Attachment 1.
(FACE SHEET)

Date 14/8/91

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Principal Investigator Dr. Steve Attridge / Dr. M. John Albert

Trainee Investigator (if any) _____

Application No. 91:010

Supporting Agency (if Non-ICDDR,B) _____

Title of Study Do Vibrio cholerae express novel surface antigens during growth in the human gut ?

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

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

1. Source of Population:
   (a) Ill subjects (Yes) No
   (b) Non-ill subjects Yes No
   (c) Minors or persons under guardianship Yes No
2. Does the study involve:
   (a) Physical risks to the subjects Yes No
   (b) Social Risks Yes No
   (c) Psychological risks to subjects Yes No
   (d) Discomfort to subjects (Yes) No
   (e) Invasion of privacy Yes No
   (f) Disclosure of information damaging to subject or others Yes No
3. Does the study involve:
   (a) Use of records, (hospital, medical, death, birth or other) (Yes) No
   (b) Use of fetal tissue or abortus Yes No
   (c) Use of organs or body fluids Yes No
4. Are subjects clearly informed about:
   (a) Nature and purposes of study (Yes) No
   (b) Procedures to be followed including alternatives used Yes No
   (c) Physical risks Yes No
   (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
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.

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

A-032076

Trainee

91-010
14/8/91

APPLICATION FOR PROJECT GRANT

1. PRINCIPAL INVESTIGATORS : Dr. Stephen R. Attridge[1]
Dr. M. John Albert[2]

2. OTHER INVESTIGATORS : Dr. Firdausi Qadri[3]
Dr. M. R. Islam[4]
Assoc. Prof. Paul A. Manning[5]
Mrs. Elena Voss[6]

[1]Senior Research Officer, [5]Associate Professor, [6]Ph.D. Student
Department of Microbiology and Immunology
The University of Adelaide
Adelaide, SA
Australia

[2]Senior Research Microbiologist, [3]Immunologist, [4]Senior Physician
International Centre for Diarrhoeal Disease Research, Bangladesh
Dhaka, Bangladesh

3. TITLE OF PROJECT : Do *Vibrio cholerae* O1 express novel surface antigens during growth in the human gut?

4. STARTING DATE : As soon as possible

5. COMPLETION DATE : Three years from starting date

6. TOTAL BUDGET REQUIRED : US$ 25,703

7. FUNDING SOURCE :

8. HEAD OF PROGRAMME : Acting Head
Laboratory Sciences Division

9. AIMS OF PROJECT

a) General aim

To study the antigens induced in *Vibrio cholerae* O1 during growth in the environment of the human gut.

b) Specific aims

1) To demonstrate the existence of "*in vivo*" antigens on *V. cholerae* 01 isolated from cholera patients and to assess in an experimental model the collective protective significance of such components.

2) To clone the genes encoding each of the "*in vivo*" antigens in order to construct isogenic mutant strains with which to evaluate the pathogenic significance of these components.

3) To assess the significance of toxin-coregulated pili as a colonization factor for El Tor strains of *V. cholerae* 01.

c) Significance

It is now clear that the virulence determinants of pathogenic bacteria are not constitutively expressed, so the identification of candidate protective antigens cannot rely simply on studies of organisms which have been grown "*in vitro*". The studies proposed will be the first to examine the antigens produced by *V. cholerae* in response to the human intestinal environment. As such, they have the potential to improve our understanding of the disease process in man and to identify novel components of vaccine significance.

10. ETHICAL IMPLICATIONS

From patients, stool, sera and jejunal juices will be studied.

11. BACKGROUND, RESEARCH PLAN AND BIBLIOGRAPHY

a) BACKGROUND

At the present time there is still no ideal cholera vaccine, i.e. one which is both highly immunogenic and protective on the one hand yet completely safe, inexpensive and convenient to administer on the other. The best of the formulations presently available is an oral inactivate vaccine developed by Holmgren's group, which comprises enormous numbers of killed bacteria together with the purified B subunit of the cholera toxin molecule. This vaccine is completely safe and reasonably effective when administered to adults in the field, conferring a protection rate of 50% over 3 years (1). It is, however, comparatively expensive to produce and the need for multiple doses is a serious practical disadvantage. In addition, protection for children below the age of 5 years is only short-term. Research continues on the causative agent of the disease, the bacterium *V. cholerae*, with the aim of identifying new virulence determinants which can be exploited as protective antigens for the development of more effective vaccines.

Colonization factors and protective antigens of *V. cholerae*

The colonization factors of *V. cholerae* have received much attention over the past 10-15 years. Experimental studies have implicated a variety of factors, including a flagellar adhesin (2,3), various molecules operationally defined as haemagglutinins (4,5), and most recently toxin-coregulated pili (TCP) (6). Of these, only TCP has been shown to promote colonization of the human gut. In human volunteer studies, Herrington *et al.* (7) compared the behaviour of an attenuated (non-toxigenic) *V. cholerae* strain with that of an isogenic TCP-

negative mutant. The mutant strain lacked the colonizing, immunizing and protective potential of its parent. By analogy with earlier experience with *Escherichia coli* pilus colonization factors, it seems likely that antibodies to TCP would protect against infection. Although this remains to be demonstrated for human disease, TCP is a protective antigen in the infant mouse cholera model (8,9). Recent experiments in our laboratory showed for the first time that antibodies to TCP are sufficient to protect infant mice from a potentially lethal inoculum of TCP-positive Classical *V. cholerae* (8,9).

