tachment 1.
ACE SHEET)

Library

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Date 29/9/96 ✓

(LO)

ncipal Investigator M. John Albert

Trainee Investigator (if any) _____

plication No. 96-018

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

le of Study Studies on the capsule of vio cholerae 0139 Bengal

Project status:
(✓) New Study
( ) Continuation with change
( ) No change (do not fill out rest of form)

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

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

Does the study involve:
(a) Physical risks to the subjects — Yes (No)
(b) Social Risks — Yes (No)
(c) Psychological risks to subjects — Yes (No)
(d) Discomfort to subjects — Yes (No)
(e) Invasion of privacy — Yes (No)
(f) Disclosure of information damaging to subject or others — Yes (No)

Does the study involve:
(a) Use of records, (hospital, medical, death, birth or other) — Yes (No)
(b) Use of fetal tissue or abortus — Yes (No)
(c) Use of organs or body fluids — Yes (No)

Are subjects clearly informed about: N.A.
(a) Nature and purposes of study — Yes No
(b) Procedures to be followed including alternatives used — Yes No
(c) Physical risks — Yes No
(d) Sensitive questions — Yes No
(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 parent or guardian (if subjects are minors) — Yes No
6. Will precautions be taken to protect anonymity of subjects — Yes No

NA

7. Check documents being submitted herewith to Committee:
   ___ Umbrella proposal - Initially submit an overview (all other requirements will be submitted with individual studies).
   ✓ Protocol (Required)
   ✓ Abstract Summary (Required)
   ___ Statement given or read to subjects on nature of study, risks, types of questions to be asked, and right to refuse to participate or withdraw (Required)
   ___ Informed consent form for subjects
   ___ Informed consent form for parent or guardian
   ___ Procedure for maintaining confidentiality
   ___ Questionnaire or interview schedule *

* If the final instrument is not completed prior to review, the following information should be included in the abstract summary:
1. A description of the areas to be covered in the questionnaire or interview which could be considered either sensitive or which would constitute an invasion of privacy.
2. Examples of the type of specific questions to be asked in the sensitive areas.
3. An indication as to when the questionnaire will be presented to the Cttee. for review.

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

M. John Albert

Principal Investigator

Trainee

ENTERED 2 1 JUN 1996

5. Whether the proposal is a collaborative one ?

Yes [✓]

No [ ]

If the answer is 'YES', the type of collaboration, name and address of the institution and name of the collaborating investigator be indicated:

Drs. Andrej Weintraub & Per Erik Jansson, Huddinge Hospital, Huddinge, Sweden.

6. Has the budget been cleared by Finance Division ?

Yes [✓]

No [ ]

If the answer is 'NO', reasons thereof be indicated: _____

7. Does the study involve any procedure employing hazardous materials, or equipments ?

Yes [ ]

No [✓]

If the answer is 'YES', fill the necessary form.

29/9/96
Date

Signature of the
Principal Investigator

CHECK-LIST FOR SUBMISSION OF PROPOSALS TO THE RESEARCH REVIEW COMMITTEE (RRC)

[Please tick (✓) the appropriate box]

1. Has the proposal been reviewed, discussed and cleared at the Division level ?

   Yes [✓]

   No [ ]

   If the answer is 'NO', please clarify the reasons: _____

2. Has the proposal been peer-reviewed externally ?

   Yes [✓]

   No [ ]

   If the answer is 'NO', please explain the reasons: _____

3. Does the proposal address gender-issues ?

   Yes [ ]

   No [✓]

   If the answer is 'NO', Please give the reasons.

   Studies will carried out in human subjects.

4. Has a funding source been identified ?

   Yes [✓]

   No [ ]

   If the answer is 'YES', please indicate the name of the donor: SIDA - SAREC

Contd... /

STUDIES ON THE CAPSULE OF *VIBRIO CHOLERAE* O139 BENGAL

ABSTRACT

Vibrid cholerae O139 Bengal is the second aetiologic agent of cholera. *V. cholerae* O139 differs from *V. cholerae* O1, the traditional aetiologic agent of cholera, by the possession of a capsule. The present project aims to define the biological significance of the capsule. By analogy with other capsulated *V. cholerae* non-O1 strains, the capsule of *V. cholerae* O139 may confer increased virulence, such as serum-resistance, resistance to phagocytosis and invasion of the intestinal epithelium on the organism. It has been reported that the capsule and the lipopolysaccharide of *V. cholerae* O139 share antigenic epitope(s). It is to be defined whether the capsule is a protective antigen for incorporation into a vaccine. For evaluation of immune response in natural infection and for protective efficacy of vaccines, capsular antigen-based assays need to be developed. For this purpose, capsule-based antigen will be prepared by interaction of *V. cholerae* O139 with phages that specifically attack capsulated *V. cholerae* O139 strains and then the degraded capsule will be conjugated to a suitable carrier. The capsule of *V. cholerae* O139 shows cross-reactivity with several bacteria and the basis of cross-reactivity will be demonstrated by structural elucidation of surface polysaccharides. Based on a unique sequence that encodes the surface polysaccharide of *V. cholerae* O139, a PCR assay will be developed for rapid diagnosis of *V. cholerae* O139 from clinical and environmental samples.

