of *V. cholerae* O1 (the traditional serogroup that causes cholera), the former differs from the latter in the possession of a CPS. By analogy with other encapsulated bacteria, the CPS of *V. cholerae* O139 will have important biological effects including increased virulence of the organism. The properties that will be studied are: serum resistance, invasion of epithelial cells, and proinflammatory cytokine production. CPS will also be assessed as a protective antigen. Recent isolates of *V. cholerae* O139 are CAMP haemolysin negative compared to earlier isolates which were positive. Comparison of clinical data of patients suggested that those infected with CAMP haemolysin positive isolates had a more severe disease compared to those infected with CAMP haemolysin negative isolates. Cloning of CAMP haemolysin gene, construction of a CAMP haemolysin negative mutant and studies with purified CAMP haemolysin will clarify the role of CAMP haemolysin in virulence. The data that will be generated by this proposal will lead to a better understanding of the virulence of *V cholerae* O139, and contribute to vaccine development.

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

Name	Professional Discipline/ Specialty	Role in the Project
1. M. John Albert, Ph.D. MRCPath	Microbiology	Overall responsibility for the project
2. A. Weintraub, Ph.D	Biochemistry/Molecular Biology	Responsible for chemical and molecular aspects.
3. F. Qadri, Ph.D	Immunology	Responsible or immunological aspect
4. M. Rahman, Ph.D	Molecular Biology	Responsible for molecular aspect
5. M. Mathan, M.D., Ph.D	Pathology	Responsible for histopathological and electron microscopical aspects.

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.

V. cholerae O139, a second aetiologic agent of cholera possesses a polysaccharide capsule (CPS). By analogy with other capsulated bacteria, and from preliminary data on serum resistance, resistance to phagocytosis, and invasion of epithelial cells, CPS seems to contribute to increased virulence of the bacterium. Moreover, isolates from the initial epidemic possesed CAMP haemolytic activity, which was absent in subsequent isolates; this activity seemed to correlate with severity of illness. Therefore, we hypothesize that both CPS and CAMP haemolysin are associated with increased virulence of *V. cholerae* O139.

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).

1. To study the effect of CPS on serum resistance, invasion of epithelial cells, and proinflammatory cytokine production.

2. To study the protective efficacy of monoclonal antibodies (MAbs) to CPS in a suckling mouse cholera model.

3. To conjugate oligosaccharides of CPS to carriers such as tetanus toxoid, B-subunit of cholera toxin, or polymerized acrylamide to generate specific capsule-based antigen to evaluate immunogenicity and protective efficacy.

4. To clone the CAMP haemolysin gene and determine its sequence.

5. To generate a CAMP haemolysin negative mutant.

6. To compare the virulence of wild type CAMP haemolysin positive strain and its isogenic mutant in the suckling mouse cholera model.

7. To purify CAMP haemolysin and determine its diarrhoeagenic activity in appropriate models.

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 references. 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).**

BACKGROUND

Since its appearance in late 1992 as a second aetiologic agent of cholera, *V. cholerae* O139 has established itself as a causative agent of endemic cholera in the Indian subcontinent. In Dhaka, Bangladesh, in recent times, the prevalence of *V. cholerae* O139 has been found to be relatively low compared to *V. cholerae* O1 El Tor, but the reverse has been found to be true in neighboring Calcutta, India (1). The reason(s) for this is not clear, but may be related to various factors including host and environmental, and changes in bacteria themselves (2).

Available evidence suggests that *V. cholerae* O139 may have been derived from *V. cholerae* O1 El Tor. There are striking similarities between *V. cholerae* O1 and O139, but there are also differences. The most important difference is the possession of a capsule by *V. cholerae* O139 which is absent in *V. cholerae* O1 (3). However, the lipopolysaccharide (LPS) and CPS have been found to be cross-reactive (4). The CPS is built up of hexasaccharide repeating units of one D-galactose residue, two colitose residues, one N-acetyl D-quinovosamine residue, one N-acetyl-D-glucosamine residue, one D-galacturonic acid residue, and one residue of phosphate. The highly unusual components are colitose, and the phosphate which is cyclic; the latter has not been described in bacterial glycans (5). The LPS molecule consists of a lipid part (lipid A) and a core oligosaccharide, both of which are similar to the ones found in *V. cholerae* O1, and with a phosphate substituted hexasaccharide (19). The structure of the hexasaccharide is identical to the structure of one repeating unit of the CPS which may explain the cross-reactivity between the LPS and CPS (6,19).

Capsules are important virulence determinants of Gram positive bacteria (e.g. *Streptococcus pneumoniae*) as well as Gram negative bacteria (e.g. *Haemophilus influenzae, Neisseriae meningitidis, Klebsiella pneumoniae*, non-O1, non-O139 *V. cholerae*). They impart increased virulence on bacteria such as serum resistance, resistance to phagocytosis, and invasiveness which lead to disseminated infections (7).

To study the biologic significance of capsule of *V. cholerae* O139, a previous proposal entitled "Studies on the capsule of *Vibrio cholerae* O139 Bengal" has been funded by SIDA/SAREC for the period, July 1996-December 1998. The objectives of this project are listed below along with the progress achieved to-date.

a) To study the effect of capsular polysaccharide (CPS) on increased virulence of *V. cholerae* O139 such as serum resistance, resistance to phagocytosis, and invasion of epithelial cells.

We have compared the phagocytic killing of *V. cholerae* O139 strains with a standard capsulated strain of *Klebsiella pneumoniae*. There was no killing of *K. pneumoniae* with 100% survival, whereas *V. cholerae* O139 isolates were partially resistant with approximately 40% survival.

b) To study the protective efficacy of monoclonal antibodies (MAbs) to CPS in a suckling mouse cholera model.

We have generated six MAbs to *V. cholerae* O139. Two have been tested for their protective efficacy in a suckling mouse cholera model, and one provided protection. The remaining four MAbs are being studied. The binding of these MAbs to CPS will be verified by immunoelectron microscopy.

c) To study the interaction between phage specific for *V. cholerae* O139 and the CPS of *V. cholerae* O139 to generate oligosaccharides.

We have previously isolated phages that specifically lyse capsulated *V. cholerae* O139. We have now studied the interaction between the CPS and one of the phages and found that the phage hydrolyzes the site between the glucosamine residue and the galacturonic acid residue and generates an oligosaccharide.

d) To conjugate oligosaccharides of CPS of *V. cholerae* O139 to carriers such as tetanus toxoid, B-subunit of cholera toxin or polymerized acrylamide to generate specific capsule-based antigen for immunodiagnostic tests.

Since LPS and CPS in *V. cholerae* O139 are cross-reactive, preliminary studies were carried out with oligosaccharide from LPS. Oligosaccharide was conjugated to bovine serum albumin and used to raise antibodies in rabbits. The antibodies reacted with both LPS and CPS of *V. cholerae* O139. The protective efficacy of these antisera will be evaluated in suckling mouse cholera model.

e) To study the structural basis of cross-reaction between the CPS of *V. cholerae* O139 and other cross-reacting bacteria.

V. cholerae O139 cross-reacts with a number of bacteria including *V. cholerae* O155 and some strains of *V. mimicus*. The structures of O-specific polysaccharides of a strain of *V. cholerae* O155 and a strain of *V. mimicus* have been determined. *V. cholerae* O155 contained a pentasaccharide repeating unit consisting of two L-fucose residues and one residue each of galactose, acetyl glucosamine and acetyl fucosamine, with the galactose residue carrying a cyclophosphate group. An unusual component, galactose cyclophosphate has been reported previously as a component of the capsular polysaccharide and the O-antigen of *V. cholerae* O139. This component appears to be responsible for the serological cross-reactivity between the two bacteria. On the other hand, the *V. mimicus* O-polysaccharide contained a tetrasaccharide repeating unit consisting of galactose and acetyl galactosamine. The cross-reactivity seems to reside in the galactose residue.

f) Based on the unique sequences encoding the surface polysaccharides of *V. cholerae* O139, design primers and develop a polymerase chain reaction (PCR) assay for detection of *V. cholerae* O139 in clinical and environmental studies.