The first antigen to be described as a protective antigen in experimental cholera models was lipopolysaccharide (LPS) (10,11). Until recently, evidence that antibodies to LPS might be protective in man was circumstantial, but direct evidence comes from a recent cholera challenge trial which assessed the protective efficacy of a candidate bivalent cholera-typhoid vaccine (12). The vaccine essentially comprised *Salmonella typhi* Ty21a bacteria expressing O-antigens cloned from *V. cholerae*. Vaccinees suffered significantly less purging and excreted fewer challenge organisms than controls. Two of eight vaccinees - the only recipients to show significant serum bactericidal responses following immunization - were completely protected against disease, indicating that antibodies to O-antigenic determinants are sufficient to mediate protection (12).

Although the killed oral vaccine developed by Holmgren's group comprises vibrios which express antigens associated with the LPS structure, they were not cultured appropriately for production of TCP. It has been suggested that the inclusion of TCP would improve the protective efficacy of this vaccine formulation (13); if so, it might be possible to reduce the number of bacteria

per dose or the number of doses required. Whether TCP is the only non-LPS protective antigen is not known.

Expression of bacterial virulence factors is not constitutive

Molecular biological studies in the past few years have greatly improved our understanding of the genetic control of virulence determinant expression. Although much remains to be learned, two things have become clear. First, virulence factors are not necessarily constitutively expressed (6,14,15). Second, global regulatory genes control the expression of sets of genes encoding, for example, products critical for pathogenicity; in this way, the synthesis of diverse virulence factors can be coordinately controlled (14,16).

Pathogens such as *V. cholerae* have evolved a variety of virulence determinants which collectively allow the bacteria to cause disease in man. However, these organisms must also cope with prolonged periods of existence outside a susceptible host, in the external environment. Production of multiple virulence factors might be not only unnecessary during this period, but might also reduce the likelihood of survival through sub-optimal allocation of limited resources. Teleologically it is more sensible to have sets of factors under the control of global regulatory genes, so that the organism synthesizes those factors most appropriate at the time.

In the case of *V. cholerae*, the production of TCP is coordinately controlled with production of the cholera toxin (hence, toxin-coregulated pili) and of at least two outer membrane proteins (6,16). A global regulatory gene, *toxR*, has been identified (6,16). The stimuli which induce expression are under investigation, but appear to include temperature, pH, osmolarity and the presence of certain amino acids (16). A recent study by Jonson *et al.* (17)

indicates that *V. cholerae* synthesize a number of novel membrane proteins during growth in the adult rabbit intestine. At least 8 "*in vivo*-specific" proteins are induced; these are immunogenic and not evident in membrane profiles of "*in vitro*" grown bacteria. This report has obvious implications for those interested in vaccine development. If any of the "*in vivo*" proteins performs a vital role in pathogenesis, neutralization of this function by antibodies might prevent infection. Clearly it is no longer sufficient to study the antigens present on bacteria which have been grown "*in vitro*" (18).

b) RESEARCH PLAN

The aim of the present proposal is to determine whether *V. cholerae* expresses novel antigens during growth in the human gut. If so, the protective significance of these components will be evaluated by assessing the protective potential of the respective antibodies in the infant mouse cholera model. These antibodies can also be used to clone the "*in vivo*-specific" antigens as a prelude to their more detailed characterization. The construction of isogenic mutant strains lacking the capacity to produce a particular "*in vivo*-antigen" will allow an assessment of the pathogenic significance of each such component.

i) Do *V. cholerae* produce novel antigens during *in vivo* growth in man?

About 40 adult cholera patients, 50% of whom will be infected with Classical biotype and the other 50% infected with El Tor biotype will be studied. *V. cholerae* will be recovered from the rice-water stools of cholera patients by differential centrifugation, using a method similar to that described by Johnson *et al.* (17). These "*in vivo*" organisms will be stored frozen in

aliquots and used to prepare antisera in rabbits. The causative bacteria will be streaked onto selective media and stored in lyophilized form. Acute and convalescent sera and jejunal fluids will also be collected. Acute specimens will be collected as soon as after admission and convalescent specimens after two weeks.

The rabbit antisera will subsequently be extensively absorbed with homologous "*in vitro*-grown" bacteria, to obtain antibodies collectively directed against any antigens exclusively produced during replication "*in vivo*". Then, following the approach of Johnson *et al.* (17), these absorbed sera will be applied to immunoblots of "*in vivo*" and "*in vitro*" grown bacterial suspensions, to detect the "*in vivo* antigens". Similar blots will be performed using patient serum and jejunal fluid samples to determine whether these antigens are immunogenic in their human host as a result of bacterial growth within the gut. It is possible that "*in vivo*" antigens will be expressed at low levels "*in vitro*". Therefore, absorption of sera with "*in vitro*-grown" bacteria is likely to remove specific low level antibodies to "*in vivo* antigens" and result in failure of experiment. To avoid this, sera from rabbits immunized with "*in vitro*-grown" bacteria will be absorbed with lipopolysaccharide antigens. The absorbed sera will be used to probe Vibrios collected from the human gut. Low level antibodies would, therefore, recognize antigens that are expressed more "*in vivo*", and this should demonstrate differences between "*in vivo*" and "*in vitro*" expressed antigens.