PROJECT PROPOSAL

1. TITLE OF PROJECT : Studies on the capsule of *Vibrio cholerae* 0139 Bengal

2. INVESTIGATORS :

M. John Albert, Ph.D.
Microbiologist and Acting Director
and
Firdausi Qadri, Ph.D.
Immunologist

Laboratory Sciences Division,
International Centre for Diarrhoeal
Disease Research, Bangladesh,
Dhaka, Bangladesh

COLLABORATING INVESTIGATORS :

Andrej Weintraub, Ph.D.
Acting Professor-in-Charge;
Department of Immunology,
Microbiology, Pathology and
Infectious Diseases; Division of
Clinical Bacteriology, Karolinska
Institute, Huddinge Hospital,
Huddinge, Sweden
and
Per Erik Jansson, Ph.D.
Professor
Clinical Research Center
Karolinska Institute
Huddinge Hospital
Huddinge, Sweden

3. DURATION OF STUDY : Two and a half years

4. BUDGET : US$ 65,475 for ICDDR,B and
US$ 68,520 for the Division of
Clinical Bacteriology and Clinical
Research Center, Karolinska
Institute, Huddinge University
Hospital, Huddinge, Sweden

5. FUNDING SOURCE : SIDA-SAREC

6. HEAD OF THE PROGRAMME: Division Director
Laboratory Sciences Division

7. AIMS OF THE PROJECT

a) General aim

To study the importance of the capsular polysaccharide of *Vibrio cholerae* O139 Bengal, the recently discovered second aetiologic agent of cholera.

b) Specific aims

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

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

3) To study the interaction between phages specific for *V. cholerae* O139 Bengal and the CPS of *V. cholerae* O139 Bengal to generate oligosaccharides.

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

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

6) 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.

c) Significance

V. cholerae O139 Bengal is a recently discovered second aetiologic agent of epidemic cholera. Although there are striking similarities between *V. cholerae* O139 and (El Tor biotype of) *V. cholerae* O1, the traditional causative agent of 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 is expected to have important biological effects such as conferment of increased virulence on the organism. Preliminary data suggest that the CPS of *V. cholerae* O139 is an important antigen; it may have structural similarities with other bacteria which may form the basis for cross-reactivity and throw light on the origin of *V. cholerae* O139. Moreover, it has become obvious that CPS-based assays need to be developed for diagnosis and immune response studies to infection and vaccination. The proposed study will help: (a) understand the biological significance of CPS, particularly its effect on increased virulence of *V. cholerae* O139, (b) develop molecular biological and immunodiagnostic tests, (c) possibly throw light on the evolution of *V. cholerae* O139, and (d) ultimately in vaccine development against cholera due to *V. cholerae* O139.

8. ETHICAL IMPLICATIONS

The study will not involve experiments in human beings, stool samples only will be collected. However, studies will be carried out in suckling mice using standard procedures.

