Attachment 1. C6 Library Date 9/3/1997

(FACE SHEET)

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Principal Investigator M. John Albert Trainee Investigator (if any) _____

Application No. 97-002 Supporting Agency (if Non-ICDDR,B) _____

Title of Study Ecological and epidemiological studies on Aeromonas spp.

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.

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

M. John Albert
Principal Investigator

_____
Trainee

PROJECT DESCRIPTION

1. TITLE OF PROJECT : Ecological and epidemiological studies on *Aeromonas* spp. in Bangladesh with special emphasis on their spread between the environment and the humans

2. Investigators:

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3. DURATION OF PROJECT : 2½ years

4. TOTAL BUDGET : US$ 117,000

5. SOURCE OF FUNDING : SIDA/SAREC, Sweden

6. HEAD OF THE PROGRAMME: Director
Laboratory Sciences Division
ICDDR,B

ABSTRACT

Aeromonas spp. are ubiquitous organisms that are present in aquatic fauna and flora, and in the intestinal tract of healthy and diarrhoeic humans. They are also causative agents of invasive diseases in humans. However, their aetiological role in human diarrhoea is not clear. This may be due to the presence of pathogenic and nonpathogenic clones among *Aeromonas* spp. which are not apparently distinguishable by the routine laboratory methods. In this project we plan to screen *Aeromonas* spp. from diarrhoeal cases, matched healthy controls, surface water source and aquatic fauna and flora. By PhP typing, predominant clones present in these various sources will be identified. These clones will be further subjected to subtyping by molecular techniques with the identification of selected virulence properties. In this way, it will be possible to identify whether *Aeromonas* spp. present in various sources are indeed of different clonal origin that can be distinguished from strains causing diarrhoeal disease in humans.

7. HYPOTHESIS

Aeromonas spp. are autochthonous flora of natural water bodies. By contact with water sources, humans acquire the organisms. However, only some strains of *Aeromonas* spp. are pathogenic and capable of causing diarrhoea. We postulate that it is possible to identify such pathogenic clones by careful typing and subsequent screening of selected types/clones for virulence properties.

8. GENERAL AIM

To define the role of *Aeromonas* spp. in the causation of diarrhoea in humans.

9. SPECIFIC AIMS

a) To isolate *Aeromonas* spp. from humans with diarrhoea, from healthy controls and from surface water sources.

b) To type the *Aeromonas* spp. by biochemical fingerprinting (PhP typing) and DNA fingerprinting to find out

   i) the diversity of clones infecting humans and those present in the surface water;

   ii) common clones infecting humans and those present in the surface water;

   iii) predominant clones in surface water and among human isolates;

iv) the prevalence of virulence properties among clones common to surface water and to humans, and among the predominant clones of human and surface water isolates.

c) From the above data draw conclusions regarding the existence of pathogenic clones and their aetiological role in the causation of diarrhoea in humans.

10. SIGNIFICANCE

Most previous studies on the ecology and pathogenicity of bacteria belonging to *Aeromonas* spp. have mainly dealt with the characterization of bacterial isolates with regard to species. Since many species within this bacterial group are very heterogeneous, and comprise different types, contradictory results have often been obtained. In the present study we want to characterize these bacteria on a clonal level, and correlate clonal data of the isolates to their possible pathogenicity. This means that we will better understand the ecology and epidemiology of *Aeromonas* spp. on the clonal level, instead of only on the species level. Since this bacterial group may contain new, emerging diarrhoeal pathogens, it is of great importance to be able to monitor their occurrence in developing countries. We will also better understand the virulence of aeromonads. This means better means to understand the epidemiology of diarrhoeal disease, and the distribution pattern in the environment, in the community and in hospitalized patients. Such information will be of great importance to develop new strategies for controlling diarrhoeal diseases in endemic areas of developing countries including Bangladesh.

11. BACKGROUND

General characteristics of *Aeromonas* spp.

According to the ninth edition of Bergey's manual, the genus *Aeromonas* belongs to the family *Vibrionaceae*, which also contains the *Vibrio* spp., *Plesiomonas*, and *Photobacterium* (Bergeys manual). The aeromonads are gram-negative, rod-shaped, fermentative bacteria, and are easily differentiated from members of the family *Enterobacteriaceae* by their positive oxidase reaction, and from *Vibrio* spp. mainly by their resistance to the vibriostatic agent O/129. Recently it has been suggested to place aeromonads in a separate family, *Aeromonadaceae* (The Third International Workshop on *Aeromonas* and *Plesiomonas*, Helsingör, Denmark 1990).