The PCR assay developed with the primer pair produced an amplicon only from *V. cholerae* O139. The assay was evaluated for diagnosis of *V. cholerae* O139 directly from 180 watery stools. All the 67 *V. cholerae* O139 culture-positive stool specimens were positive by PCR, and the remaining specimens which had either other recognized pathogens or no pathogens, were all negative by PCR.

The following publications have resulted from this project so far.

1. Albert MJ, Islam D, Nahar S, Qadri F, Falklind S, Weintraub A. Rapid detection of *V. cholerae* O139 Bengal from stool specimens by PCR. J Clin Microbiol 1997; 35:1633-5.

2. Landersjo C, Weintraub A, Ansaruzzaman M, Albert MJ, Widmalm G. 1998. Structural analysis of the O-antigenic polysaccharide from *Vibrio mimicus* N-1990. Eur J Biochem 1998; 251:986-90.

3. Senchenkova N, Zatonsky GV, Shashkov AS, Knirel YA, Jansson P-E, Weintraub A, Albert MJ. Structure of the O-antigen of *V. cholerae* O155 that shares a putative D-galactose-4, 6-cyclophosphate-associated epitope with *V. cholerae* O139 Bengal. Eur J Biochem 1998; 254:58-62.

4. Knirel YA, Senchenkova SN, Jansson PE, Weintraub A. More on the structure of *Vibrio cholerae* O22 lipopolysaccharide. Carbohydr Res (in press).

5. Albert MJ, Qadri F, Bhuyan NA, Ahmed SM, Ansaruzzaman M, Weintraub A. Phagocytosis of *V. cholerae* O139 Bengal by human polymorphonuclear leukocytes (submitted for publication).

6. Linnerborg M, Weintraub A, Albert MJ, Widmalm G. Phage hydrolysis of capsular polysaccharide from *V. cholerae* O139 (submitted for publication).

The present proposal is a continuation of the previous proposal which ends in December 1998. We intend to complete the unfinished work remaining in the previous project, and undertake additional objectives. The total objectives are stated in the present proposal.

Serum resistance

It has been reported that *V. cholerae* O139 is serum resistant, but the mechanism of resistance has not been investigated (4,8). Complement can be activated by classical pathway, alternative pathway or both. C3 is the central component of the complement system. Activation of complement will result in one or two products: i) Conversion of C3 into opsonic protein C3b or iC3b which activates the C5 cascade ii) C5 cascade results in the formation and assembly of C5b-9 membrane attack complex (MAC) capable of lysing susceptible bacteria. Serum resistance may be either due to the inability of capsulated bacteria to activate complement or after activating complement system and binding C3b, unable to form MAC,
because C3b binds far from the target cell membrane (9). Therefore, the mechanism of serum resistance needs to be investigated for *V. cholerae* O139.

Invasion of epithelial cells

Oral challenge of adult rabbits resulted in invasion of intestinal epithelial cells by *V. cholerae* O139, whereas this was absent after challenge with *V. cholerae* O1 (10). In an ultrastructural study of the upper small intestinal mucosal biopsy, more infiltration of polymorphonuclear leukocytes was seen in O139 patients compared to O1 patients (11). Moreover, a significantly higher proportion of O139 patients has been found to have faecal leukocytes compared to O1 patients (12). In a preliminary study of invasiveness of *V. cholerae* O139, some isolates invaded HEp-2 monolayer as documented by the gentamicin-survival assay. However, invasion studies with non-polarized cells such as HEp-2 do not approach the in vivo situation, since these cells lack brush border, and do not permit translocation *per se*. On the contrary, cell lines such as Caco-2 and HT-29, which are human colonic cell derived cell lines, form polarized monolayers with apical and basolateral surfaces separated by tight junctions, thereby providing a permeability barrier. This model is believed to more closely approximate bacteria-target cell interactions that may occur in vivo. In this model, epithelial cells are seeded onto a permeable filter support treated with collagen. The epithelial surface adherent to the filter forms the basolateral surface, non-adherent surface forms the apical surface. Separating these surfaces are tight junctions which provide impermeability and their polarity. Transepithelial permeability is studied by measuring electrical resistance across the monolayers; disruption of tight junction results in decrease in resistance. Using this model of infection, the translocation of invasive bacteria such as *Salmonella typhimurium* and *Campylobacter jejuni* has been studied. These bacteria translocated across the epithelial layer and could be recovered in the medium bathing the basolateral surface. Ultrastructural study of the polarized epithelium correlated with morphological changes seen in animal infection models indicating that this in vitro system is useful to study pathogens that interact with human intestinal epithelium (13,14). Therefore, this model appears to be appropriate to investigate the potential invasiveness of *V. cholerae* O139.

Production of proinflammatory cytokines

Bacterial products such as LPS and CPS have the ability to elicit or modulate the release of cytokines from host cells in both in vivo and in vitro models. The CPS of a variety of bacteria including *S. pneumoniae, S. aureus* and *V. vulnificus* all stimulate proinflammatory cytokines, TNF-α, IL-1, and IL-6, which play a pivotal role in host response to infection (15,16). Since CPS is a major surface antigen of *V. cholerae* O139 and cross-reactive with LPS, it is probable that this polysaccharide may play a role in the release of proinflammatory cytokines. Also infection of human colonic epithelial cell lines (Caco-2, HT-29) with invasive bacteria, results in coordinate expression and upregulation of an array of proinflammatory cytokines (17). Cytokines expressed in response to bacterial invasion have a well-documented role in chemotaxis and activation of inflammatory cells in the intestinal mucosa (18). Therefore, the possibility of production of proinflammatory cytokines needs to be studied in colonic epithelial cell lines upon infection with *V. cholerae* O139.

Conjugation of oligosaccharides of CPS to carriers

The LPS is not a suitable antigen for immunization since it has toxic properties. One way to convert a polysaccharide antigen to a good immunogen is to conjugate oligosaccharides to protein carriers. The CPS of *V. cholerae* O139 is a large molecule and not suitable for conjugation. It is, however, possible to

depolymerize the CPS and use oligosaccharides for conjugation. Chemical depolymerization of the CPS is not possible since the antigen possess both alkali-labile (uronic acid) and acid-labile (colitose)

residues which upon treatment are either destroyed or lost. Another possibility to generate oligosaccharides preserving specificity is to use enzymatic methods. Bacteriophages specific for encapsulated *V. cholerae* O139 indicate that these may have an enzyme with CPS degrading capability. We have tested the phages and found that one of them possesses an enzyme that hydrolyzes the linkage between the N-acetyl glucosamine and the galacturonic acid. We have been able to isolate oligosaccharides corresponding to two repeating units of the CPS. In addition, we are in the process of synthesizing one complete repeating unit of the CPS. The generated oligosaccharides as well as the synthetic repeating unit will be covalently conjugated to carrier proteins such as tetanus toxoid, cholera toxin B-subunit, diphtheria toxoid or to bovine serum albumin. In addition, we will generate conjugates with detoxified LPS and the above mentioned carrier proteins since the LPS possesses an epitope cross-reacting with the CPS, and it should be possible to generate immune response against CPS using LPS-conjugates.