9. BACKGROUND, EXPERIMENTAL DESIGN AND BIBLIOGRAPHY

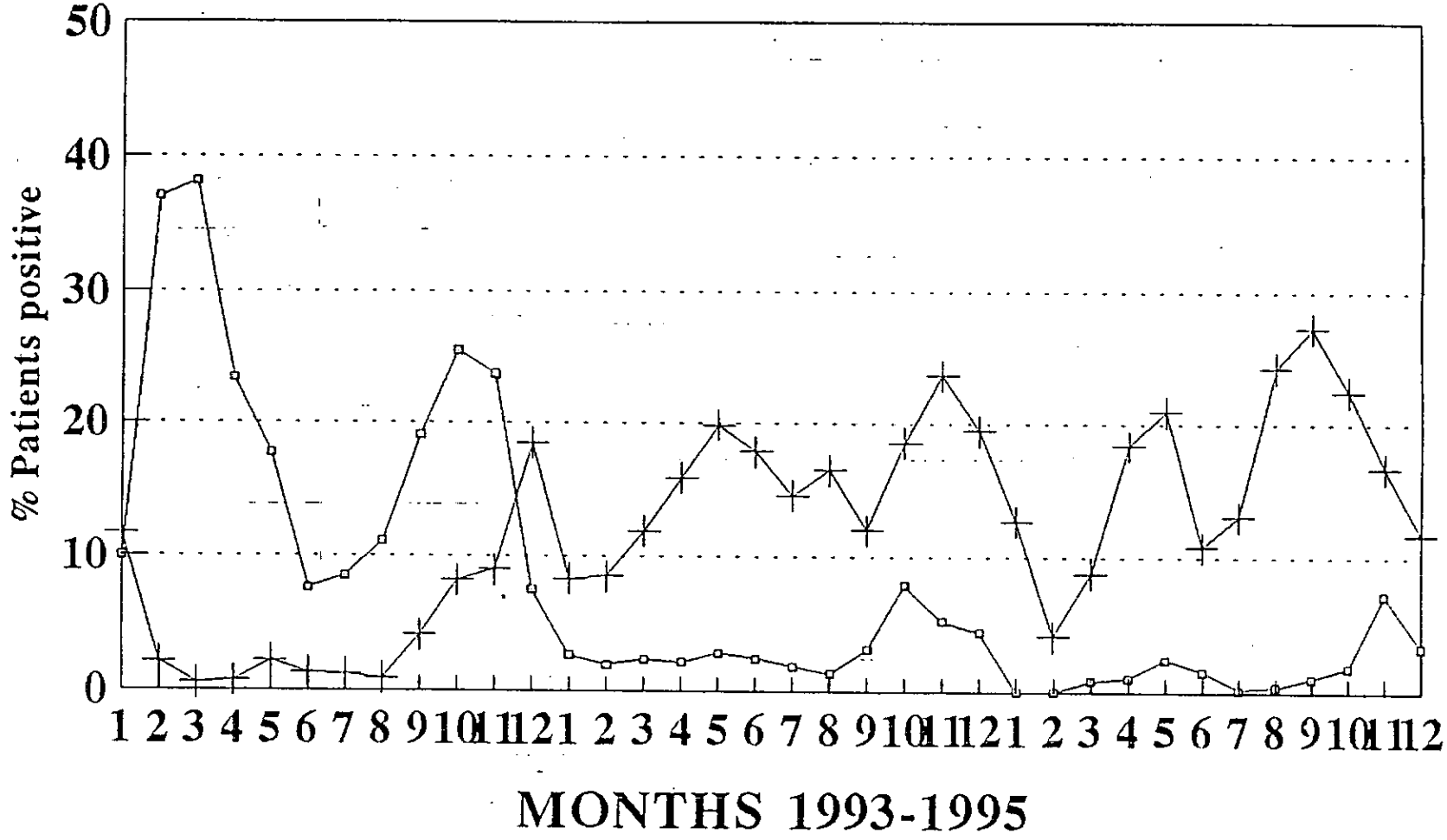
BACKGROUND

Cholera is an acute secretory diarrhoea and is endemic in most of the developing world. The traditional causative agent of cholera is *V. cholerae* O1. There are two biotypes (classical and El Tor) and two serotypes (Inaba and Ogawa) of *V. cholerae* O1 (1). Of the seven pandemics of cholera the world has experienced so far, the fifth and sixth pandemics were due to the classical biotype of *V. cholerae* O1, whereas the current, seventh pandemic, is due to the El Tor biotype of *V. cholerae* O1 (2). Non-O1 *V. cholerae* serogroups were known to be the causative agents of sporadic cases of diarrhoea and extra-intestinal infections, but not the causative agents of epidemic cholera (3). Therefore, it was surprising when *V. cholerae* O139 Bengal, a non-O1 serogroup emerged as a second aetiologic agent of cholera in late 1992 in the Indian subcontinent (4). In a short period since its emergence, *V. cholerae* O139 has invaded several countries in Asia, and cholera cases (due to *V. cholerae* O139) have also been exported to several developed countries (4). Therefore, *V. cholerae* O139 has come to be considered as the probable aetiologic agent of the "eighth pandemic" of cholera (4).

The percentage isolation of *V. cholerae* O1 and *V. cholerae* O139 at the Clinical Research and Service Centre (CRSC) of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) is shown in Figure 1. The CRSC at Dhaka treats approximately 100,000 patients a year for diarrhoea. Of these, a 4% systemic sample of patients (every twenty-fifth patient) (surveillance patients) is cultured for enteric pathogens including *V. cholerae* (5). The data presented in the figure represent isolation rate of *V. cholerae* in the surveillance patients. In 1992, cholera was caused by

SEASONALITY

Vc O139 vs. Vc O1



SURVEILLANCE SYSTEM

Figure 1

5A

V. cholerae O1 only. In 1993, the first year of *V. cholerae* O139 epidemic, the isolation of *V. cholerae* O139 predominated. However, beginning in 1994 and continuing through 1995, *V. cholerae* O1 has re-emerged as the predominant strain. The reason(s) for the diminished isolation of *V. cholerae* O139 is not understood. *V. cholerae* O139 (or even *V. cholerae* O1) would have undergone changes in the environmental persistence that would have allowed *V. cholerae* O1 to re-emerge as the predominant strain. However, it is important to note that *V. cholerae* O139 continues to cause cholera albeit at a low level. It is difficult to predict the future course of *V. cholerae* O1 and O139 infections; both strains may coexist with one strain as the dominant strain or one strain may completely replace the other.

The similarities between *V. cholerae* O139 Bengal and *V. cholerae* O1 El Tor biotype are numerous. These extend to morphology, culture, possession of virulence antigens and the type of disease they produce among other properties (4). There are also differences, the most important of which is the possession of a capsule by *V. cholerae* O139 Bengal (6), a biopolymer that is absent in *V. cholerae* O1.

Several gram-positive and gram-negative bacteria possess capsules (7). The property of capsule production is also found among non-O1 *V. cholerae* (8) and variation in capsule production determines colonial morphology. Capsulated bacteria produce opaque colonies and bacteria in translucent colonies are either non-capsulated or minimally capsulated (8). Capsulated bacteria are also serum resistant due to inability to fix complement and resist phagocytosis (8). The latter two properties will confer on the organism the ability to cause invasive disease such as septicaemia, especially in immunocompromised patients, a property common among several non-O1 *V. cholerae*

serogroups (9). Again, among non-O1 *V. cholerae* serogroups, heavily capsulated strains are more likely to cause septicemia than strains that are minimally capsulated (10). Since *V. cholerae* O139 is reported to have a thin capsule (11), this may explain why the incidence of septicemia in *V. cholerae* O139 infection is low (12-14).

Since *V. cholerae* O139 isolates are capsulated, they produce opaque colonies, and phase variation to translucent colony types is also seen (11). Preliminary studies suggest that *V. cholerae* O139 are serum-resistant but detailed data are lacking. On intradermal inoculation, *V. cholerae* O139 produced bacteremia in suckling mouse (11) and on oral challenge of adult rabbits, invasion of intestinal epithelial cells by *V. cholerae* O139 was seen, whereas these properties were absent with *V. cholerae* O1 (15). In a preliminary ultrastructural study of the upper small intestinal mucosal biopsies of *V. cholerae* O139 patients, more infiltration of polymorphonuclear leukocytes was seen as compared to that seen in *V. cholerae* O1 patients (16). In keeping with this observation, a significantly higher proportion of *V. cholerae* O139 patients has been found to have faecal leukocytes as compared to *V. cholerae* O1 patients (17). This may indicate the ability of *V. cholerae* O139 to invade intestinal mucosa. In a preliminary study of invasiveness of *V. cholerae* O139, some isolates were found to invade HEp-2 cell monolayers as documented by the gentamicin-surival assay and by demonstration of intracellular bacteria by electron microscopy (18); surprisingly, this property of invasion was also found among some isolates of *V. cholerae* O1. However, this needs to be validated in a more appropriate cell line, such as Caco-2 which is a human intestinal derived cell line (19). The effects of metabolites that inhibit actin polymerization, microtubule formation,

receptor-mediated endocytosis, endosome acidification and receptor recycling also need to be studied to define the exact mechanism of cellular invasion by bacteria.