Taxonomy

Aeromonas spp. are separated into two different groups, where the first group consists of psychrophilic and non-motile *A. salmonicida*, which cannot grow at 37°C and are not pathogenic to human, but are important fish pathogens. The second group consists of mesophilic and motile *Aeromonas* spp. that are found in human infections. This group was originally subdivided into the phenospecies *A. hydrophila*, *A. sobria* and *A. caviae* based on their biochemical reactions (Popoff, 1984), and later other phenospecies were added to the list. However, by using DNA hybridization techniques, isolates within these phenospecies have been shown to display such a wide variation in DNA relatedness that they should in fact be of different species (Popoff *et al.*, 1981). A new classification scheme of *Aeromonas* into 14 different genospecies

or DNA hybridization groups (HGs) has thus been defined (Carnahan *et al.*, 1991). However, the identification of *Aeromonas* to the genospecies level requires access to special identification techniques and, therefore, cannot be performed in any laboratory, and for this reason many laboratories perform identification to the phenospecies level only.

Isolation, identification and typing of *Aeromonas* spp.

Aeromonas spp. can be isolated on suitable selective media, usually containing ampicillin, such as Ampicillin-Dextrin agar (ADA) (Havelaar *et al.*, 1987; Havelaar *et al.*, 1988), sheep blood agar with ampicillin, or starch-ampicillin agar. Identification to the species level can be, e.g. performed by applying selected biochemical tests (Altwegg *et al.*, 1990; Altwegg *et al.*, 1991), by using species specific probes (Dorsch *et al.*, 1994), or by commercially available identification kits such as API or Biolog. For accurate identification of aeromonads to the genospecies level, DNA hybridization techniques are of course preferable, but too difficult to be applicable on large numbers of isolates. However, new sophisticated techniques have been developed that show good correlation to DNA hybridization techniques, such as gas-liquid characterization of fatty acid methyl esters (FAMEs) (Osterhout *et al.*, 1991; Huys *et al.*, 1994), or AFLP analysis (Vos *et al.* 1995; Huys *et al.*, 1996; Janssen *et al.*, 1996).

In order to be able to study the epidemiology of aeromonads, isolates have to be typed below the pheno- or genospecies level. Several different typing methods can be used for *Aeromonas* spp., e.g. molecular methods, such as ribotyping, RAPD, plasmid profiling, AFLP (Hänninen *et al.*, 1995; Huys *et al.*, 1996), chemotyping, such as SDS-PAGE (Mulla *et al.*, 1993; Millership *et al.*,

1993; Hänninen *et al.*, 1995), or phenotyping (Kühn *et al.*, 1992; Hänninen *et al.*, 1995). A serotyping scheme also exists for *Aeromonas* spp. (Shimada *et al.*, 1991).

Distribution of *Aeromonas* spp.

Aeromonas spp. are widely distributed in fresh water (Kaper *et al.*, 1981; Araujo *et al.*, 1989), drinking water supplies (Havelaar *et al.*, 1990; Krovacek *et al.*, 1992; Huys *et al.*, 1995), sewage, wastewater (Araujo *et al.*, 1990; Havelaar *et al.*, 1990), and soil (Osborn *et al.*, 1993). In drinking water, certain strains of *Aeromonas* spp. have shown a remarkably high ability to persist over long periods of time (Kühn *et al.*, 1992; Kühn *et al.*, 1996b). They are common in sea-food, such as shellfish, oysters, and fish (Abeyta *et al.*, 1986), and have been isolated from a number of food products of animal origin (Buchanan *et al.*, 1985; Kirov *et al.*, 1993), and also from vegetables (Buchanan *et al.*, 1985; Berrang *et al.*, 1989). They have also been isolated in high numbers from processed and cooked food, which have been stored at low temperatures (Fricker *et al.*, 1989), probably due to their ability to multiply at low temperatures.

Diseases caused by *Aeromonas* spp.