If the absorbed rabbit antisera identify novel membrane proteins, their protective activities will be assessed in Adelaide, using the infant mouse cholera model (8). Because the proteins of interest will not be present on the ("*in vitro*-grown") challenge bacteria, the normal procedure of pre-

treating the challenge inoculum with the antisera of interest might not result in protection of the infant mice. Thus, our previous studies showed that the protective potential of antibodies to TCP was greatly reduced if the challenge organisms were TCP-negative at the time of administration (8). In this event, antibodies could be administered intraperitoneally, prior to oral challenge to ensure their continued availability during the course of infection (e.g. ref. 19). The protective significance of the separate "*in vivo*-antigens" can be assessed following the cloning of the various structural genes (see below).

ii) Cloning of genes encoding *in vivo*-antigens

Because the "*in vivo*-antigens" are not produced during "*in vitro*" culture, cloning of the structural genes will require the use of expression vectors. This approach would involve partially digesting the bacterial DNA with restriction enzymes, such as *Sau*3A or *Taq*I and cloning into vectors with regulatable promoters. The *p*UC plasmids are suitable for this purpose - expression can be induced by the addition of IPTG. The plasmids would be maintained in a *lacI*^{q} strain to prevent inappropriate expression. [We have used this approach for cloning antigens of *Giardia lamblia*.] An alternative vector is one which contains the *trc* promoter (a hybrid *trp-lac* promoter) which can also be controlled in the same way but gives much higher expression levels.

The absorbed antisera specific for "*in vivo*-antigens" will allow isolation of the clones of interest, which can then be used to prepare antisera specific for the cloned antigens. These can be tested for protective activity as described above. In addition, adult female mice can be immunized with the clones prior to mating; challenge of the resulting offspring with the

homologous strain will allow an evaluation of the protective significance of the cloned antigen. This modification of the infant mouse cholera model has been previously used in our laboratory (20); its advantage is that antibodies are being continually transmitted to the suckling mice in the milk. Therefore, even if "*in vivo*" production of the antigen is somewhat delayed, the antibodies will still be available.

Each of the cloned genes can also be inactivated by insertion of an antibiotic resistance cartridge, prior to homologous recombination of the defective gene into the chromosome. [If the cloned DNA fragments are not sufficiently large to allow ready allelic exchange by homologous recombination, then they can be used as probes to identify larger fragments from gene libraries which we have constructed.] This will allow the derivation of isogenic mutant strains, each different from its parent by its inability to synthesize a single "*in vivo*-antigen". The pathogenic significance of each such antigen can then be evaluated by comparing its colonization and persistence with its parent strain in competition experiments (3,6) in infant mice. Briefly, this involves the feeding of a mixed (parent/mutant) inoculum to infant mice. At various times after dosing, the animals are sacrificed and intestinal homogenates prepared and plated onto differential media to allow separate enumeration of parent and mutant organisms.

iii) The colonization factors of El Tor *V. cholerae*

The present proposal would also provide an opportunity to test excreted vibrios for the presence of TCP. Studies in our (8) and other (21) laboratories suggest that the inductive stimuli for TCP synthesis might be different for strains of the two biotypes of *V. cholerae*. Detailed

immunoblotting and electron-microscopic (EM) analyses have consistently detected TCP on organisms of Classical biotype which have been appropriately cultured "*in vitro*" (6,8,21). However, these same methods have been unable to detect TCP expression by numerous El Tor strains cultured under identical conditions (8,21). Southern hybridization analyses indicate that El Tor strains do possess DNA homologous to that encoding TCP in strains of Classical biotype (9,13) and it is, therefore, assumed that the addition of TCP to Holmgren's oral inactivated vaccine will result in improved protective efficacy against strains of either biotype. The underlying assumption is that El Tor strains will be induced to express TCP by undefined stimuli present in the human gut. Very recently, Sun *et al.* (19) reported that two *V. cholerae* strains of El Tor biotype produced TCP "*in vitro*" following culture in specialized media. We have subsequently cultured eight other strains of this biotype using these media, but have been unable to detect TCP expression (Voss and Attridge, unpublished). In addition, Johnson *et al.* (17) failed to detect the production of TCP during the growth of El Tor bacteria in the rabbit gut. Clearly, further studies are required to determine the significance of TCP as a colonization factor of El Tor *V. cholerae*.

We have prepared a source of antibodies to TCP by extensive absorption of a polyclonal rabbit antiserum, and have confirmed the restricted specificity of this reagent by immunoblotting. The absorbed serum detects TCP on wild-type strains of Classical biotype, but not on isogenic mutant strains unable to produce TCP. This reagent would initially be used to examine the presence of TCP on Classical vibrios excreted in the cholera stool. On the basis of both experimental (6,8) and clinical (7) studies, such samples would be expected to be positive, but it is possible that TCP expression is "switched off" late in

infection. Also possible, but perhaps unlikely, is the denaturation of TCP during passage of vibrios through the large bowel.

If excreted Classical vibrios are indeed TCP-positive, it would then be possible to determine the TCP status of excreted El Tor *V. cholerae*, using both colony and Western-blotting. In addition, suspensions of both biotypes would be applied to EM grids, for subsequent EM and immuno-EM inspection in Adelaide. Should the El Tor samples be TCP-negative, the approach outlined above will allow an investigation of the colonization factors produced by vibrios of this biotype.

c) BIBLIOGRAPHY

1) Clemens JD, Sack DA, Van Loon F, Chakraborty J, Ahmed F, Rao MR, Harris JR, Khan MR, Yunus M, Huda N, Stanton BF, Kay BA, Walter S, Eeckels, R, Svennerholm A-M and Holmbren J. (1990) Field trial of oral cholera vaccines in Bangladesh: results from three-year follow-up. Lancet i:270-273.