CAMP haemolysin

The CAMP effect (named after the first letters of the names of the discoverers, Christie, Atkins and Munch-Petersen) is a synergistic haemolysis of sheep erythrocytes produced by a cohaemolysin of group B streptococci (*S. agalactiae*) in the diffusion zone of ß-toxin (sphingomyelinase) of *Staphylococcus aureus* (20). Subsequently, it has been found that in addition to group B streptococci, several other bacteria including *Listeria monocytogenes*, *Aeromonas, Actinobacillus pleuropneumoniae*, and vibrios produce the CAMP haemolysin (21). The CAMP factor has been purified from *S. agalactiae* by hydrophobic interaction chromatography and chromatofocussing. It has a molecular weight of 25k Da with a pI of 8.9 (22). CAMP factor from *S. agalactiae* was lethal when injected intravenously in rabbits (23). Injection of CAMP factor significantly lowered the lethal dose of group B streptococci (24). CAMP factor also binds immunoglobulins non-specifically via the Fc portion (25). CAMP gene has been cloned and expressed from *S. agalactiae, S. uberis* and *A. pleuropneumoniae*. In *S. agalactiae*, the CAMP gene was located on a 3.6 kb fragment of the chromosome which expressed a CAMP protein of mol. wt. 20,000 to 23,000. This mol. wt was slightly lower than the mol. wt. of CAMP factor from group B streptococci. This may be due to processing in *E. coli* host (26). In *S. uberis*, the CAMP gene was located on a 3.2 kb fragment of the chromosome. Sequencing suggested an ORF that could encode a 256-residue polypeptide with a mol.wt. of 28,363. The N-terminus of the predicted sequence showed a secretory signal sequence. The predicted amino acid sequence was highly homologous to the *S. agalactiae* CAMP protein. In immunoblot, the recombinant protein had a mol.wt of 28,000. CAMP protein of *S. uberis* was cross-reactive with *S. agalactiae* CAMP protein (21). In *A. pleuropneumoniae*, the CAMP gene was located on a 1.8 kb DNA fragment. It expressed a protein of 27 KDa on immunoblot developed with antibodies to CAMP factor of *S. agalactiae*. The cross-reactivity was reciprocal as antibodies to cloned CAMP haemolysin cross-reacted with CAMP factor from *S. agalactiae* (27).

The CAMP haemolytic activity has been used for presumptive diagnosis of *V. cholerae* O1 infection. El Tor biotype is always positive, classical biotype is negative, but some non-O1, non-O139 strains produce a weak atypical zone of haemolysis (28). *V. cholerae* O139 isolates were tested for CAMP haemolysis. All the 1993-94 isolates and the majority of 1995 isolates were CAMP positive, and TMP-SMX and vibriostatic compound O/129 resistant. However, the subsequent isolates exhibited opposite

characteristics. Comparison of clinical data of patients infected with CAMP$^+$, resistant isolates, and CAMP$^-$, susceptible isolates showed that, the patients infected with the former group of isolates had a disease of greater severity (2). The genetic aspect of resistance to TMP-SMX (and streptomycin and O/129) has been investigated by Waldor *et al.* who showed that it was encoded by a conjugative transposon named SXT element. Since in the suckling mouse model, the wild type resistant isolates and their isogenic susceptible mutants exhibited similar colonizing abilities, the conjugative transposon does not appear to contribute to virulence (29). However, the role of CAMP haemolysin in *V. cholerae* virulence has not been investigated. This can be done by studying the diarrhoeagenic potential of purified CAMP haemolysin, and by making CAMP haemolysin mutant and then comparing the virulence of wild type parent and mutant.

Preliminary results on cloning of CAMP gene

The amino acid sequences of the CAMP factor gene of *Streptococcus uberis* (20) and *Streptococcus agalactiae* (30) were compared to find homologous areas. Three primers were selected and ambiguous bases were used where matching bases were not obtained. The primers where used in a seminested PCR that gave a product of 386 bp in the positive control *S. uberis*.

This seminested PCR was used to investigate if an analogous CAMP factor gene was present in isolates of *V. cholerae* O139. One CAMP-test positive isolate of *V. cholerae* O139 from 1993, and one CAMP-test negative isolate of *V. cholerae* O139 from 1996, were tested together with *V. cholerae* O1 El Tor (CAMP-test positive) and *V. cholerae* O1 classical (CAMP-test negative). All of the tested isolates gave a single PCR fragment of expected length.

In order to examine if the amplified fragment was the correct gene, the PCR products were cloned, sequenced and compared with the published sequences of *S. uberis* and *S. agalactiae*. The results of the comparison are in progress. In order to verify the results, the sequencing will be repeated. If the PCR products show analogy to the CAMP factor gene, attempts to sequence the complete gene will be made.

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).

Experimental procedures

For this study, the following bacterial strains will be available.

V. cholerae O139 wild type strains which will be used as test organisms.

The following strains will be used as controls in various experiments. *V. cholerae* O139, spontaneous mutant that has lost the ability to produce capsule, but still reacts with O139 antiserum. Tn*phoA* mutant that has lost the ability to synthesize CPS and LPS, and do not react with O139 antiserum. *V. cholerae* O1 strains, *E. coli* DH 5α, *S. uberis*.

Various strains of *Klebsiella pneumoniae*: strain that does not activate complement; strain that activates complement up to C3b, but does not form membrane attack complex C3b-9; and a serum sensitive strain. *Shigella flexneri* 2a and *Salmonella typhimurium*.

In all the tests, appropriate controls will be used.

1.1. Serum resistance

The assay will be carried out as described previously with pooled normal human serum (NHS) (4). Viable bacteria will be enumerated after incubation of a standard dose of young culture with sera for 1 h at 37°C. Serum resistance will be calculated as 100 x ratio of number of colony forming units obtained after cells are incubated in unheated serum divided by the number of colony forming units obtained after the cells are incubated in heat-inactivated serum (56°C/30 min).

To study the effect of classical pathway (CP) of complement, serum will be first treated with 10 mM ethylene-glycoltetraacetic acid (EGTA) in the presence of 5 mM $MgCl_2$, and serum resistance test carried out (31). To test for the involvement of alternative pathway (AP) of complement, serum will be heated at 50°C/20 min before testing for resistance (32).

1.1a. Inhibition of serum resistance

The NHS will be treated with whole bacterial cell, purified LPS, and CPS separately. The absorbed serum will then be tested for serumcidal activity using a serum sensitive strain of *K. pneumoniae*. To verify whether inhibition of serum bactericidal activity is due to depletion of complement, the absorbed serum will be tested for complement activity using sensitized RBC and for complement components C_1q and C3 by ELISA (9).

1.1b. Activation of complement

Activation of complement by *V. cholerae* O139 will be studied by quantifying the activation products C3b and MAC (C5b-9). Bacteria will be incubated with NHS, washed with PBS, incubated with anti-C3b or anti-C5b-9 and washed with PBS again. The bacteria will be incubated with protein A-alkaline phosphatase. Colour reaction will be developed by paranitrophenyl phosphate and optical density measured (9).

1.2. Invasion of epithelial cells

The assay will be carried out using the human colonic epithelial cell line, Caco-2, in normal monolayer as well as in polarized monolayer in transwell filters for translocation. In the normal monolayer assay, a standard dose of bacteria will be incubated with the monolayer. After the infection period, the monolayer will be incubated in presence of gentamicin to kill extracellular bacteria. The gentamicin-surviving progeny will be enumerated after lysis of the monolayer (33,34). The invasive property will be verified by transmission electron microscopy. After infection with bacteria and incubation in the presence of gentamicin, cells will be fixed with glutaraldehyde in cacodylate buffer, stained with uranyl acetate, and examined by transmission electron microscope for intracellular bacteria. The effect of metabolic inhibitors on the invasion of Caco-2 cells by *V. cholerae* O139 will be examined as described previously in the quantitative cell culture assay containing gentamicin. The inhibitors that will be used are: cytochalasin D, chloroquine, ammonium chloride, dansylcadaverine, and colchicine.

Translocation of bacteria across polarized monolayer of Caco-2 cells will be carried out as described previously (13). Transwell filter units containing porous membranes will be seeded with Caco-2 cells and incubated in tissue culture medium for 10-14 days until a complete polarized monolayer is formed. Bacteria will be inoculated into apical layer. Translocation will be monitored by culturing the basolateral medium periodically over a period.