Several *Aeromonas* species have been described as fish pathogens (Austin *et al.*, 1993; Esteve *et al.*, 1994; Paniagua *et al.*, 1990; Olivier *et al.*, 1992). *Aeromonas salmonicida* causes two severe diseases among cultured fish: furunculosis (caused by the subspecies *A. salmonicida* var. *salmonicida*) and infectious dermatitis (caused by the subspecies *A. salmonicida* var. *achromogenes*). According to the definition, *A. salmonicida* cannot grow at

37°C and is therefore insignificant as a human pathogen; however, DNA hybridization studies have shown that *A. salmonicida* belongs to hybridization group 3, a genospecies that is also found in human faecal material (Kuijper *et al.*, 1991).

Among humans, *Aeromonas* spp. have been associated with a wide spectrum of diseases (Janda *et al.*, 1988; Wadström *et al.*, 1991), such as gastrointestinal infections (Aziz *et al.*, 1986; Khardori *et al.*, 1988; Deodhar *et al.*, 1991; Havelaar *et al.*, 1992; Kirov, 1993; Begue *et al.*, 1994) including travellers' diarrhoea (Hanninen *et al.*, 1995), septicaemia (Khardori *et al.*, 1988; Janda *et al.*, 1988; Janda *et al.*, 1994), peritonitis (Munoz *et al.*, 1994), and wound infections that are often associated with accidents in aquatic environments (Khardori *et al.*, 1988; Semel *et al.*, 1990).

Pathogenicity of *Aeromonas* spp.

Bacteria belonging to *Aeromonas* spp. have been identified as common enteric pathogens from several countries, with a reported isolation rate in stools from diarrhoeal patients of 1.4% (Nigeria) (Ogunsanya *et al.*, 1994), 8.7% (tourists from Morocco with travellers diarrhoea) (Hanninen *et al.*, 1995), 3% (Peru) (Begue *et al.*, 1994), or 1.8% (India) (Deodhar *et al.*, 1991). Most of these studies reported *Aeromonas hydrophila*, HG1 as the most frequently occurring *Aeromonas* species (Begue *et al.*, 1994; Deodhar *et al.*, 1991, Ogunsanya *et al.*, 1994); however, among tourists to Morocco with travellers diarrhoea, *A. veronii*, biotype sobria, and *A. caviae* were the most common species isolated (Hanninen *et al.*, 1995).

Although aeromonads are often isolated from clinical specimens, the question whether they should be regarded as pathogens or not, remains unanswered (Golik

et al., 1990; Janda, 1991; Krovacek *et al.*, 1994; Pin *et al.*, 1995). *Aeromonas* spp. are known to produce many different extracellular toxins, such as cytotoxin and enterotoxins (Namdari *et al.*, 1990; Houston *et al.*, 1991; Majeed *et al.*, 1994), as well as adhesins (Cahill, 1990). It has also been shown that pathogenicity can be associated with certain phenospecies and/or DNA hybridization groups (HGs) of *Aeromonas* (Janda and Kokka, 1991; Kokka *et al.*, 1991; Singh and Sanyal, 1992), and also that, within the same HG, clinical isolates more frequently produce virulence factors than environmental isolates (Kirov *et al.*, 1994). These findings indicate that, like among many *Enterobacteriaceae*-species (e.g. *Escherichia coli*), only certain clones of these bacteria are pathogenic to humans, and this might be one of the reasons why they can be found in the intestinal floras of both healthy and diseased individuals, and why they are relatively rarely encountered in human disease, compared to their abundance in food and water.

Aeromonas spp. in Bangladesh

In Bangladesh, *Aeromonas* spp. are abundant in aquatic environments (Islam *et al.*, 1992), and in patients with diarrhoea (Aziz *et al.*, 1986; Hoque *et al.*, 1994; Hossain *et al.*, 1991; Hossain *et al.*, 1992). As much as 12.1% of stools from infants with diarrhoea contained *Aeromonas* spp., compared to only 1.6% in healthy control infants (Hossain *et al.*, 1992). In a recently concluded case-control study at the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), Dhaka, 12.2% of children with diarrhoea and 4.8% of matched healthy children yielded *Aeromonas* spp. (Albert *et al.*, unpublished data).