2) Eubanks ER, Geuntzel MN and Berry LJ. (1977) Evaluation of surface components of *Vibrio cholerae* as protective immunogens. Infect Immun 15:533-538.

3) Attridge SR and Rowley D. (1983) The role of the flagellum in the adherence of *Vibrio cholerae*. J Infect Dis 147:864-872.

4) Manning PA. (1987) Involvement of cell envelope components in the pathogenesis of *Vibrio cholerae*: targets for cholera vaccine development. Vaccine 5:83-87.

5) Finn TM, Reiser J, Germanier R and Cryz SJ. (1987) Cell-associated haemagglutinin-deficient mutant of *Vibrio cholerae*. Infect Immun 55:942-946.

6) Taylor RK, Miller VL, Furlong DB and Mekalanos JJ. (1987) Use of *phoA* gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. Proc Natl Acad Sci USA 84:2833-2837.

7) Herrington DA, Hall RH, Losonsky G, Mekalanos JJ, Taylor RK and Levine MM. (1988) Toxin, toxin-coregulated pili, and the *ToxR* regulon are essential for *Vibrio cholerae* pathogenesis in humans. J Exp Med 168:1487-1492.

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

9) Sharma DP, Stroeher UH, Thomas CJ, Manning PA and Attridge SR. (1989) The toxin-coregulated pilus (TCP) of *Vibrio cholerae*: molecular cloning of genes involved in pilus biosynthesis and evaluation of TCP as a protective antigen in the infant mouse model. Microbial Pathogen 7:437-448.

10) Noeh SH and Rowley D. (1972) Protection of infant mice against cholera by antibodies to three antigens of *Vibrio cholerae*. J Infect Dis 126:41-47.

11) Svennerholm A-M and Holmgren J. (1976) Synergistic protective effect in rabbits of immunization with *Vibrio cholerae* lipopolysaccharide and toxin-toxoid. Infect Immun 13:735-740.

12) Tacket CO, Forrest B, Morona R, Attridge SR, LaBrooy J, Tall BD, Reymann M, Rowley D and Levine MM. (1990) Safety, immunogenicity, and efficacy against cholera challenge in humans of a typhoid-cholera hybrid vaccine derived from *Salmonella typhi* Ty21a. Infect Immun 58:1620-1627.

13) Taylor R, Shaw C, Peterson K, Spears P and Mekalanos J. (1988) Safe, live *Vibrio cholerae* vaccines? Vaccine 6:151-154.

14) Miller JF, Mekalanos JJ and Falkow S. (1989) Coordinate regulation and sensory transduction in the control of bacterial virulence. Science 243:916-922.

15) Maurelli AT. (1989) Temperature regulation of virulence genes in pathogenic bacteria: a general strategy for human pathogens? Microbial Pathogen 7:1-10.

16) Miller VL and Mekalanos JJ. (1988) A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J Bacteriol 170:2575-2583.

17) Jonson G, Svennerholm A-M and Holmgren J. (1989) *Vibrio cholerae* expresses cell surface antigens during intestinal infection which are not expressed during *in vitro* culture. Infect Immun 57:1809-1815.

18) Smith H. (1990) Pathogenicity and the microbe *in vivo*. J Gen Microbiol 138:377-393.

19) Sun D, Mekalanos JJ and Taylor RK. (1990) Antibodies directed against the toxin-coregulated pilus isolated from *Vibrio cholerae* provide protection in the infant mouse experimental cholera model. J Infect Dis 161:1231-1236.

20) Attridge SR and Rowley D. (1983) Prophylactic significance of the non-lipopolysaccharide antigens of *Vibrio cholerae*. J Infect Dis 148:931-939.

21) Hall RH, Vial PA, Kaper JB, Mekalanos JJ and Levine MM. (1988) Morphological studies on fimbriae expressed by *Vibrio cholerae* 01. Microbial Pathogen 4:257-265.

12. PUBLICATIONS OF PRINCIPAL INVESTIGATORS (LAST 5 YEARS)

a) Dr. S. R. Attridge

1) Sharma D, Attridge SR, Hackett J and Rowley D. (1987) Non-lipopolysaccharide protective antigens shared by Classical and El Tor biotypes of *Vibrio cholerae*. J Infect Dis 155:716-723.

2) Hone D, Morona R, Attridge SR and Hackett J. (1987) Construction of defined *galE* mutants of *Salmonella* for use as vaccines. J Infect Dis 156:167-174.

3) Hackett J, Attridge SR and Rowley D. (1988) Oral immunization with live, avirulent *fla* strains of *Salmonella* protects mice against subsequent oral challenge with *Salmonella typhimurium*. J Infect Dis 157:78-84.

4) Hone DM, Attridge SR, Forrest B, Morona R, Daniels D, LaBrooy JT, Bartholomeusz RCA, Shearman DJC and Hackett J. (1988) A *galE via* (Vi-negative) mutant of *Salmonella typhi* Ty2 retains virulence in man. Infect Immun 56:1326-1333.

5) Attridge SR, Hackett J, Morona R and Whyte P. (1988) Towards a live oral vaccine against enterotoxigenic *Escherichia coli* of swine. Vaccine 6:387-389.

6) Hone D, Attridge SR, van den Bosch L and Hackett J. (1988) A chromosomal integration system for stabilization of heterologous genes in *Salmonella* based vaccine strains. Microbial Pathogen 5:407-418.