1.3. Proinflammatory cytokine production

The ability to induce proinflammatory cytokine production will be ascertained using live cells, and purified CPS and LPS with polymorphonuclear leukocytes from human peripheral blood, and also by inoculating colonic epithelial cell lines, Caco-2 and HT-29.

1.3a. Assay with peripheral blood mononuclear cells (PBMCs)

Whole blood will be collected from healthy volunteers (laboratory staff including the PI) and PBMCs separated by Ficoll-Paque gradient centrifugation. PBMCs will be stimulated with varying concentrations of CPS and LPS and live cells for varying periods of time. The supernatant tissue culture medium will be assayed for selected proinflammatory cytokines such as TNF-α, IL-1 and IL-6 by commercially available ELISA (16).

1.3b. Assay with colonic epithelial cell lines, Caco-2 and HT-29

Live bacterial cells will be inoculated into Caco-2 and HT-29 monolayers in tissue culture medium as in invasion assay. After 1 h interaction the monolayer will be washed, and replaced with tissue culture medium containing gentamicin to prevent extracellular bacterial multiplication. Supernatants will be harvested after 7 h and assayed for the above cytokines by ELISA (17).

2. Protective efficacy of MAbs to CPS in suckling mouse model

Suckling mouse model will be used to study the protective efficacy of monoclonal antibodies directed against the CPS of *V. cholerae* O139.

The 50% lethal dose (LD_{50}) of a wild type strain of capsulated clinical isolate of *V. cholerae* O139 will be determined in the infant mouse cholera model as described previously (35). The ability of MAbs to protect against cholera will be determined by incubating the MAbs with *V. cholerae* O139 and challenging a group of infant mice with antigen-antibody mixture.

2a. Localization of binding of MAbs to *V. cholerae* O139

This will be carried out using the MAbs against *V. cholerae* O139. The binding of MAbs to CPS will be verified by immunoelectron microscopy and gold labelling by standard procedures (36).

3. Isolation and purification of *V. cholerae* O139 LPS and CPS

V. cholerae strain AI-1838 is grown in a 30 L fermentor (Belach AB, Stockholm, Sweden) in a rich tryptone-yeast extract (TY-medium) as described (37) to late logarithmic phase. The culture is checked for purity by Gram-stain and inoculation on blood- and L-agar at the end of the growth cycle and the bacteria killed with formaldehyde (1% wt/vol). After incubation at 4°C overnight, the bacteria are collected by centrifugation (8.000 X *g*, 4°C, 20 min) and washed with phosphate buffered saline (PBS, 0.01 M, pH 7.3). Pelleted bacteria are then suspended in water and extracted with hot phenol-water (38), followed by phenol-chloroform-petroleum ether (PCP) extraction (39,40). Two fractions are obtained: one PCP-soluble and one PCP-insoluble. The PCP-soluble fraction (containing LPS) is further processed as follows. The chloroform and petroleum ether are removed by evaporation and the LPS precipitated from the phenol phase by 6 volumes of diethyl ether-acetone (1:5 v/v) (41). The PCP-insoluble fraction (containing the CPS) is air-dried, suspended in water and dialyzed against distilled water for three days at 4°C. The CPS is further purified by treatment with RNase and DNase as well as with proteinase K (Sigma Chemical Company, St. Louis, MO. USA) followed by repeated ultracentrifugations (2 x 100.000 x *g*, 16 h at 4°C). The CPS present in the supernatant after ultracentrifugations is lyophilized and used for further studies.

3a. Generation of oligosaccharides

In order to generate oligosaccharides from *V. cholerae* O139 CPS, the earlier described bacteriophages will be used (42). Due to the high specificity of these phages for capsulated strains, it is likely that enzyme(s) is present with a glycosidase activity. If so, specific oligosaccharides may be generated by incubation of purified CPS with purified phages. If the CPS is hydrolyzed, the oligosaccharides may be separated by gel-permeation chromatography. Since the CPS is of built of carbohydrates, it is a poor immunogen. Conjugation of the whole native CPS to protein is possible, but due to its large molecular weight, the immunogenicity of the conjugate may not increase. Chemical methods for degradation of large polysaccharides are available. However, the V. cholerae O139 CPS contains both acid-labile (colitose) and alkali-labile (galacturonic acid) components. Enzymatic degradation of the CPS will generate oligosaccharides that are easy to characterize without the risk of losing important epitopes. The oligosaccharides will be characterized by ^{1}H-NMR and ^{13}C-NMR as well as by mass spectrometry. The purified oligosaccharides will then be conjugated to different carriers depending on the purpose of the conjugate i.e. (a) acrylamide-CPS conjugates will be used as antigens in enzyme immunoassay; (b) protein-CPS conjugates (bovine serum albumin, cholera toxin B-subunit or tetanus toxoid) will be used as immunogens for generation of specific antibodies. We have now generated small amounts of oligosaccharides by incubation of bacteriophage with purified CPS. After the incubation at 37°C, oligosaccharides were isolated by gel-permeation chromatography. Initial analyses of the oligosaccharides show that the major fraction corresponds to two repeating units. It is also evident that the enzyme present in the bacteriophage is specific for the linkage between the N-acetyl glucosamine and the galacturonic acid. Attempts to conjugate this oligosaccharide to protein carrier are in progress.

Another approach for generation of antibodies and for generation of specific diagnostic antigens is to couple a detoxified LPS molecule to different carriers. The rationale behind this is that the LPS molecule contains a single repeat identical to that of the CPS. Since the LPS is an endotoxin, it is important to detoxify it. This can be achieved by O-deacetylation using anhydrous hydrazine. Following controlled oxidation, we will introduce reactive groups in the inner core region of the molecule to be used in conjugation.

The conjugates will be used to immunize rabbits and the antiserum will be tested for protective efficacy in infant mouse cholera model against challenge with virulent *V. cholerae* O139.

4. Cloning and sequencing of the CAMP haemolysin gene in *V. cholerae* O139

A seminested PCR will be adapted to amplify a 386 bp fragment of CAMP gene. Oligonucleotide primers will be designed from the conserved region of *S. urbers* CAMP gene to amplify a similar fragment of CAMP gene of *V. cholerae*. The amplified product will be ligated into pCRII vector. The ligated product was transformed into *E. coli* INVF'α cell using a TA cloning kit (Invitrogen, San Diego, CA, USA). The transformants will be screened by PCR using M13 universal and reverse primers. Plasmid DNA will be extracted from the positive clones and sequenced by di-deoxy chain termination method. DNA sequencing will be performed with an automated laser fluorescent DNA sequencer (A.L.F. Pharmacia Biotech, Uppsala, Sweden) and the obtained sequence will be analyzed for its homology with CAMP factor of other bacteria using GCG software package. A lambda library will be constructed using ECoRI digested (3-5 kb) chromosomal DNA from a CAMP haemolysin positive test strain of *V. cholerae* O139 (43). The library will be screened with the p^{32}-labelled cloned CAMP gene

fragment. Positive clones will be screened twice, subsequently cloned and complete nucleotide sequence of CAMP-gene will be determined. The 5' flanking region of CAMP gene sequence will be determined from clones carrying this region.

5. Construction of CAMP haemolysin negative isogenic mutant

Full-length CAMP gene along with the 5' flanking region will be amplified and cloned in pCRII vector using TA cloning kit. Full-length cloned gene will be used for construction of isogenic mutant by homologous recombination. The 5'end along with the promoter region of the CAMP-gene will be replaced with a kanamycin resistant gene (44). The resulting plasmid will be transformed into *E. coli* DHα and the positive transformants selected on kanamycin. Plasmid DNA will be extracted from the positive clones and electroporated into wild type parent *V. cholerae* O139 strain. The mutants will be selected on kanamycin plate and analyzed for CAMP haemolysin production. Clones which are negative for CAMP haemolysin will be used for further study.