Preliminary results

At present in ICDDR,B, a collaborative project supported by SAREC on "Biochemical fingerprinting in the epidemiological studies of diarrhoeal pathogens in Bangladesh" is ongoing. Within the framework of this project, we have developed a simple biochemical fingerprinting method (PhP typing) for various bacteria that are known to spread between environment and humans, such as *Vibrios* (Kühn *et al.*, 1996a), including *V. cholerae* (Ansaruzzaman *et al.*, 1996), *Aeromonas* (Kühn *et al.*, 1992), and various enterobacteria (Möllby *et al.*, 1993). Since this method is so simple to use, it can, without any problems, be used in studies involving several thousands of isolates, and is, therefore, particularly suitable to study bacterial populations from environmental samples.

Among our important findings was that *Aeromonas* spp. from patients with diarrhoea, the diversity was much lower than among environmental strains, indicating that only a minority of *Aeromonas* strains found in the environment could be found in humans with diarrhoea (Kühn *et al.*, 1996c). We also found a cytotoxin producing PhP type that was dominant among patient isolates but was absent among environmental strains. By applying FAME analysis and a highly discriminating DNA fingerprinting method (AFLP), we could further verify that these isolates belonging to this PhP type were highly related, all representing *A. hydrophila* HG1, and thus are possibly derived from a pathogenic, *A. hydrophila* clone. However, the total incidence of toxin producing isolates was higher among environmental isolates than among patient isolates, and thus, if the strains would not have been subjected to typing, this finding could never have occurred. These data also indicate a very useful way to study bacteria that are found in both the environment and in

disease, but yet are of uncertain pathogenicity. By typing a large number of isolates from diseased individuals, from healthy individuals, and from environmental samples, it is possible to obtain information on the prevalence of certain groups among diseased individuals. Isolates representing such groups can then be further characterized, e.g. typed by molecular methods, or characterized with regard to virulence factors.

In another ongoing project, at the Karolinska Institute, we are now studying the *Vibrio* floras isolated from healthy and diseased fish, and comparing it to isolates from other marine organisms and from water, using a combination of several different typing methods. We have shown, together with research groups in Denmark, France, Spain, Belgium, and UK, after analyzing more than 800 *Vibrio* isolates with several typing methods (ribotyping, serotyping, plasmid typing, LPS typing, Biolog-typing, API typing and FAME analysis) that the PhP typing method has a high concordance with other methods used in that project, and in addition, the highest discriminatory power among unrelated isolates. We have also found, by using data from combined typing, that the *Vibrios* belonging to the same species, but isolated from different sources (different fish species, algae, water and different geographical regions) seem to consist of different strains (Kühn *et al.*, 1996), a finding which indicates that also among these bacteria there may exist clones with selected pathogenicity for different animal species, and thus also possibly different pathogenicity for humans.

12. WORK PLAN

Aeromonas spp. isolates will be obtained from a number of different sources as indicated below.

a) **Dhaka hospital surveillance study**

ICDDR,B's hospital situated in Dhaka treats approximately 100,000 patients a year for diarrhoea. Of these, a 2% systematic sample (every 50th patient) is studied in-depth including detection for most of the diarrhoeal pathogens. The diarrhoeal pathogens studied include: *Salmonella* spp., *Shigella* spp., *V. cholerae*, *Campylobacter* spp., *Aeromonas* spp., *Plesiomonas shigelloides*, diarrhoeagenic *E. coli* (enterotoxigenic, enteropathogenic, enteroinvasive, enterohaemorrhagic, enteroaggregative and diffuse adherent), rotavirus, *Cryptosporidium parvum*, *Giardia lamblia* and *Entamoeba histolytica*.