7) Forrest BD, LaBrooy JT, Attridge SR, Boehm G, Beyer L, Morona R, Shearman DJC and Rowley D. (1988) A candidate live oral typhoid/cholera hybrid vaccine is immunogenic in humans. J Infect Dis 159:145-146.

8) Kotlarski I, Pope M, Doherty K and Attridge SR (1989) The *in vitro* proliferative response of lymphoid cells of mice infected with *Salmonella enteritidis* 11RX. Immunol Cell Biol 67:19-29.

9) Sharma DP, Thomas CJ, Hall RH, Levine MM and Attridge SR. (1989) Significance of toxin-coregulated pili as protective antigens of *Vibrio cholerae* in the infant mouse model. Vaccine 7:451-456.

10) Dent L, Spencer L, Attridge SR and Finlay-Jones J. (1989) Difference in the phenotypes of cells mediating anti-tumour immunity at various stages of tumour progression. Immunol Cell Biol 67:403-412.

11) Sharma DP, Stroeher UH, Thomas CJ, Manning PA and Attridge SR. (1989) The toxin-coregulated pilus (TCP) of *Vibrio cholerae*: molecular cloning of genes involved in biosynthesis and evaluation of TCP as a protective antigen in the infant mouse model. Microbial Pathogen 7:437-448.

12) Attridge SR and Rowley D. (1990) Cholera. *In*: Principles of Bacteriology, Virology and Immunity (MT Parker and LH Collier, eds.), vol. 3, 8th ed. Topley and Wilson, pp.459-468.

13) Attridge SR, Daniels D, Morona J and Morona R. (1990) Surface coexpression of *Vibrio cholerae* and *Salmonella typhi* O-antigens of Ty21a clone EX210. Microbiol Pathogen 8:177-188.

14) Tacket CO, Forrest B, Morona R, Attridge S, LaBrooy J, Tall BD, Reymann M, Rowley D and Levine MM. (1990) Safety, immunogenicity and efficacy against cholera challenge in man of a typhoid-cholera hybrid vaccine derived from *S. typhi* Ty21a. Infect Immun 58:1620-1627.

15) Bartowsky EJ, Attridge SR, Thomas CJ, Mayrhofer G and Manning PA. (1990) Analysis of the role of the P plasmid in the attenuation of *Vibrio cholerae*. Infect Immun 58:3129-3134.

16) Collins LV, Attridge SR and Hackett J. (1991) Mutations at *rfc* or *pmi* attenuate the virulence of *Salmonella typhimurium* for the mouse. Infect Immun (in press).

17) Attridge SR, Dearlove C, Beyer L, vanden Bosch L, Howles A, Hackett J, Morona R, LaBrooy J and Rowley D. (1991) Characterization and immunogenicity of EX880, a *Salmonella typhi* Ty21a-based clone which produces *Vibrio cholerae* O-antigen. Infect Immun (in press).

18) Attridge SR. (1991) Oral immunization with *Salmonella typhi* Ty21a-based clones expressing *Vibrio cholerae* O-antigen: serum bactericidal antibody responses in man, in relation to pre-immunization antibody levels. Vaccine (submitted for publication).

b) Dr. M. J. Albert

1) Albert MJ. (1986) Significance of cryptosporidium and other enteric pathogens in developing countries. Lancet 1:921-922.

2) Bishop RF, Tzipori S, Coulson B, Unicomb L, Albert MJ and Barnes GL. (1986) Heterologous protection against rotavirus-induced diseases in gnotobiotic piglets. J Clin Microbiol 24:1023-1028.

3) Albert MJ, Unicomb L and Bishop RF. (1987) Cultivation and characterisation of human rotaviruses with "super-short" RNA patterns. J Clin Microbiol 25:183-185.

4) Albert MJ, Unicomb L, Tzipori S and Bishop RF. (1987) Isolation and serotyping of animal rotaviruses and antigenic comparison with human rotaviruses. Arch Virol 93:123-130.

18) Albert MJ, Alam K, Islam M, Montanaro J, Rahman H, Haider K, Hossain MA, Kibriya AKMG and Tzipori S. (1991) *Hafnia alvei*: a probable diarrhoeal pathogen of humans. Infect Immun 59:1507-1513.

19) Faruque SM, Haider K, Albert MJ, Ahmed QS, Nahar S and Tzipori S. A comparative study of gene probes and standard bioassays to identify diarrhoeagenic *Escherichia coli* in Bangladeshi paediatric diarrhoeal patients. J Med Microbiol (In press).

20) Albert MJ, Alam K, Ansaruzzaman M, Montanaro J, Islam M, Faruque S, Haider K, Bettelheim K and Tzipori S. (1991) Localised adherence and attaching-effacing properties of non-enteropathogenic serotypes of *Escherichia coli*. Infect Immun 59:1864-1868.

21) Haider K, Albert MJ, Hossain A and Nahar S. Contact haemolytic activities of enteroinvasive *Escherichia coli* and shigellae. J Med Microbiol (In press).

22) Albert MJ, Leach A, Asche V, Hennessy J and Penner J. Epidemiology of *Campylobacter jejuni* and *Campylobacter coli* infections in hospitalised patients in Central Australia. J Clin Microbiol (submitted).

23) Albert MJ, Haider K, Nahar S, Kibriya AKMG and Hossain MA. (1991) Multiresistant *Salmonella typhi* in Bangladesh. J Antimicrob Chemother (in press).