An alternate strategy for making CAMP haemolysin negative mutant is by using a suicide vector. Approximately, 0.7 kb 3' fragment of the CAMP gene will be amplified and cloned in PCR 2.1 vector using a standard protocol. The cloned gene fragment will be cleaved, purified, and ligated in suicide vector, pJM 703.1 (49). The ligation product will be transformed into λ pir lysogen of *E. coli* strain SM10. The pJM 703.1 derivative will be transformed into *V. cholerae* strain by conjugation (50). The transconjugants will be screened for CAMP haemolysin production. Mutants obtained will be used for further study.

6. Comparison of virulence of wild type CAMP haemolysin positive strain and its isogenic mutant

Virulence assays will be done by suckling mouse colonization competition assay (45) and by determination of LD_{50} for suckling mouse (46). In the competition assay, a 1:1 ratio of wild type strain and its isogenic mutant will be orally inoculated into a group of 3- to 5-day suckling mice. After 20 h, the mice will be sacrificed and the relative number of each colony type determined by plating dilutions of intestinal homogenate. In the LD_{50} assay, varying dilutions of the parent and mutant strains will be inoculated with suckling mice and LD_{50} calculated by Reed and Muench formula (47).

7. Purification of CAMP haemolysin and determination of its diarrhoeagenic potential

The strategy used for purification of CAMP factor from *S. agalactiae* will be adopted. The various steps involved are ultrafiltration (Amicon hollow fiber dialyzer), ammonium sulphate precipitation to 75% saturation, hydrophobic interaction chromatography with octyl-sepharose CL-4B (Pharmacia), and chromatofocussing on polybuffer exchanger PBE 94 (Pharmacia) column. Purity will be checked by SDS-PAGE. Finally, purified protein will be obtained by PAGE under non-denaturating conditions (22).

The biological activity of purified CAMP haemolysin will be assessed by tube haemolysis as described previously (27). Varying concentrations of the purified protein in Tris-HCl-NaCl will be mixed with *S. aureus* crude ß-toxin and sheep RBC in the same buffer.

The diarrhoeagenic potential of the purified haemolysin will be assessed by suckling mouse cholera model, rabbit ileal loop assay, and Ussing chamber assay. In the Ussing chamber assay, rabbit ileal

mucosa will be mounted in Lucite chamber. Defined quantities of purified CAMP haemolysin will be added to both mucosal and serosal sides and short circuit current (ISc) measured (48).

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 equipments that will be required for the study. For field studies, describe the field area including its siz population, and means of communications. (TYPE WITHIN THE PROVIDED SPACE).

This is a laboratory based study which will be carried out with stored clinical isolates. The project will require tissue culture facilities, small laboratory animals, molecular biological facilities, and immunochemical analysis. Some facilities are available in ICDDR,B and other in Huddinge Hospital, Karolinska Institute.

Data Analysis

Describe ans for data analysis. Indicate whether data will be analyzed by the investigators themselves or by other professionals. Specify what statistical softwares 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).

The significance of difference in the LD50s of the wild type strain and its CAMP negative isogenic mutant will be deduced by Student t test.

Ethical Assurance for Protection of Human Rights

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.

This study does not involve human subjects; only stored bacterial isolates from diarrhoeal stools will be used.

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.

Standard animal assays such as adult rabbit ileal loop assay and suckling mouse assay will be used to investigate the diarrhoeagenic potential of bacterial isolates. There is no alternative to using these animal models to investigate the virulence of wild type strains and genetically engineered mutants. However, standard animal ethical guidelines will be complied with for these 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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24. Fehrenbach FJ, Jurgens D, Ruhlmann J, Sterzik B, Özel M. Role of CAMP-factor (protein B) for virulence. In: Fehrenbach FJ, Alouf JE, Falmagne P, Goebel W, Jeljaszewicz J, Jurgens D, Rappuoli R, eds. Bacterial protein toxins. Stuttgart: Gustov Fischer Verlag, 1988: 351-357.

25. Jurgens D, Sterzik B, Fehrenbach FJ. Unspecific binding of group B streptococcal cocytolysin (CAMP factor) to immunoglobulins and its possible role in pathogenicity. J Expl Med 1987; 165:720-732.

26. Schneewind O, Friedrich K, Lutticken R. Cloning and expression of the CAMP factor of group B streptococci in *Escherichia coli*. Infect Immun 1988; 56:2174-2179.

27. Frey J, Perrin J, Nicolet J. Cloning and expression of a cohemolysin, the CAMP factor of *Actinobacillus pleuropneumoniae*. Infect Immun 1989; 57:2050-2056.

28. Lesmana M, Subekti D, Tjaniadi P, Pazzaglia G. Modified CAMP test for biogrouping *Vibrio cholerae* O1 strains and distinguishing them from strains of *Vibrio cholerae* non-O1. J Clin Microbiol 1994; 32:235-237.

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41. Qureshi N, Takayama K, Ribi E. Purification and structural determi-nation of nontoxic lipid A obtained from the lipopolysaccharide of *Salmonella typhimurium*. J Biol Chem 1982; 257:11808-11815.

42. Albert MJ, Bhuiyan NA, Rahman A, Ghosh AN, Hultenby K, Weintraub A, Nahar S, Kibriya AKMB, Ansaruzzaman M, Shimda T. Phage specific for *Vibrio cholerae* O139 Bengal. J Clin Microbiol 1996; 34:1843-1845.

43. Maniatis T, Fritsch EF, Sambrook J. Molecular cloning: a laboratory manual. Spring Harbor, NY: Cold Spring Harbor Laboratory, 1982.

44. Häse CC, Finkelstein RA. Cloning and nucleotide sequence of the *Vibrio cholerae* hemagglutinin/protease (HA/protease) gene and construction of an HA/protease-negative strain. J Bacteriol 1991; 173:3311-3317.

45. Waldor MK, Mekalanos JJ, ToxR regulates virulence gene expressed in non-O1 strains of *Vibrio cholerae* that cause epidemic cholera. Infect Immun 1994; 62:72-78.

46. Attridge SR, Rowley D. The role of flagellum in the adherence of *Vibrio cholerae*. J Infect Dis 1983; 147:864-872.

47. Reed LJ, Muench H. A simple method of estimating fifty percent endpoints. Am J Hyg 1938; 27:493-497.

48. Saha P, Koley H, Mukhopadhyay AK, Bhattacharya SK, Nair GB, Ramakrishna BS, Krishnan S, Takeda T, Takeda Y. Nontoxigenic *Vibrio cholerae* O1 serotype Inaba biotype El Tor associated with a cluster of cases of cholera in southern India. J Clin Microbiol 1996; 34:1114-1117.

49. Miller MV, Mekalanos JJ. A novel suicide vector and its use in construction of insertion mutations: osmoregulations of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires toxR. J Bacteriol 1998;170:275-2583.

50. Maniatis T, Fritsch EF, Sambrook J. Molecular cloning: a laboratory manual. Cold Spring Harbor Laoraory. Cold Spring Harbor, N.Y.

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 findings will be disseminated through the Inter Divisional Scientific Forum and Annual Scientific Conference of ICCDR,B, international conferences and international peer-reviewed scientific journals.

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 is a collaborative project between Laboratory Sciences Division of ICDDR,B and Department of Immunology, Microbiology, Pathology and Infectious Diseases, Division of Clinical Bacteriology, Karolinska Institute, Huddinge, Sweden. This project will be funded by SIDA/SAREC.

Title: : Studies on virulence of *V. cholerae* 0139 Bengal

PI : M John Albert (ICDDR,B)

Collaborator : Andrej Weintraub (Huddinge Hospital)

Division of work between ICDDR,B and Huddinge Hospital.