Most of the surveillance patients are children below 5 years of age. For the present study, stool isolates from surveillance patients below 5 years of age and matched neighbourhood control children without diarrhoea will be utilized. Once the house of a case child is located, a bottle will be spun on the floor and when it stops, the first house at which direction the open end of the bottle points will be checked to locate a matched child. If a matched child is not available in the house, the next house will be checked, and so on. The age-matching of the control child will be within six months of a case child. The non-diarrhoeal control children will not have a history of diarrhoea for the preceding two weeks of the specimen collection.

b) **Epidemiologic and ecologic study of *V. cholerae***

This is a National Institute of Health (NIH), USA-funded project based at ICDDR,B. The duration of the project is 5 years from July 1996. The PI of the project is Professor R. Bradley Sack, based in The Johns Hopkins University, USA. The co-investigators are M.J. Albert, M.S. Islam, S.M. Faruque and A.K. Siddique. In this project, *V. cholerae* isolates from

clinical cases and environmental samples are being studied. However, from the same samples, we will also isolate *Aeromonas* spp. and utilize them for the present study. A brief description of the project is as follows:. Four sentinel surveillance centres are being set up in the north, centre, south and western parts of Bangladesh to study cholera. Each centre has a population of approximately 200,000, and is served by a primary health care clinic. Physicians will visit the surveillance centres every two weeks for three successive days and study all cases of diarrhoea. Rectal swabs will be collected for the study of *V. cholerae*. In addition, surface water, sediment and zooplankton and phytoplankton will be collected every two weeks from two selected ponds and two river sites from each surveillance area. These water sources are used by the population for washing, bathing and also for cooking. Specimen collection from patients and the environment will take place at the same time. It is estimated that at least 800 cases of diarrhoea will be seen from all four sentinel sites during a year.

13. LABORATORY METHODS

Isolation of *Aeromonas* spp. from rectal swabs

Rectal swabs will be transported in Cary-Blair medium at ambient temperature to the laboratory. They will be directly inoculated onto taurocholate-tellurite-gelatin agar (TTGA, Monsur's medium) (Monsur, 1961) as well as after enrichment for 8 hours at 37^{O}C in bile-peptone-water (BPW). Oxidase positive colonies will be identified as *V. cholerae* or *Aeromonas* spp. by standard methods (Janda *et al.*, 1995). In ICDDR,B laboratory, it has been shown that the isolation rate of *Aeromonas* spp. using BPW enrichment and plating on TTGA is superior to isolation on ampicillin-blood agar (unpublished data).

Isolation of *Aeromonas* spp. from environmental samples

Surface water will be collected from 5 cm below the surface using pre-sterilized 1 litre bottles. Sediment samples will be collected by means of a core sampler and stored in 4 oz glass bottles. Using a 20 µm mesh size net, plankton samples will be collected from water samples. Using a larger mesh plankton net, zooplankton and phytoplankton will be separated in the laboratory. All samples will be transported to the laboratory in an ice box in an insulated foam box with ice packs.

The plankton samples will be homogenized in a tissue grinder. Ten ml of plankton homogenate, 50 ml of water, and 1 g of sediment will be enriched separately in BPW and subcultured onto TTGA. Suspected *Aeromonas* spp. colonies will be identified as above.

Estimated number of isolates available for the study

We plan to collect *Aeromonas* spp. isolates during a one-year period from the above mentioned sources.

Dhaka hospital surveillance: From an estimated 2000 patients, 1600 will be paediatric patients. At a 10% isolation rate, 1600 padiatric patients will yield 160 isolates.

Dhaka Shishu Hospital control children: At a 4% isolation rate, 1600 control children will yield 64 isolates.

Field sentinel surveillance patients: At a 8% isolation rate, 800 patients from all four surveillance sites will yield 64 isolates.

Environmental isolates: At least one sample each of surface water, sediment, phytoplankton and zooplankton from each of 2 ponds and 2 rivers from 4 sentinel sites samples, 26 times a year, will yield 1248 samples. A previous study has shown that isolation of *Aeromonas* spp. from environment is as high as 37% (Islam *et al.*, 1992). At this isolation rate, 1248 samples will yield 462 isolates. Since the number of environmental isolates is disproportionately higher compared to human isolates, every third isolate will be selected for further study. This will yield approximately 150 isolates. All the *Aeromonas* spp. isolates will be stored in Luria broth with 15% glycerol at -70^{o}C until studied.