24) Albert MJ, Ansaruzzaman M, Faruque SM, Haider K, Qadri F, Islam M and Tzipori S. An outbreak of keratoconjunctivitis due to *Salmonella weltevreden* in a guinea pig colony. J Clin Microbiol (under revision).

25) Albert MJ, Alim ARMA, Ansaruzzaman M and Mitra A. Fluorescent antibody test for rapid diagnosis of *Shigella dysenteriae* 1 infection. Diagnos Microbiol Infect Dis (submitted).

26) Haider K, Albert MJ, Nahar S and Kibriya AKMG. Plasmid-associated resistance to pivmecillinam in *Shigella flexneri* and *Shigella* boydii. J Antimicrob Chemother (in press).

27) Hossain MA, Kabir I, Albert MJ, Alam K, Kibriya AKMG and Alam AN. *Campylobacter jejuni* bacteremia in Bangladeshi children: review of six cases. Rev Infect Dis (submitted).

28) Haider K, Faruque SM, Shahid N, Albert MJ, Nahar S, Malek A and Alam AN. Clinical and microbiological features of enteroaggregative *Escherichia coli* infections in Bangladeshi children. (submitted)

29) Albert MJ, Tee W, Leach A, Asche V and Penner JL. Comparison of a blood-free medium and a filtration technique for the isolation of *Campylobacter* spp. from diarrhoeal stools of hospitalized patients in Central Australia. J Clin Microbiol (submitted).

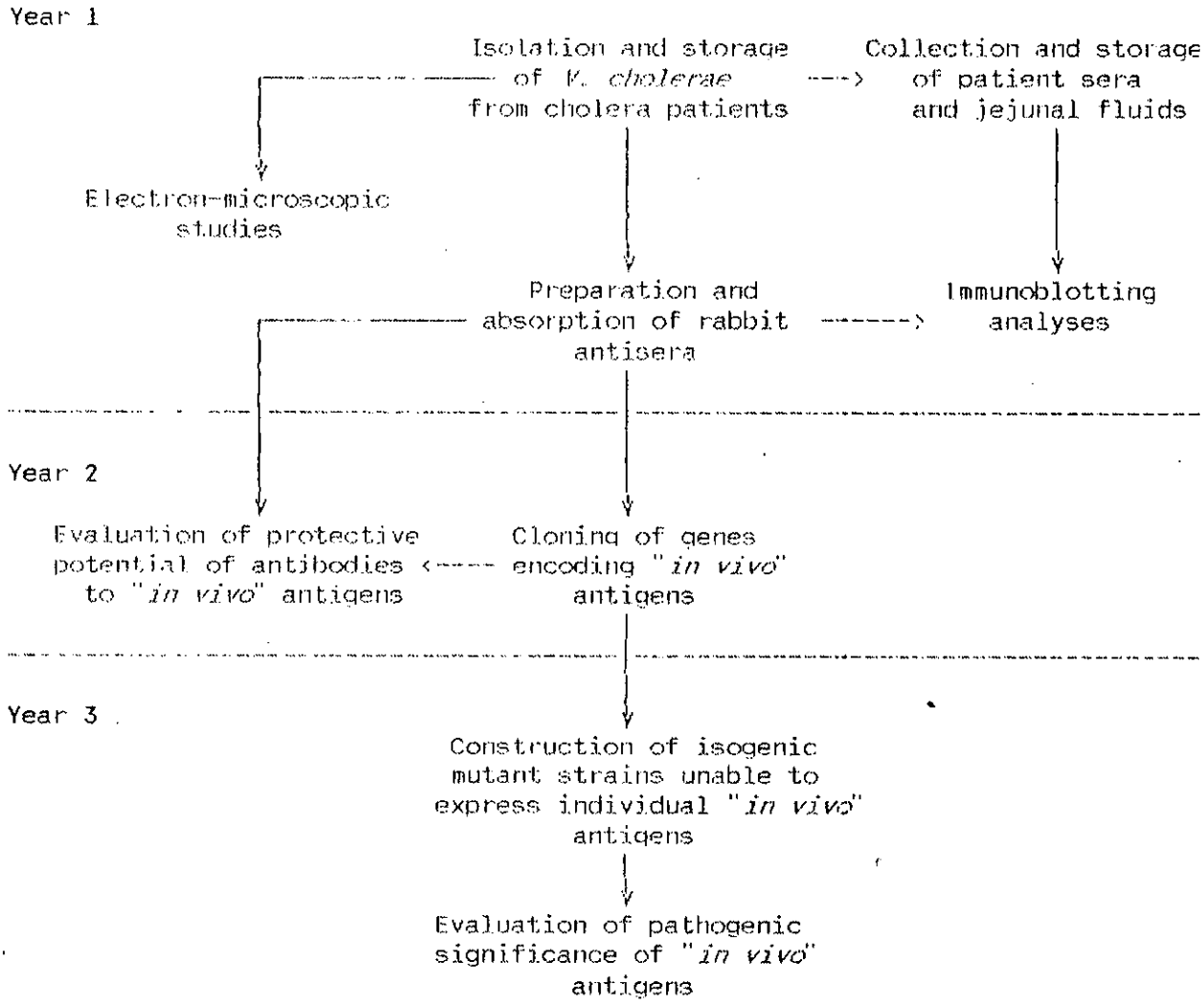
30) Faruque SM, Haider K, Alim AKMA, Baqui AH, Ahmad QS and Albert MJ. An evaluation of the DNA probe as compared to HeLa cell adherence assay in identifying enteroaggregative *Escherichia coli* in children with diarrhoea in Bangladesh. Curr Microbiol (submitted).