The structure of the CPS of *V. cholerae* O139 has been determined. 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 (20). The highly unusual components are colitose, and the phosphate which is cyclic, the latter has not been previously described in bacterial glycans. The lipopolysaccharide (LPS) and CPS of *V. cholerae* O139 are cross-reactive (21). Based on studies conducted on wild type capsulated isolates and Tn*phoA* mutants defective in LPS/CPS synthesis with monoclonal antibodies, it was postulated that CPS might be a polymerized form of LPS (21). Structural studies on the LPS obtained from a *V. cholerae* O139 Bengal strain showed that the molecule in addition to the *V. cholerae* O1 lipid A-core region contains a single repeat unit identical to that of the CPS (22). This explains the cross-reactivity between the LPS and CPS of *V. cholerae* O139 Bengal. However, another study has disputed this finding and reported that CPS and LPS are antigenically different (23).

V. cholerae O139 Bengal shows cross-reactivity with *V. cholerae* serogroups O22 and O155 (24), some strains of *Aeromonas trota* (25), *Vibrio mimicus* (26), *Escherichia coli* serogroup O55 and *Salmonella greenside* (20). The nature of the cross-reactivity between *V. cholerae* O139 and *V. cholerae* serogroups O22 and O155, and *A. trota* is on an ab-ac type basis, where "a" is the common antigenic epitope and "b" and "c" are unique antigenic epitopes (24,25). The LPS structures of *E. coli* O55 and *S. greenside* are known. Their cross-

reactivity with *V. cholerae* O139 may be due to the presence of a common epitope containing a trisaccharide composed of colitose, galactose and an N-acetyl glucosamine (20). We have recently completed a study on the structure of the O-antigenic polysaccharide of the cross-reacting *A. trota* strain (27). The cross-reactivity is most likely due to a common tetrasaccharide identical to that found in *V. cholerae* O139 CPS except for the cyclic phosphate. It is noteworthy that this epitope is only present on the most distal part of the LPS molecule (27). The structural basis of cross-reactivity with *V. cholerae* serogroups O22 and O155, and *V. mimicus* is yet to be determined.

We have generated a synthetic analogue of CPS of *V. cholerae* O139 which when coupled to a carrier protein showed reactivity with rabbit polyclonal antiserum to *V. cholerae* O139 in an enzyme immunoassay (28). The specificity of this analogue needs to be further studied, and if found specific, can be coupled to a suitable carrier to be used as an antigen in immune response studies. It should also be possible to use the synthetic analogue in a receptor blockade therapy which can be explored in the infant mouse cholera model.

In our laboratories, we have derived several monoclonal antibodies specific for *V. cholerae* O139 and these belong to IgG and IgM classes of immunoglobulins (29,30). The specific epitopes that these monoclonals bind to on *V. cholerae* O139, are yet to be determined. If some of these monoclonals bind to CPS, their effectiveness to prevent cholera by passive protection in an infant mouse cholera model can be evaluated. This will demonstrate whether CPS is a critical antigen to be incorporated in a potential vaccine.

Data so far suggest that *V. cholerae* O139 might be derived from *V. cholerae* O1 El Tor. Studies on the genetics of O-antigen synthesis in *V. cholerae* O139 suggested that 14 of 17 genes involved in the O-antigen synthesis and Ogawa serotype modification in *V. cholerae* O1 are absent in *V. cholerae* O139. The three remaining genes *rfb Q*, *R* and *S* were present in a modified locus. The sequence of these genes resembled transposases of a number of insertion sequence elements. It is likely that this insertion sequence recombined with a set of novel genes is responsible for LPS/CPS synthesis in O139 serogroup. Acquisition of these novel genes would have led to an incompatibility in LPS synthesis that selected for deletion events that eliminated *V. cholerae* O1 region (31).

Recently, Comstock *et al.* generated Tn*phoA* mutants of *V. cholerae* O139 which abolished both O-antigen and capsule production (32). By mapping the insertion site and extended PCR analysis, a region of approximately 35 kb long not present in *V. cholerae* O1 was identified in *V. cholerae* O139 (33). Exploiting the similarity of LPS antigen of *Salmonella greenside* with that of LPS/CPS of *V. cholerae* O139, we designed a pair of primers (5'-GTC ATT ATT AAA ACT GC-3' and 5'-GCG TTA TAG GTA TCA TC-3') for PCR detection of *V. cholerae* O139. In a preliminary screening of isolates, the primer pair generated an amplicon of 417 kb from O139 isolates only, not from *V. cholerae* O1. However, these primers will have to be further tested with control isolates for sensitivity and specificity. If proven sensitive and specific, the PCR will prove to be an invaluable adjunct in the diagnosis of *V. cholerae* O139 infection. Combined with immunomagnetic bead capture of *V. cholerae* O139 with the help of specific monoclonal antibodies, the sensitivity of PCR can be increased in clinical and environmental monitoring of *V. cholerae* O139. With the availability of sequence data from the gene bank (31,33) of the gene(s)

encoding the surface polysaccharide of *V. cholerae* O139, it should now be possible to match our primer sequences to identify the position of the gene that is being amplified. It may correspond to a glycosyl transferase that is involved in the surface polysaccharide synthesis (PA Manning, personal communication). In case our primer sequences are found not to be satisfactory on further screening of isolates, it should be possible to design new primers for PCR using the gene bank data.

Even though *V. cholerae* O139 shares striking similarities with *V. cholerae* O1 El Tor, Mukerjee's phage specific for El Tor vibrios, phage 5, does not attack *V. cholerae* O139. Mukerjee's phage specific for classical biotype of *V. cholerae* O1, phage IV also does not attack *V. cholerae* O139 (34). We sought phages that will specifically attack *V. cholerae* O139, from the stools of patients infected with *V. cholerae* O139 and identified several phages belonging to *Podoviridae* family. These phages specifically attacked capsulated wild type *V. cholerae* O139 isolates that formed opaque colonies on Luria agar and were serum resistant. *V. cholerae* O139 isolates that lost the ability to produce capsule, hence formed translucent colonies and serum-sensitive, were not attacked. It is obvious that the receptor(s) for the phages are present on the capsule of *V. cholerae* O139 (35). The phage-capsule interaction needs to be studied to see whether an enzyme(s) may degrade the capsule analogous to the interaction between phage CP-T_1 and *V. cholerae* O1 (36), and phage P22 and *Salmonella* (37). If so, the phages can be used to degrade CPS to generate oligosaccharides for conjugation to a carrier to generate antigens for immunodiagnostic tests.