Work that will be done in ICDDR,B:

Objective 1: Studies on serum resistance, invasion of epithelial cells, and proinflammatory cytokine production.

Objective 2: Studies on protective efficacy of monoclonal antibodies in the suckling mouse cholera model (part of this objective).

Objective 5: Generation of CAMP haemolysin negative mutant.

Objective 6: Comparison of virulence of CAMP hemolysin positive strain and its isogenic mutant in the suckling mouse cholera model.

Objective 7: Purification of CAMP haemolysin; determination of diarrhoeagenic activity (part of this objective).

Work that will be done in Huddinge Hospital

Objective 2: Immuno electron microscopy studies to determine the location of binding sites of antibodies (part of this objective).

Objective 3: Conjugation of CPS to carriers to generate CPS-based antigen.

Objective 4: Cloning of the CAMP gene and determination of its sequence.

Objective 7: Purification of CAMP haemolysin (part of this objective).

a:vcholera.MJA

Biography of the Investigators

Give biographical data in the following table for key personnel including the Principal Investigator. Use a photocopy of this page for each investigator.

Name	Position	Date of Birth
M. John Albert	Research Microbiologist	28/5/1950

Academic Qualifications (Begin with baccalaureate or other initial professional education)

Institution and Location	Degree	Year	Fild of Study
Christian Medical College, Martandam; University of Madurai	B.Sc.	1968	Biology
JIPMER, Pondicherry; University of Madras	M.Sc.	1970	Medical Microbiology
Christian Medical College Hospital Vellore; University of Madras	Ph.D	1979	Medical Microbiology
Royal College of Pathologists, London .	MRCPath	1994	Medical Microbiology

Research and Professional Experience

Concluding with the present position, list, in chronological order, previous positions held, experience, and honours. Indicate current membership on any professional societies or public committees. List, in, chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. (DO NOT EXCEED TWO PAGES, USE CONTINUATION SHEETS).

Research Microbiologist, Laboratory Sciences Division, ICDDR,B, May 1989-May 1992.

Research Microbiologist and Head, Department of Laboratory Research, Laboratory Sciences Division, ICDDR,B, June 1992-May 1994.

Acting Division Director, Laboratory Sciences Division, ICDDR,B, June 1994-November 1996.

Interim Division Director, Laboratory Sciences Division, ICDDR,B, December 1996-December 1997.

Research Microbiologist, Laboratory Sciences Division, ICDDR,B, January 1998-present.

Member ,Australian Society for Microbiology

Selected publications of Dr. M.J. Albert

1. Naka A, Yamamoto K, Albert MJ, Honda T. *Vibrio cholerae* O139 produces a protease that is indistinguishable from the hemagglutinin/protease of *V. cholerae* O1 and non-O1. FEMS Immunol Med Microbiol 1995; 11:87-90.

2. Stroeher UH, Jedani KE, Dredge BK, Morona R, Brown MH, Karageorgos LE, Albert MJ, Manning P. Genetic rearrangement in the *rfb* regions of *Vibrio cholerae* O1 and O139. Proc Natl Acad Sci USA 1995; 92:10374-10378.

3. Albert MJ, Bhuiyan NA, Rahman A, Ghosh AN, Hultenby K, Weintraub A, Nahar S, Kibriya AKMG, Ansaruzzaman M, Shimada T. Phage specific for *Vibrio cholerae* O139 Bengal. J Clin Microbiol 1996; 34:1843-1845.

4. Knirel Y, Senchenkeuva SN, Jansson P-E, Weintraub A, Ansaruzzaman M, Albert MJ. Structure of the O-specific polysaccharide of *Aeromonas trota* strain cross-reactive with *Vibrio cholerae* O139 Bengal. Eur J Biochem 1996; 238:160-165.

5. Albert MJ. Epidemiology and molecular biology of *Vibrio cholerae* O139 Bengal. Indian J Med Res 1996; 104:14-27.

6. Faruque ASG, Fuchs GJ, Albert MJ. Changing epidemiology of cholera due to *Vibrio cholerae* O1 and O139 Bengal in Dhaka, Bangladesh. Epidemiol Infect 1996; 116:275-278.

7. Dhar U, Bennish ML, Khan WA, Seas C, Khan EH, Albert MJ, Salam MA. Clinical features, antimicrobial susceptibility and toxin productions in *Vibrio cholerae* infection : comparison with *Vibrio cholerae* 01 infection. Trans R Soc Trop Med Hyg 1996; 90:402-405.

8. Nandy RK, Albert MJ, Ghose AC. Serum antibacterial and antitoxin responses in clinical cholera caused by *Vibrio cholerae* O139 Bengal and evaluation of their importance in protection. Vaccine 1996; 14:1137-1142.

9. Falklind S, Stark M, Albert MJ, Uhlén M, Lundeberg J, Weintraub A. Cloning and sequencing of a region of *Vibrio cholerae* O139 Bengal and its use in PCR-based detection. J Clin Microbiol 1996; 34:2904-2908.

10. Ansaruzzaman M, Albert MJ, Kühn I, Faruque SM, Siddique AK, Möllby R. Differentiation of *Vibrio cholerae* O1 isolates with biochemical fingerprinting and comparison with ribotyping. J Diarrhoeal Dis Res 1996; 14:248-254.

11. Lesmana M, Albert MJ, Subekti D, Richie E, Tjaniadi P, Waltz S, Lebron CI. Simple differentiation of *Vibrio cholerae* O139 from *V. cholerae* O1 and non-O1 and non-O139 by modified CAMP test. J Clin Microbiol 1996; 34:1038-1040.

12. Nakasone N, Iwanaga M, Yamashiro T, Nakashima K, Albert MJ. *Aeromonas trota* strains which agglutinate with *Vibrio cholerae* O139 Bengal antiserum, possess a serologically distinct fimbrial colonization factor. Microbiology 1996; 142:309-313.

13. Islam MS, Alam MJ, Begum A, Rahim Z, Felsenstein A, Albert MJ. Occurrence of culturable *Vibrio cholerae* O139 Bengal with *ctx* gene in various components of aquatic environment in Bangladesh. Trans R Soc Trop Med Hyg 1996; 90:128.

14. Faruque SM, Ahmed KM, Alim ARMA, Siddique AK, Albert MJ. Emergence of a new clone of toxigenic *Vibrio cholerae* O1 El Tor displacing *V. cholerae* O139 Bengal in Bangladesh. J Clin Microbiol 1997; 35:624-630.

15. Albert MJ, Islam D, Nahar S, Qadri F, Falklind S, Weintraub A. Rapid detection of *Vibrio cholerae* O139 Bengal from stool by polymerase chain reaction. J Clin Microbiol 1997; 35:1633-1635.

16. Qadri F, Johnson G, Begum YA, Wenneras C, Albert MJ, Salam MA, Svennerholm A-M. Immune response to the mannose-sensitive hemagglutinin in patients with cholera due to *Vibrio cholerae* O1 and O139. Clin Diagn Lab Immunol 1997; 4:429-434.

17. Faruque SM, Ahmed KM, Siddique AK, Zaman K, Alim ARMA, Albert MJ. Molecular analysis of toxigenic *Vibrio cholerae* O139 Bengal isolated in Bangladesh between 1993 and 1996: evidence for the emergence of a new clone of the Bengal vibrios. J Clin Microbiol 1997; 35:2299-2306.

18. Qadri F, Wenneras C, Albert MJ, Hossain J, Mannoor K, Begum YA, Mohi G, Salam MA, Sack RB, Svennerholm A-M. Comparison of immune responses in patients infected with *Vibrio cholerae* O139 and O1. Infect Immun 1997; 65:3571-3576.