31) Peron-Henry D, Herrmann JE, Blacklow NR, Albert MJ and Erlich J. Incidence of rotaviruses and enteric adenoviruses in Aboriginal children from Central Australia. Presented at the American Society for Microbiology, Annual Meeting 1990: Annaheim, California. Abstract No. C-128, p.365.

32) Albert MJ. (1986) Enteric adenoviruses : brief review. Arch Virol 88:1-17.

33) Albert MJ. Epidemiology of rotavirus infection in children in Indonesia. In: Kurstak E and Thongcharoen P (eds). Virus diseases in Asia. Mahidol University, Bangkok, Thailand 1988:262-255.

13. FLOW CHART



The studies to be performed in the first year of the project will be primarily conducted at ICDDR,B, Dhaka. The gene cloning, strain construction and animal experiments will be performed in Adelaide in years two and three.

14. ITEMIZED SPECIFIC TASKS FOR EACH LISTED INVESTIGATOR

Most of the actual experimental work will be performed by Mrs. Elena Voss, whose Ph.D. thesis will be centered upon the studies outlined here, and a Research Assistant (to be appointed).

Dr. S.R. Attridge	–	Supervisor of Mrs. Elena Voss
	–	Will assist with animal studies designed to evaluate pathogenic and protective significance of "*in vivo*" antigens
Dr. M.J. Albert and Dr. F. Qadri	–	Will supervise Research Assistant in Dhaka
	–	Will oversee collection of *V. cholerae*, serum and jejunal fluid samples, preparation of absorbed rabbit antisera and immunoblotting analyses
Dr. M. R. Islam	–	Care of patients and collection of samples
Assoc. Prof. P.A. Manning	–	Will provide molecular biological expertise necessary for cloning of genes encoding "*in vivo*" antigens and construction of isogenic mutant strains
Mrs. Elena Voss	–	In Adelaide, will perform electron-microscopic examination of grids prepared in Dhaka, cloning of genes encoding "*in vivo*" antigens and construction of mutant strains lacking capacity to express individual "*in vivo*" components.
	–	Infant mouse experiments to evaluate pathogenic and protective significance of "*in vivo*" antigens

15. BUDGET

We seek funds to cover the cost of experiments to be performed in Dhaka during the first year of this project. Subsequent experiments in Adelaide will be financed by NH&MRC grants held by Drs. Attridge and Manning.

a) Personnel		
Research Assistant, salary for one year	US$	3,000
b) Operating costs		
Reagents for immunoblotting		7,000
Media ...		1,000
Disposable lab-wares ...		7,500
Rabbits for production of antibodies		1,000
Recruitment of patients for convalescent specimen collection		1,000
c) Capital equipment		
Transblot SD system (Bio-Rad)		1,500
Incubation tray ...		200
Incubation tray lid ...		53
d) Travel		
Air-fare ...		1,650
Living expenses ...		1,300
e) Correspondence and computing		
Fax messages, postage of manuscript by courier mail, photocopying and computing of patient data		500
Total for one year	US$	25,703

16. JUSTIFICATION OF BUDGET

a) Personnel

The work to be performed in Dhaka will require the services of a Research Assistant for a year. Initially this person will be responsible for collection, preparation, storage and cataloguing of bacterial and serum samples. Subsequently "*in vivo*" organisms will be used to generate rabbit antisera which will then be extensively absorbed prior to use in immunoblotting analyses. Patient serum samples will also be tested to determine whether the "*in vivo*" antigens are immunogenic.

b) Operating costs

These are costs of disposables which are essential for carrying out the study.

c) Capital equipment

We have a very old immunoblot apparatus in the laboratory which needs to be replaced in order to carry out this work.

d) Travel

We seek one return economy airfare from Adelaide to Dhaka to enable Dr. S. Attridge to visit Bangladesh. Living expenses relate to projected visit of 1 and 6 weeks duration.

e) Correspondence and computing

These are necessary expenditures for successful completion of the project.

MJA:mh/M6C:NOVSUR.PRT

RESPONSE TO DR. SCIORTINO'S COMMENTS

To avoid the possibility of absorbing out desired antibodies with "*in vitro* grown" bacteria, the antisera will be absorbed with lipopolysaccharide as suggested by the referee. This modification has now been included in the protocol.

We do not agree with the second comment that undue emphasis has been placed upon TCP. Our approach will allow the cloning of "*in vivo* antigens" whatever their nature. While it appears that TCP is not very immunogenic in man, it is also clear that this structue is vital for the colonizing and immunizing potential of an attenuated, oral cholera vaccine. There remains considerable doubt, however, as to whether TCP is as important for the pathogenesis of El Tor strains as that of classical biotype. The experiments we have proposed will clarify this issue, which has obvious vaccine significance.

Project title: Do Vibrio cholerae 01 express novel surface antigens during growth in the human gut?

Principal Investigator(s):

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	X		
Potential value to field of knowledge	X		

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 [ ]

Name of Referee: Tatsuo Yamamoto

Position: Assistant Prof. of Medicine

Institution: Department of Bacteriology, School of Medicine, Juntendo University

Tatsuo Yamamoto
Signature

July 15, 1991
Date

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 they are justified.