10. EXPERIMENTAL DESIGN

For this study, the following *V. cholerae* O139 Bengal strains are available: wild type strains; spontaneous translucent mutants that have lost the ability to produce capsule, but reacts with O139 antiserum; and Tn*phoA* mutants that lost the ability to synthesize CPS and LPS and do not react with O139 antiserum.

Serum resistance test: This test will be carried out as described previously (21). Both pooled guinea pig sera and pooled adult human sera will be used. The 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 100X the ratio of the 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. Capsulated strains of *Klebsiella pneumoniae* will be used as positive controls, and *V. cholerae* O1 strains will be used as negative controls.

Phagocytosis test: This test will be carried out as described previously (38). A standard dose of young bacterial culture will be incubated with polymorphonuclear leukocytes obtained from the blood of healthy adult volunteers. The incubation mixture will also contain pooled human serum from adult healthy volunteers. After appropriate incubation time, viable bacteria will be enumerated. The controls included will be same as those in the serum resistance test.

LD_{50} in mice: This test will be carried out as described previously (8) to determine the relative virulence of capsulated wild type strains and non-

capsulated variants of *V. cholerae* O139. Groups of mice will be injected intradermally with serial dilutions of culture, and 50% lethal dose (LD_{50}) will be calculated. Bacteremia will be checked for by culturing blood.

Invasion of Caco-2 cells: This assay will be carried out as described previously (39). Instead of HEp-2 cells, Caco-2 cells will be used. A standard dose of bacteria will be incubated with Caco-2 cell monolayer. After 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.

The invasive property of bacteria will be verified by transmission electron microscopy. After infection with bacteria and after incubation in presence of gentamicin, the cells will be fixed with glutaraldehyde in cacodylate buffer, stained with uranyl acetate and examined by transmission electron microscopy for intracellular bacteria. An invasive strain of *Shigella flexneri* 2a will be used as a positive control and a nonpathogenic human faecal isolate of *Escherichia coli* will be used as a negative control.

The effect of metabolic inhibitors on the invasion of Caco-2 cells by *V. cholerae* O139 Bengal will be examined as described previously in the quantitative cell culture assay containing gentamicin (39). The inhibitors that will be used are: cytochalasin D, chloroquine, ammonium chloride, dansylcadaverine and colchicine.

Localization of attachment of monoclonal antibodies to *V. cholerae* O139 Bengal: This will be carried using a set of monoclonal antibodies (MAbs) against *V. cholerae* O139 generated in our laboratories. The purpose of this experiment is to identify MAbs that will specifically bind to CPS. This

experiment will be carried out by immunoelectron microscopy and gold labelling by standard procedures (40).

Suckling mouse cholera model: This model will be used to study the protective efficacy of MAbs directed against the CPS of *V. cholerae* O139, and the synthetic analogue of CPS.

The 50% lethal dose (LD_{50}) of a wild type strain of capsulated clinical isolate of *V. cholerae* O139 Bengal will be determined in the infant mouse cholera model as described previously (41). The ability of MAbs to protect against cholera due to *V. cholerae* O139 will be determined by incubating MAbs with *V. cholerae* O139 and challenging a group of infant mice with the antigen-antibody mixture. To evaluate the protective efficacy of synthetic analogue of CPS, infant mice will be orally fed with the synthetic analogue prior to challenge with *V. cholerae* O139.

PCR assay for detection of *V. cholerae* O139: Current PCR assay referred to previously will be extended to include a large number of different bacterial species to test its sensitivity and specificity. In addition, acute watery diarrhoeal stools including those from cholera patients will be screened for detection of *V. cholerae* O139. The conditions of PCR assay are: separation of strands at 94^{o}C/30 sec, annealing of primers at 50^{o}C/30 sec and primer extension at 72^{o}C/1 min.

We will also try to increase the sensitivity of PCR by immunomagnetic separation (IMS) of bacteria first, and then conducting the PCR on captured bacteria (42). The specific MAbs to *V. cholerae* O139 will be used for IMS.

Determination of capsule-deficient *V. cholerae* O139 isolates in patients: This will be determined in 50 consecutive cholera patients infected with

V. cholerae O139. Stools are routinely plated onto the primary isolation medium, taurocholate-tellurite-gelatin agar (43). From each patient, about 10 randomly picked *V. cholerae* O139 colonies will be subjected to susceptibility to a *V. cholerae* O139 phage. Colonies not attacked by the phage will be considered as capsule deficient (This test has been validated previously [35]). This will be further confirmed by testing some isolates for serum susceptibility. Serum susceptible *V. cholerae* O139 are capsule deficient (21). The presence of LPS in the capsule-deficient strains will be confirmed by SDS-PAGE and silver staining (44). The proportion of patients with only capsule-deficient bacteria, and the proportion of capsule-deficient colonies in mixed infections with both capsulated and non-capsulated colonies will be determined. These data will be correlated with clinical severity of infections in patients. The clinical severity will be judged based on objective criteria such as degree of dehydration, frequency of purging and requirement of fluid replacement therapy (45). This portion of the study will be done in conjunction with an approved protocol entitled "Further evaluation of the oral ETEC vaccine and studies on the immune responses in acute watery diarrhoea" (protocol no. 96-014, PI: F. Qadri).

Generation of oligosaccharides: In order to generate oligosaccharides from *V. cholerae* O139 CPS, the above described phages will be used. 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 oligosacharides may be generated by incubation of purified CPS with purified phages and the resulting oligosaccharides could than be separated by gel-permeation chromatography. Since the CPS is built up 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 easy to characterize without the risk of losing important epitopes. The oligosaccharides will be characterized by 1H-NMR and 13C-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. (i) acrylamide-CPS conjugates will be used as antigens in enzyme immunoassay; (ii) protein-CPS conjugates (bovine serum albumin, cholera toxin B-subunit or tetanus toxoid) will be used as immunogens for generation of specific antibodies.