19. Albert MJ, Bhuiyan NA, Talukder KA, Faruque ASG, Nahar S, Faruque SM, Ansaruzzaman M, Rahman M. Phenotypic and genotypic changes in *Vibrio cholerae* O139 Bengal. J Clin Microbiol 1997; 35:2588-2592.

20. Ehara M, Shimodori S, Kojima F, Ichinose Y, Hirayama T, Albert MJ, Supawat K, Honma Y, Iwanaga M, Amako K. Characterization of filamentous phages of *Vibrio cholerae* O139 and O1. FEMS Microbiol Lett 1997; 154:293-301.

21. Kjellberg A, Weintraub A, Albert MJ, Widmalm G. Structural analysis of the O-antigenic polysaccharide from *Vibrio cholerae* O10. Eur J Biochem 1997; 249:758-761.

22. Ehara M, Iwami M, Ichinose Y, Hirayama T, Albert MJ, Sack RB, Shimodori S. Induction of fimbriated *Vibrio cholerae* O139. Clin Diagn Lab Immunol 1998;5:65-69.

23. Landersj OC, Weintraub A, Ansaruzzaman M, Albert MJ, Widmalm G. Structural analysis of the O-antigen polysaccharide from *Vibrio mimicus* N-1990. Eur J Biochem 1998; 251:986-990.

24. Albert MJ, Faruque ASG, Mahalanabis D. Association of *Providencia alcalifaciens* with diarrhea in children. J Clin Microbiol 1998; 36:1433-1435.

25. Elliott SJ, Srinivas S, Albert MJ, Alam K, Robins-Browne RM, Gunzburg ST, Mee BJ, Chang BJ. Characterization of the roles of hemolysin and other toxins in enteropathy caused by α-hemolytic *Escherichia coli* linked to human diarrhoea. Infect Immun 1998; 66:2040-2051.

26. Basu A, Mukhopadhyay AK, Sharma C, Jyot J, Gupta N,Ghosh A, Bhattacharya SK, Takeda Y, Faruque ASG, Albert MJ, Nair GB. Heterogeneity in the organization of the ctx element in strains of *Vibrio cholerae* O139 Bengal isolated from Calcutta, India, and Dhaka, Banlgadesh, and its plausible link to the dissimilar incidence of the O139 cholera in the two locales. Microb Pathog 1998; 24:175-183.

27. Qadri F, Mäkelä PH, Holmgren J. Albert MJ, Mannoor K, Kantele A, Saha D, Salam MA, Kantele JM. Watery diarrhea in an endemic area induces a circulating antibody-secreting cell response with homing potentials to both mucosal and systemic tissues. J Infect Dis 1998; 177:1594-1599.

28. Senchenkova SN, Zatonsky GV, Shashkov AS, Knirel YA, Jansson P-E, Weintraub A, Albert MJ. Structure of the O-antigen of *Vibrio cholerae* O155 that shares a D-galactose-4,6-cyclophosphate epitope with *V. cholerae* O139 Bengal. Eur J Biochem 1998; 254:58-62.

Principal Investigator: Last, first, middle _Albert MJ

Detailed Budget for New Proposal

Project Title: Studies on virulence of *Vibrio cholerae* O139 Bengal

Name of PI: M. John Albert

Protocol Number: Name of Division: Laboratory Sciences Division

Funding Source: SIDA/SAREC Amount Funded (direct): 155,296 Total: 196,861 Overhead (%) 13.6 (Swedish part only)

Starting Date: 1/1/99 Closing Date: 31/12/2001

Strategic Plan Priority Code(s): 11 (100%), 92 (100%)

Sl. No	Account Description	Salary Support			US $ Amount Requested		
	Personnel	**Position/Grade**	**Effort%**	**Salary Rate**	**1st Yr**	**2nd Yr**	**3rd Yr**
1.	Senior scientific Officer (1828-3)	NO B	45%	1084	5845	6147	6454
2.	Senior Scientific Officer (378-0)	NO B	45%	1104	5962	6260	6573
3.	Research Officer (1949-7)	GS V	100%	430	5160	5418	5688
4.	Sr. Research Officer (3571-7)	GS VI	75%	510	4590	-	-
	Sub Total				21566	17825	18715
	Consultants				2000	2000	2000
	Local Travel				300	300	300
	International Travel				5200	5200	5200
	Sub Total				7500	7500	7500
	Supplies and Materials (Description of Items)						
1.	Bacteriological media				5000	5000	5000
2.	Chemicals				4000	4000	4000
3.	Chromatographic reagents				2000	2000	2000
4.	Immunological reagents				2566	2377	2350
5.	Tissue culture media				1000	2000	2000
6.	Molecular Biology reagents				3000	6000	6000
7.	Electron microscopy reagents				500	500	500
8.	Glass ware				549	1070	500
	Sub Totals				18615	22947	22350

	Other Contractual Services			
	Repair and Maintenance	500	500	500
	Rent, Communications, Utilities	500	500	500
	Training Workshop, Seminars	-	-	-
	Printing and Publication	600	600	600
	Staff Development	-	-	-
	Sub Total	1600	1600	1600

	Interdepartmental Services	**1st Yr**	**2nd Yr**	**3rd Yr**
	Computer Charges			
	Pathological Tests			
	Microbiological tests			
	Biochemistry Tests			
	X-Rays			
	Patients Study			
	Research Animals	350	350	350
	Biochemistry and Nutrition			
	Transport			
	Xerox, Mimeographs etc.			
	Staff Clinic charges	**600**	**600**	**600**
	Sub Total	950	950	950
	Other Operating Costs	1000	400	-
	Capital Expenditure Minicell-ERS resistance system	-	1728	-
	GRAND TOTAL	**51231**	52950	**51115**

TOTAL DIRECT COST $ 155,296

Budget for Division of Clinical and ORAL Bacteriology, Karolinska Institute, Huddinge, Sweden.

Sl. No	Account Description	Salary Support			US $ Amount Requested		
	Personnel	**Position/Grade**	**Effort %**	**Salary Rate**	**1st Yr**	**2nd Yr**	**3rd Yr**
1.	Laboratory technician (incl social charges)		30%		9438	9938	10438
	Sub Total				9438	9938	10438
	Overhead (13.6%)				1284	1352	1420
	Consultants						
	Local Travel						
	International Travel				2875	2938	3125
	Sub Total				13597	14228	14983
	Supplies and Materials (Description of Items)						
1.	Bacteriological media				900	500	500
2.	Chemicals				1500	1200	1300
3.	Chromatographic reagents and columns				1900	1300	1400
4.	Immunological reagents				400	380	360
5.	Tissue culture media				900	458	528
6.	Molecular Biology reagents				2000	1500	1940
7.	Electron microscopy reagents				400	350	350
8.	Glass ware				500	500	500
9	Literature				1000	1250	1500
10	**Overhead**(13.6%)				1292	1012	1140
11	**Sub Totals**				10792	8450	9516
	TOTAL				24389	22678	24500

Conversion 1 US $ = 8 SEK
Total = $ 71566

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.

ICDDR,B part

Salary support is budgeted for three support staff who are essential to carry out the work.

Supplies and materials include bacteriological media, and reagents for immunology, tissue culture, chromatography, molecular biology and electron microscopy. These items are essential for the work.

International travel includes air fare and per diem. This is required for the consultant pathologist, Dr. M. Mathan, to make several trips to the National Institute of Cholera and Enteric Diseases (NICED), Calcutta, India, where electron microscopic work will be done. Travel money is also required for Dr. Albert to make trips to the collaborative laboratory in Sweden.

The capital item is required for measurement of electrical resistance of tissue culture system in bacterial translocation assays across cell monolayer.

Huddinge Hospital part:

The expenses relate to salary support of a support staff, chemicals and reagents and travel. Immunochemical and some molecular aspects of the work will be done in Huddinge; the reminder of the work will be done in 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)

1. Ecological and epidemiological studies on *Aeromonas* spp. in Bangladesh with special emphasis on their spread between the environment and the humans. July 1996-December 1998, SIDA/SAREC, US $ 117,000; Principal Investigator.