(Use additional pages if necessary)

The data which will be obtained during the course of the research project seems creative, and very informative. The research schedule seems appropriate. Since I could not find any data about personal ability of the investigator(s) (e.g., publications), I can not judge the investigator's ability to carry out the project. If the investigator(s) has enough ability to reach the goal, I feel, this research proposal should be accepted.

Project title: Do Vibrio cholerae 01 express novel surface antigens during growth in the human gut?

Principal Investigator(s): .....

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 to 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 Referee: C.V. Sciortino, Jr., Ph.D.

Position: Chief of Microbiology & Asst. Professor

Institution: Veterans Administration and University of Louisville Medical School

C.V. Sciortino

Signature

7/16/91

Date

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 they are justified.

(Use additional pages if necessary)

The experiments described herein, take an informative and logical approach to answering a lingering question, "are vibrios different when grown in the human gut?" This question has recently become more important due to several reports that vibrios may have developed unique *in vivo* regulatory systems for pili and outer membrane proteins. Although the PI's research approach is not entirely novel, it is feasible and likely to provide some firm answers to fill the gaps in the current knowledge and understanding of cholera immunity.

The scientific approach is valid and up-to-date. The experimental design (although not detailed) appears to have only one major flaw. The PI states that the expression of cholera virulence factors is not constitutive and it is under a unique regulatory control, but in the experimental design he/she overlooks that the genetic regulatory factors for virulence components are unknown. Therefore, it occurs that conditions of *in vitro* growth often induce low level production of virulence factors *in vitro* (such as cholera toxin and TCP). There is some concern that the PI doesn't take into consideration that *in vivo* antigens are expressed at low levels *in vitro*. Therefore, adsorption of sera with *in vitro* grown bacteria, is likely to remove specific low level antibodies to *in vivo* antigens and result in failure of the experiment. For example, the TCP pilus is minimally expressed in some vibrios when grown *in vitro* (as pointed out by the PI). Nevertheless, the TCP pilus is an "*in vivo*" regulated component. Also, in cholera-immune individuals there is minimally detectable antibody to the TCP pilus. Because TCP antibody levels are low and easily adsorbed from polyclonal sera, the studies described in this proposal would have overlooked TCP altogether if the *in vitro* grown organism used to adsorb sera was phenotypically TCP$^+$. **To avoid this error**, experiments should include the use of LPS-adsorbed sera from rabbits immunized with *in vitro* grown bacteria. This adsorbed serum should be used to probe vibrios collected from the human gut. Low level antibodies would therefore recognize antigens that are increased *in vivo*, and easily demonstrate differences between *in vivo* and *in vitro* expressed antigens.

It is also recommended that less emphasis should be placed on studies concerning the TCP pilus. Recent reports indicate that in clinical and experimental cholera, the TCP pilus does not contribute demonstrably to the vibriocidal response. In fact, cholera immunity occurs in the absence of detectable TCP antibody.

The funds requested for support appear reasonable. The time limits described to perform experiments also appear reasonable.

CWV
7/16/91

আন্তর্জাতিক উদরাময় গবেষণা কেন্দ্র
মহাখালী, ঢাকা।

সম্মতি পত্র

আপনি ভিব্রিও কলেরা নামক জীবাণু দ্বারা সংঘটিত কলেরা নামক ভয়াবহ রোগে আক্রান্ত। আমরা এই জীবানু সম্বন্ধে ব্যাপক অনুসন্ধান চালাইতে চাই যা আমাদিগকে ভয়াবহ কলেরা নামক রোগের হাত থেকে রোগ প্রতিরোধ কল্পে একটি কার্যকরী ভ্যাকসিন তৈরী করতে সহায়ক হবে। আপনি সম্মত হলে আপনাকে এই পরিপ্রেক্ষিতে দুই দফায় ৭ মি: লি: (এক চা চামচের চেয়ে কিছু বেশী) রক্ত পরীক্ষা নিরীক্ষার জন্য প্রদান করতে হবে। ভর্তীর সময় একবার এবং দুই সপ্তাহ পরে আর একবার দিতে হবে। উল্লেখ্য এই রক্ত প্রদানে আপনার শারীরিক কোন ক্ষয়ক্ষতির সম্ভাবনা নাই। আপনাকে এই প্রসংগে একটি ছোট সারকারী থলে সমন্বলিত সরু প্লাষ্টিক নল গিলতে হবে, যাহার দ্বারা আপনার পেটের অন্ত্রের রস পরীক্ষা নিরীক্ষার জন্য টানা হবে। ইহাতে আপনার জন্য হয়ত সাময়িক অসুবিধা হতে পারে। আপনাকে দুই সপ্তাহ পরে পরবর্তী পরীক্ষা নিরীক্ষার জন্য আর একবার এই হাসপাতালে আসতে হবে। আপনি চাইলে আপনার যাতায়ত খরচও হাসপাতাল থেকে প্রদান করা হবে।

উপরোক্ত শর্তাবলী আপনার কাছে গ্রহনযোগ্য বলিয়া মনে হ'লে নিম্নে আপনার সবাক্ষর বা টিপসই প্রদান করুন। আপনি এই গবেষনায় অংশগ্রহনে রাজি না থাকলেও আপনাকে উপযুক্ত চিকিৎসা প্রদান করা হবে।

গবেষকের সবাক্ষর

তারিখ:_____

রুগীর সবাক্ষর/টিপসই

তারিখ:_____

সবাক্ষীর সবাক্ষর

তারিখ:_____