Another approach for generation of antibodies and for generation of specific diagnostic antigen 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 for use in conjugation.

Cross-reactivity with other bacteria: Both *V. cholerae* O22 and O155 as well as one strain of *V. mimicus* have been shown to react with polyclonal anti-*V. cholerae* O139 antibodies. In addition, some strains belonging to the *Aeromonas trota* species showed similar reactivity. To explain the cross-reactivities, the strains will be grown in fermentor and the LPS extracted by hot-phenol/water. After dialysis and lyophilization, the LPS will be

subjected to chemical and immunochemical analyses. The chemical analyses will include: sugar- and methylation analyses; specific degradations; combined gas chromatography - mass spectrometry; fast-atom bombardment mass spectrometry; and 13C-NMR and 1H-NMR (20).

11. BIBLIOGRAPHY

1. Kay BA, Bopp CA, Wells JG. Isolation and identification of *Vibrio cholerae* O1 from faecal specimens. In: Wachsmuth IK, Blake PA, Olsvik O, editors. *Vibrio cholerae* and Cholera: Molecular to Global Perspectives. Washington DC: American Society for Microbiology, 1994: 3-25.

2. Barua D. History of cholera. In: Barua D, Greenough WB III, editors. Cholera. New York: Plenum Publishing Corp., 1992: 1-36.

3. Janda JM, Powers C, Bryant RG, Abbott SL. Current perspectives on the epidemiology and pathogenesis of clinically significant *Vibrio* spp. Clin Microbiol Rev 1988; 1:245-267.

4. Albert MJ. *Vibrio cholerae* O139 Bengal. J Clin Microbiol 1994; 32:2345-2349.

5. Stoll BJ, Glass RI, Huq MI, Khan MU, Holt JE, Banu H. Surveillance of patients attending a diarrhoeal disease hospital in Bangladesh. Br Med J 1982; 285:1185-1188.

6. Weintraub A, Widmalm G, Jansson P-E, Jansson M, Hultenby K, Albert MJ. *Vibrio cholerae* O139 Bengal possesses a capsular polysaccharide which may confer increased virulence. Microb Pathog 1994; 16:235-241.

7. Cross AS. The biologic significance of bacterial encapsulation. Curr Top Microbiol Immunol 1990; 150:87-95.

8. Johnson JA, Panigrahi P, Morris JG Jr. Non-O1 *Vibrio cholerae* NRT36S produces a polysaccharide capsule that determines colony morphology, serum resistance and virulence in mice. Infect Immun 1992; 60:864-869.

9. Safrin S, Morris JG Jr, Adams M, Pons V, Jacobs R, Conte JE Jr. Non-O1 *Vibrio cholerae* bacteremia: a case report and review. Rev Infect Dis 1988; 10:1012-1017.

10. Johnson JA, Joseph A, Panigrahi P, Morris JG Jr. Frequency of encapsulated versus unencapsulated strains of non-O1 *Vibrio cholerae* isolated from patients with septicemia or diarrhea or environmental strains [Abstract B-277]. 92nd Gen Meet Am Soc Microbiol 1992.

11. Johnson JA, Salles CA, Panigrahi P, Albert MJ, Wright AC, Johnson RJ, Morris JG Jr. *Vibrio cholerae* O139 synonym Bengal is closely related to

Vibrio cholerae O1 El Tor but has important differences. Infect Immun 1994; 62:2108-2110.

12. Jesudason MV, Cherian AM, John TJ. Blood stream invasion by *Vibrio cholerae* O139 Bengal. Lancet 1993; 342:431.

13. Boyce TG, Mintz ED, Greene KD, Wells TG, Hockin JC, Morgan D, Tauxe RV. *Vibrio cholerae* O139 Bengal infections among tourists to southeast Asia: An intercontinental foodborne outbreak. J Infect Dis 1995; 172:1401-1404.

14. Khan AM, Albert MJ, Sarker SA, Bhattacharya MK, Azad AK. Septicemia due to *Vibrio cholerae* O139 Bengal. Diagn Microbiol Infect Dis 1995; 22:337-338.

15. Koley H, Ghosh AN, Paul M, Ghosh AR, Ganguly PK, Nair GB. Colonization ability and intetinal pathology of rabbits orally fed with *Vibrio cholerae* O139 Bengal. Indian J Med Res 1995; 101:57-61.

16. Albert MJ. Unpublished data.

17. Khan MA, Rabbani GH, Fuchs G. Faecal leucocytes in cholera due to *Vibrio cholerae* O139 Bengal [Letter]. J Diarrhoeal Dis Res 1996; 14:46-47.

18. Albert MJ. Unpublished data.

19. Panigrahi P, Tall BD, Russell RG, Detolla LJ, Morris JG Jr. Development of an *in vitro* model for study of non-O1 *Vibrio cholerae* virulence using Caco-2 cells. Infect Immun 1990; 58:3415.

20. Knirel YA, Paredes L, Jansson P-E, Weintraub A, Widmalm G, Albert MJ. Structure of the capsular polysaccharide of *Vibrio cholerae* O139 synonym Bengal containing D-galactose 4,6-cyclophosphate. Eur J Biochem 1995; 232:391-396.

21. Waldor MK, Colwell R, Mekalanos JJ. The *Vibrio cholerae* O139 serogroup antigen includes an O-antigen capsule and lipopolysaccharide virulence determinants. Proc Natl Acad Sci USA 1994; 91:11388-11392.

22. Cox AD, Brisson J-R, Varma V, Perry MB. Structural analysis of the lipopolysaccharide from *Vibrio cholerae* O139. Carbohydrate Res 1996; 290:43-58.