PhP typing of *Aeromonas* spp. isolates

Aeromonas spp. stock will be subcultured onto McConkey agar, and colonies transferred directly into the automated PhP biochemical fingerprinting plates, which are read kinetically as described earlier (Möllby *et al.*, 1993). According to the data obtained from the PhP system, the isolates will be subdivided into phenotypes, and isolates representing relevant phenotypes can be selected for further studies (Fig. 1). The diversity of bacteria belonging to the relevant groups are calculated (Bianchi *et al.*, 1982; Atlas *et al.*, 1984; Kühn *et al.*, 1991) and compared between bacterial populations from environmental samples, healthy controls and patients with diarrhoea. A lower diversity among bacteria from patients with diarrhoea than among those isolated from other sources is a first indication of the predominance of certain clones, and isolates representing PhP types predominating among patients will be carefully studied.

Species identification of *Aeromonas* spp.

The phenospecies and hybridization group analysis of PhP types of interest will be carried out by fatty acid methyl ester (FAME) profile and amplified fragment length polymorphism (AFLP) analysis in the laboratory of Drs. G. Huys and K. Kersters at the University of Gent, Belgium. The methods have been descried previously (Huys *et al.*, 1995, 1996b).

Subtyping of *Aeromonas* spp.

Initially, a small collection of strains from the different sources will be subtyped by ribotyping (Faruque *et al.*, 1995), polymorphism in the 16S-23S spacer regions of bacterial rRNA genes by the use of PCR (Kostman *et al.*, 1992) and enterobacterial repetitive intergenic consensus (ERIC) PCR (Rivera *et al.*, 1995). The method that gives maximum discrimination between strains will be chosen subsequently to subtype PhP types of interest.

Based on the data on PhP type, genospecies, hybridization group and subtype, clones predominant in human infection, in the environment and common to both environment and humans will be determined. Selected virulence properties listed below will be determined in these clones. The association of any virulence property(ies) with particular clones will be ascertained. This will help determine the pathogenic potential of particular clones.

Determination of virulence properties

The virulence properties that will be determined are cytotoxin, enterotoxin, haemolysin, HEp-2 cell adherence, HEp-2 cell invasiveness and autoagglutination.

Cytotoxin and enterotoxin: The organisms will be grown in double-strength tryptic soy broth as shaker cultures at 37°C for 20 hours. Cell-free filtrates will be tested in Vero cells for cytotoxin. For enterotoxin production, organisms will be grown in Casamino Acids-yeast extract (CAYE) broth as shaker cultures at 37°C for 20 hours. Cell-free filtrates will be tested in adrenal tumour Y1 cells for cholera toxin-like enterotoxin.

Haemolysin: The organisms will be grown in heart infusion broth as stationary culture at 37°C for 20 yours. The culture supernatant will be tested for haemolytic activity using sheep erythrocytes.

HEp-2 cell adherence and invasiveness will be carried out as described previously (Watson *et al.*, 1985; Neves *et al.*, 1994).

Autoagglutination will be done as described previously (Janda *et al.*, 1987).

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15. TIME-FRAME

First 14 months:

Collection of *Aeromonas* isolates from children with diarrhoea, healthy controls and environmental samples, and PhP typing of isolates.

Last 10 months:

Subtyping of selected PhP types, determination of clones of interest and report writing.

16. ITEMIZED TASKS OF INVESTIGATORS

M. John Albert	:	Coordinate all work
M. Sirajul Isalm A.S.G. Faruque	|	Responsible for collection of environmental samples and stool samples from children
Shah M. Faruque	:	Responsible for molecular subtyping
A.K. Siddique	:	Responsible for collection of stool samples from diarrhoeal patients in the field and of clinical and epidemiological data
R. Bradley Sack	:	Overall coordinator of NIH cholera project
Roland Möllby Inger Kühn Mohammad Katouli	|	Advice on PhP typing
G. Huys K. Kersters	|	Responsible for subtyping by FAME profile and AFLP

17. BUDGET

SUMMARY

	ICDDR,B	Karolinska Institute	Total
July-Dec, 1996	0	10,000	10,000
1997	30,000	25,000	55,000
1998	27,000	25,000	52,000
Total US$	57,000	60,000	117,000

There is no budget allocation for ICDDR,B for July-December 1996.