2. Studies on the capsule of *Vibrio cholerae* O139 Bengal. July 1996-December 1998, SIDA/SAREC, US $ 133,955; Principal Investigator.

3. Epidemiologic and ecologic study of *Vibrio cholerae* . September 1996-August 2001, National Institutes of Health, USA, US $ 4,700,000; Co-Principal Investigator.

4. Effect of vitamin A and zinc supplementation on immune response to oral cholera vaccination in Bangladeshi children. May 1998-July 1999; Thrasher Research Fund, USA; US $ 100,201; Principal Investigator.

Reviewer # 1

Title: Studies on virulence of Vibrio cholerae 0139 Bengal

Summary of Referee's Opinions: Please see the following table to evaluate the various aspects of the proposal by checking the appropriate boxes. Your detailed comments are sought on a separate, attached page.

	Rank Score		
	High	Medium	Low
Quality of Project	✓		
Adequacy of Project Design	✓		
Suitability of Methodology	✓		
Feasibility within time period	✓		
Appropriateness of budget	✓		
Potential value of field of knowledge	✓		

CONCLUSIONS

I support the application:

a) without qualification ☑

b) with qualification ☐

- on technical grounds ☐
- on level of financial support ☐

I do not support the application ☐

Detailed Comments

Please briefly provide your opinions of this proposal, giving special attention to the originality and feasibility of the project, its potential for providing new knowledge and the justification of financial support sought; include suggestions for modifications (scientific or financial) where you feel thay are justified.

(Use additional pages if necessary)

Title: n/p

PI: n/p

Reviewer:

Scientific suggestion: Experimental procedures 1 2.

Inversion of epithelial cells: Ruthenium red should be present in both fixative and washing solutions (CB) at a final concentration of 0.075% (Luft, 1971).

Reviewer # 2

Title: Studies on virulence of Vibrio cholerae 0139 Bengal

Summary of Referee's Opinions: Please see the following table to evaluate the various aspects of the proposal by checking the appropriate boxes. Your detailed comments are sought on a separate, attached page.

	Rank Score		
	High	Medium	Low
Quality of Project	X		
Adequacy of Project Design	X		
Suitability of Methodology		X	
Feasibility within time period	X		
Appropriateness of budget *			
Potential value of field of knowledge	X		

*Please see page 3.

CONCLUSIONS

I support the application:

a) without qualification [X]

b) with qualification [ ]

- on technical grounds [ ]
- on level of financial support [ ]

I do not support the application [ ]

Detailed Comments

Please briefly provide your opinions of this proposal, giving special attention to the originality and feasibility of the project, its potential for providing new knowledge and the justification of financial support sought; include suggestions for modifications (scientific or financial) where you feel thay are justified.

(Use additional pages if necessary)

Title:

Studies on virulence of *Vibrio cholerae* O139 Bengal.

PI:

M. John Albert

Reviewer: ....

This proposal is adequately designed and well written. Like for *Vibrio cholerae* O1, cholera enterotoxin is considered the major virulence factor of *V. choleare* O139. However, it is important to assess the role of other possible virulence factors of this organism for vaccine development. If the investigators achieve the goals of the proposed project, pathogenic significance of two of the possible virulence factors, a capsule and CAMP hemolysin, may be clarified. The investigators are also going to assess if the capsule can be a protective antigen.

I have minor comments on some of the methods written in the proposal as follows:

(cntinued to the next page)

(continued from the previous page)

1. The method of cloning the CAMP hemolysin gene seems to be one of the reasonable approaches. However, there is no guarantee that this approach always works. The investigators might also consider other possible approaches of cloning if the above does not seem to work after several trials.

2. As stated in the proposal, not all strains of *V. choleare* O139 are CAMP (phenotype) positive. After determining the CAMP hemolysin gene sequence of one strain, the investigators may wish to examine the distribution and structure of the gene in selected strains. Then, they may verify that the 5' region is the best part for replacement with a kanamysin-resistance gene to obtain an isogenic, CAMP negative mutant.

3. If I understand correct, they plan to select the isogenic, CAMP negative mutant by a double crossover event. In addition, they do not add long flanking sequences to the mutated gene. It may require extensive effort to obtain the isogenic mutant. Suicide vector-based allelic exchange methods are available for isogenic mutant construction in vibrios. This method is more efficient than the proposed method.

4. The investigators state that data analysis is simple and straightforward. Do they know the results of their experiments already? They are going to compare the wild-type and mutant stains in the suckling mouse competition assay and determine LD_{50} to assess the role of CAMP hemolysin. They are also going to examine purified hemolysin in various in vivo and in vitro models. Appropriate statistical analysis of the data may be required.

I do not have enough knowledge on the salary and price of supplies and materials in Bangladesh and Sweden. I therefore do not judge the appropriateness of the budget.

Response to reviewers' comments

Reviewer 1: This reviewer did not have any specific comments.

Reviewer 2: As suggested by the reviewer, the suicide vector strategy is now included as an alternative strategy for making CAMP haemolysin negative mutant.

Data analysis will be carried out by Student t test.

International Centre for Diarrhoeal Disease Research, Bangladesh
CENTRE FOR HEALTH AND POPULATION RESEARCH

INTERIM/FINAL

SUMMARY COMPLETION FORM FOR PROTOCOLS

Title : Studies on virulence of Vibrio cholerae 0139 Bengal.

Investigator(s) : Dr. G. B. Nair

Protocol No. : 99-001 **Budget Code :** 208511

Findings (Abstract) : Attached in a separate sheet

Policy Implications: This is an experimental laboratory based study that will have no policy implications.

Dissemination plans: Publications and conferences

Date: Jan 30, 2001

Signature of the P.I.

Findings (Abstract)

V. cholerae O139 is a capsulated bacteria which caused epidemics in 1992-1993 in several Asian countries and spread to other parts of the world. In this study, the virulence properties of this bacterium were studied considering its serum resistance, invasion of epithelial cells, production of proinflamatory cytokines and CAMP haemolysin.

Normal invasion assay both in Caco-2 cells and Hep-2 cell indicated low level of invasion and the counts were comparable. In the translocation study, capsulated *V. cholerae* O139 translocated across the monolayer with more number than non-capsulated *V. cholerae* O139. None of the supernatants of Caco-2 cell monolayer with capsular polysaccharide (CPS), lipopolysaccharide (LPS) or live cells of *V. cholerae* O139 elicited TNFα or IL-1β.

The conjugation of oligosaccharides to different carriers was not performed yet. The reason for that is that, until recently, we did not have access to sufficient amounts of the oligosaccharides.in order to test different conjugation methods. The standard method used for conjugation of oligosaccharides to carriers (reductive amination) is not suitable due to the nature and structure of the oligosaccharides. We have now isolated oligosaccharides corresponding to 1, 2 and 3 repeating units. The amounts that are available now are of the order 20-50 mg of each oligosaccharide. This gives us sufficient amount to develop new conjugation methods.

We tried transposon mutagenesis for characterization of CAMP gene. A number of *V. cholerae* O139 isolates were screened for CAMP haemolysin and four CAMP positive isolates were selected for TnphoA mutagenesis. After mutagenesis, all transcojugates were tested for CAMP haemolysis test. Initially we got 10 mutants that were of CAMP negative phenotype, however most of them were not stable and reverted to CAMP positive after few subculture. Stable transconjugates were then screened for the presence of insertion of kanamycin resistance gene of TnphoA in the *V. cholerae* chromosome by southern blot hybridization and all transconjugates were negative in hybridization indicating TnphoA is not integrated in the chromosomal DNA. As we failed to clone CAMP gene the subsequent objectives on CAMP gene was not done.