23. Sengupta DK, Boseman-Finkelstein M, Finkelstein RA. Antibody against the capsule of *Vibrio cholerae* O139 protects against experimental challange. Infect Immun 1996; 64:343-345.

24. Shimada T, Arakawa E, Itoh K, Nakazato T, Okitsu T, Yamai S, Kusum M, Nair GB, Takeda Y. Two strains of *Vibrio cholerae* non-O1 possessing somatic (I) antigen factors in common with *V. cholerae* serogroup O139 synonym "Bengal". Curr Microbiol 1995; 29:331-333.

25. Albert MJ, Ansaruzzaman M, Shimada T, Rahman A, Bhuiyan NA, Nahar S, Qadri F, Islam MS. Characterization of *Aeromonas trota* strains that cross-react with *Vibrio cholerae* O139 Bengal. J Clin Microbiol 1995; 33:3119-3123.

26. Albert MJ. Unpublished data.

27. Knirel YA, Senchekova, SN Jansson P-E, Weintraub A, Ansaruzzaman M, Albert MJ. Structure of the O-specific polysaccharide of an *Aeromonas trota* strain cross-reacting with *Vibrio cholerae* O139 Bengal. Submitted for publication.

28. Weintraub A. Unpublished data.

29. Qadri F, Azim T, Chowdhury A, Hossain J, Sack RB, Albert MJ. Production, characterization, and application of monoclonal antibodies to *Vibrio cholerae* O139 synonym Bengal. Clin Diagn Lab Immunol 1994; 1:51-54.

30. Weintraub A. Unpublished data.

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

32. Comstock LE, Maneval D Jr, Panigrahi P, Joseph A, Levine MM, Kaper JB, Morris JG Jr, Johnson JA. The capsule and O antigen in *Vibro cholerae* O139 Bengal are associated with a genetic region not present in *Vibrio cholerae* O1. Infect Immun 1995; 63:317-323.

33. Comstock LE, Johnson JA, Michalski JM, Morris JG Jr, Paper JB. Cloning and sequence of a region encoding surface polysaccharide of *Vibrio cholerae* O139 and characterization of the insertion site in the chromosome of *Vibrio cholerae* O1. Mol Microbiol 1995: (in press).

34. Albert MJ, Ansaruzzaman M, Bardhan PK, Faruque ASG, Faruque SM, Islam MS, Mahalanabis D, Sack RB, Salam MA, Siddique AK, Yunus M, Zaman K. Large epidemic of cholera-like disease in Bangladesh caused by *Vibrio cholerae* O139 synonym Bengal. Lancet 1993; 342:387-390.

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

36. Guidolin A, Manning PA. Bacteriophage CP-T_1 of *Vibrio cholerae*: identification of the cell surface receptor. Eur J Biochem 1985; 153:89-94.

37. Eriksson V, Svenson SB, Lonngren J, Lindberg AA. *Salmonella* phage glycanases: substrate specificity of the phage P_{22} endo-rhamnosidase. J Gen Virol 1979; 43:503-511.

38. Qadri F, Haque MA, Hossain A, Azim T, Alam K, Albert MJ. Role of *Shigella dysenteriae* type 1 slime polysaccharide in resistance to serum killing and phagocytosis. Microb Pathog 1993; 14:441-449.

39. Albert MJ, Ansaruzzaman M, Bhuiyan NA, Neogi PKB, Faruque ASG. Characterization of invasion of HEp-2 cells by *Providencia alcalifaciens*. J Med Microbiol 1995; 42:186-190.

40. Sharma DP, Thomas C, Hall RH, Levine MM, Attridge R. Significance of toxin-coregulated pili as protective antigens of *Vibrio cholerae* in the infant mouse model. Vaccine 1989; 7:451-456.

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

42. Islam D, Lindberg AA. Detection of *Shigella dysenteriae* type 1 and *Shigella flexneri* in feces by immunomagnetic isolation and polymerase chain reaction. J Clin Microbiol 1992; 30:2801-2806.

43. Monsur KA. A highly selective gelatin-taurocholate tellurite medium for the isolation of *Vibrio cholerae*. Trans R Soc Trop Med Hyg 1961; 55:440-442.

44. Hitchcock PJ, Brown TM. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polysaccharide gels. J Bacteriol 1983; 154:269-277.

45. World Health Organization. A manual for the treatment of diarrhoea. Geneva: World Health Organization. 6-7.

12. TIME-FRAME

a)	To carry out specific aims 1 and 2	...	6 months
b)	To carry out specific aims 3 and 4	...	12 months
c)	To carry out specific aims 5 and 6	...	12 months

13. TASK OF INVESTIGATORS

a) M. John Albert and F. Qadri

- To carry out specific aims 1, 2 and 6.

b) A. Weintraub and P-E Jansson

- To carry out specific aims 3, 4 and 5

MJA:mh/J8F:CAPVC.PRT

ICDDR,B Budget for studies on the Capsule of *Vibrio cholerae* 0139 Bengal

IN US$

	1997	1998	Total
Personnel			
M. John Albert (5%)	5110	5365	10475
Research Assistants (100%)x2	7600	8135	15735
Subtotal	12710	13500	26210
Supplies and Reagents			
Animals	1000	1000	2000
Molecular Biology Reagents	6500	6500	13000
Tissue culture reagents	2000	2000	4000
Electron microscopic study	800	800	1600
Airfreight of reagents	1000	1000	2000
Subtotal:	11300	11300	22600
Other contractual			
Rent, communication, utility	200	465	665
Printing and Publication	800	800	1600
Subtotal:	1000	1265	2265
Interdepartmental Services			
Media	2000	2000	4000
Land Transport	100	100	200
Medical Illustration	200	200	400
Staff Clinic	100	100	200
Library Services	100	100	200
Subtotal:	2500	2500	5000
Travel			
International Travel	3000	3000	6000
Per diem	1600	1800	3400
Subtotal:	4600	4800	9400
Total:	32110	33365	65475

Note: Budget requested for two years only (Jan 1997 - Dec 1998)

The budget has no overhead. Core personnel salary over two year is $ 10,475 i.e. Dr. Albert's salary.