DETAILED BUDGET

a) Budget for July-December, 1996

Cost items	SAREC contribution administered by ICDDR,B	SWE Inst.for SWE Inst.	Total
Salaries	0	4,000	4,000
Consumables	0	4,000	4,000
Literature	0	0	0
Overhead	0	2,000	2,000
Subtotal	0	10,000	10,000
Equipment	0	0	0
Travel	0	0	0
Subsistence grants	0	0	0
Unforeseen	0	0	0
TOTAL US$	0	10,000	10,000

For ICDDR,B no budget is allocated for July-December, 1996

b) Budget for full year 1997

Cost items	SAREC contribution administered by ICDDR,B	SWE Inst.for SWE Inst.	Total
Salaries*	11,462	14,286	25,748
Consumables	15,588	2,657	18,245
Literature	450	200	650
Overhead (20%)	0	4,000	4,000
Subtotal	27,500	21,143	48,643
Equipment	0	0	0
Travel + per diem	2,500	2,857	5,357
Subsistence grants	0	0	0
Unforeseen	0	1,000	1,000
Total US$	30,000	25,000	55,000

*Salaries of technical staff only are budgeted

c) Budget for full year 1998

Cost items	SAREC contribution administered by ICDDR,B	SWE Inst.for SWE Inst.	Total
Salaries*	12,035	14,286	26,321
Consumables	13,015	2,657	15,672
Literature	450	200	650
Overhead (20%)	0	4,000	4,000
Subtotal	25,500	21,143	46,643
Equipment	0	0	0
Travel + per diem	1,500	2,857	4,357
Subsistence grants	0	0	0
Unforeseen	0	1,000	1,000
Total US$	27,000	25,000	52,000

*Salaries of technical staff only are budgeted

MJA:mh/J8G:ECHOLR2.PRT

RESPONSE TO REFEREES' COMMENTS

Referee 1

It has been pointed out that in the itemized task, it was not indicated as to who will be responsible for collecting and analyzing clinical data. This is the task of Dr. A.K. Siddique, which is now indicated on page 24.

Another point was the multiplication of *Aeromonas* spp. during transport. This does not matter as we are not relying on quantitative data. We will also be analyzing these isolates from the primary plate as well as after enrichment.

Referee 2

There were no major comments from this referee.

Referee # 1

Title: "Ecological and epidemiological studies on Aeromonas spp. in Bangladesh with special emphasis on their spread between the environment and the humans"

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 of field of knowledge	X		

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: H

Institution:

Detailed Comments

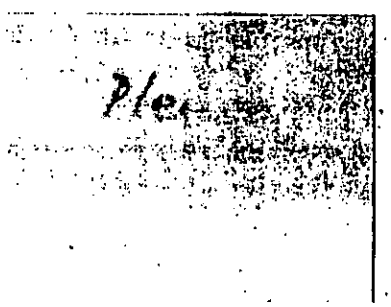
Please briefly provide your opinions of this proposal, giving special attention to the originality and feasibility of the project, its potential for providing new knowledge and the justification of financial support sought; include suggestions for modifications (scientific or financial) where you feel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

(Use additional pages if necessary)

Title:

PI:

Reviewer:



"Ecological and epidemiological studies on *Aeromonas* spp. in Bangladesh with special emphasis on their spread between the environment and the humans"

Reviewer:

DETAILED COMMENTS

General

Despite of numerous previous studies, the clinical significance of *Aeromonas* species is still open. The study now planned to be carried out at the International Centre for Diarrhoeal Disease Research has surely an important impact to better understanding the potential clonal nature of clinically important *Aeromonas* isolates. The study is a well planned continuation to that work published by the same investigators very recently (in February 1997) in the Journal of Clinical Microbiology. However, there are a few points I would like to address to the study group for consideration.

Collection and analyzing the isolates

1. The collection of *Aeromonas* isolates is very well organised to reflect the epidemiology of clinical as well as environmental strains throughout the year and in various geographical areas of the country.

   The investigators estimate that a total of almost 450 *Aeromonas* isolates will be collected. A subset of isolates, selected on the basis of results obtained by one typing method (phenotyping by PhP method), will subsequently be characterized by other typing methods and by determination of certain virulence properties. I assume that the investigators have already carefully considered that this selection do not bias their final results and conclusions.

2. The study plan includes a good plan for collecting and thorough typing of the strains. There is also a list of the investigators who are responsible for itemized tasks. However, no investigator seems to be responsible for collecting and analyzing clinical data of the diarrhoeal patients and compiling these results with the characteristics of the isolates.

3. A critical point is the quality of a stool sample taken from a diarrhoeal patient; microbial findings often differ with differing quality of the specimens. Everybody who has been working at a routine clinical microbiological laboratory and cultured routinely taken stool specimens knows that a sample sent to the laboratory as a diarrhoeal specimen may, in fact, not be a diarrhoeal specimen at all. Thus, I am suggesting that the quality of the specimens should somehow be recorded.

4. *Aeromonas* spp. may multiply at ambient temperature as well as in the fridge in different transport tubes, including Cary-Blair medium. Thus, I find it important that the preservation and transport time of each specimen is as equal as possible before the culture.

5. There is a general opinion that a diarrhoeal stool specimen commonly contains abundantly the pathogen(s) causing that diarrhoea. Thus, an organism detected after enrichment only may not be a cause of the diarrhoea. Indeed, if the quality of the enrichment medium is high it will, during an 8 hour incubation, enrich e.g. 10 *Aeromonas* cells (which may have nothing to do with diarrhoeal symptoms) to an abundant growth (10^8 cells/tube). Thus, I would be worried about analyzing only isolates collected after enrichment. The similar situation concerns *Salmonella* findings. Their number as causative agents in most studies is overestimated because of the traditional use of enrichment for the *Salmonellae* but not for other enteric pathogens. Therefore, I would consider analyzing the isolates detected on the primary culture plate separately from those only found after enrichment.

The time period of the study

It has been planned to collect and type by the PhP method the estimated 438 isolates during the first 18 month period. The one year collection time of the strains seems to be very reasonable. However, I find 6 more months to be quite a long time for the PhP typing; I would propose only 2 months. On the other hand, 6 months for characterizing further the selected PhP types and for writing reports might be far too short; I would extend that latter period.

The budget

It is very difficult to say anything about the budget of the project. It seems to be higher than I would be expecting if calculating the costs according to the Finnish price level. Still, nothing has been budgeted for the FAME and AFLP profiling of the strains.

Slightly more than half (US$ 60,000) of the total budget goes to the investigator(s) of the Karolinska Institute who are responsible for the advice on PhP typing. As far as I can see, the PhP typing being an automated method is not as laborous as some other parts (ribo- or ERIC-typing and determination of virulence properties) of the study. Especially, the budget for the salaries of the Swedish Institute seems to be quite high, the budget for the consumables, however, very reasonable. On the other hand, the budget of consumables for the ICDDR,B is quite high.

Referee # 2

Title: Ecological and epidemiological studies on Aeromonas spp. in Bangladesh with special emphasis on their spread between the environment and the humans.

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:

Institution

Detailed Comments

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

(Use additional pages if necessary)

Title: ECOLOGICAL AND EPIDEMIOLOGICAL STUDIES ON AEROMONAS Spp. IN BANGLADESH WITH SPECIAL EMPHASIS ON THEIR SPREAD BETWEEN THE ENVIRONMEN AND THE HUMANS

PI: M. JOHN ALBERT AND OTHERS

Reviewer:

Detailed comments:

Aeromonas are an interesting group of enteric pathogens whose significance has never really been clearly understood. The reason for this hiatus in our knowledge on *Aeromonas* spp. is because the organisms reside both in the environment and are also associated with clinical infection. Most previous studies have not clearly addressed this issue and therefore, the clinical status of *Aeromonas* spp. has remained in limbo.

The proposal submitted by Dr. Albert and co-investigators is an excellently designed study which goes into the crux of defining this difference using state of the art molecular techniques. The importance of such a project is that it will be able to define traits which clearly differentiate between environmental and pathogenic *Aeromonas* spp.

Aeromonas spp. *per se* are not the most important of enteropathogens in developing countries like Bangladesh where *Vibrio cholerae* and diarrhoeagenic *Escherichia coli* transcend all other enteropathogens in terms of incidence. However, the importance of studying *Aeromonas* lies in the fact that these species which are ubiquitous in the environment contribute in genetic transfers which inevitably take place in the environment as has been shown by Dr. Albert and co-workers who have demonstrated cross-reactivity in diagnostic antisera prepared against *Aeromonas* with *Vibrio cholerae* O139.

In view of the above, I strongly support the proposal without any reservations and I look forward to see the results of this project.