25/4/96

Division of Clinical Bacteriology and Clinical Research Center,
Karolinska Institute, Huddinge Hospital, Sweden
Component of budget in US$

	1996	1997	1998	Total
Salary (Lab Technician 25%)	9429	10000	10700	30129
Chemicals and reagents	4857	11000	8500	24357
Overhead(12%)	1714	2520	2300	6534
Subtotal:	16000	23520	21500	61020
Travel to Bangladesh including per diem	0 0	3500	4000	7500
Total:	16000	27020	25500	68520

Note: Budget requested for 2½ years (July 1996 - December 1998)

J3/SARECBGT.MJA

REVIEWER #1

Title: "Studies on the Capsule of Vibrio cholerae O139 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 ☐

Name of Refe

Signature: ..
Position: Pr
Institution:

This is a very ambitious proposal which aims to examine a broad range of questions relating to the pathogenesis of *V. cholerae* O139, the efficacy of specific LPS and capsular MAbs, the chemistry and structure of the O139 O-antigen and capsule and PCR development for epidemiology. All of these areas are very worthwhile and important but I am concerned that it embarks on much more than can be realistically accomplished in the time-frame of the proposal.

Specific Comments

1. P.5. It is not true to say that capsule and complement resistance "will confer on the organism the ability to cause invasive disease". This may happen but is not a natural consequence otherwise there would have been rampant septicemia in O139 patients.

2. P.6. How was invasion by O139 examined? These experiments need to be examined carefully and not just bacterial counts.

3. Some of the chemical data are very difficult to assess because the relevant publications and information are not available to the reviewer.

4. P.8. Work in a number of laboratories has already shown that MAbs to O139 polysaccharide are highly protective in the infant mouse model. Do the applicants really propose to use mouse MAbs for passive protection?

5. P.9. Professor Weintraub has already been informed which gene is being amplified by the PCR.

6. The experimental approaches are satisfactory.

7. Given the ready phase variation observed in O139 strains, would it not be appropriate to see if other virulence traits may also be affected by the variation.

8. P.13. It would be worth confirming that the capsule-deficient strains do still have the O139 LPS pattern by SDS-PAGE and silver staining.

9. The budget is not excessive, however, I believe that the applicants may have to prioritise the work to be done because of the ambitious nature of the proposal.

REVIEWER #2

Title: "Studies on the Capsule of Vibrio cholerae O139 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 ☐

Name of Refe-

Signature:

Position:

Institutio

Detailed comments

Title: Studies on the Capsule of Vibrio cholerae)139 Bengal

Since the presence of capsular polysaccharide is a majou difference between strains of Vibrio cholerae O1 and O139, many investigators have been paying the attention to the capsule. Therefore, many competitions will be seen throughout the world against this project. The originalities of this project are found in its methodology as using specific phage, monoclonal antibodies, carrier proteins for capsular oligosaccharide, etc. Although the feasibility depends on the capability of the investigators and availability of the laboratory equipments necessary, I have to believe that the priject must be feasible. This study would give a great impact to understand pathogenesis of of V. cholerae O139, leading to the development of effective vaccine.

Reviewer:

Response to reviewer # 1

1. General: The reviewer commented that the project is very ambitious, and it may not be possible to accomplish all the objectives within the stated time-frame.

   Response: This is a collaborative proposal among three different laboratories. The two collaborating laboratories in Sweden have a lot of resources which are not apparent in the proposal. Some of the work has progressed well since writing this proposal. We are confident that all the objectives can be realized within the stated time-frame.

Specific comments:

1. P.5. It is not true to say that capsule and complement resistance "will confer on the organism the ability to cause invasive disease". This may happen but is not a natural consequence otherwise there would have been rampant septicemia in O139.

   Response: This is clarified in the last para on page 6 and in the first para on page 7. Bacteremia is dependent upon the degree of capsulation. Since the capsule of *V. cholerae* 0139 is thin, this may explain why bacteremia is rare in *V. cholerae* 0139 infection. Even in non-O1 *V. cholerae* infections, bacteremia occurs only in immunocompromised subjects.

2. P.6. How was invasion by O139 examined? These experiments need to be examined carefully and not just bacterial counts.

Response: Invasion was examined by gentamicin survival and by electron microscopy which are standards. See lines 14-17, para 2, page 7.

3. Some of the chemical data are very difficult to assess because the relevant publications and information are not available to the reviewer.

Response: These data have been published in appropriate international journals.

4. P.8. Work in a number of laboratories has already shown that MAbs to O139 polysaccharide are highly protective in the infant mouse model. Do the applicants really propose to use mouse MAbs for passive protection?

Perhaps, this work is going on in the reviewer's laboratory. Certainly, there are no published data. The aim of the exercise is to find out whether capsule is a protective antigen. See lines 6-7, para 3, page 9.

5. P.9. Professor Weintraub has already been informed which gene is being amplified by the PCR.

Response: The reviewer has informed that the gene corresponds to glycosyl transferase. See lines 3-5, para 1, page 11.

6. The experimental approaches are satisfactory.

Response: No comments

7. Given the ready phase variation observed in O139 strains, would it not be appropriate to see if other virulence traits may also be affected by the variation.

Response: Other virulence traits that can be examined are toxin production and colonization factors. These are being studied by an M.Sc student. Hence we do not intend to duplicate this study.

8. P.13. It would be worth confirming that the capsule-deficient strains do still have the O139 LPS pattern by SDS-PAGE and silver straining.

This will be done. See lines 8-9, para 1, page 15.

9. The budget is not excessive, however, I believe that the applicants may have to prioritise the work to be done because of the ambitious nature of the proposal.

Response: This has been addressed

Reviewer # 2.

There were no specific comments from the reviewer.

51/Response.rev/MJA:kn