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ACE SHEET) ETHICAL REVIEW COMMITTEE, ICDDR,B. DHAKA 1212

incipal Investigator Dr. Shah M.Faruque Trainee Investigator (if any) _____

plication No. 93/033 Supporting Agency (if Non-ICDDR,B) _____

le of Study Characterization of epide- Project status:
c strains of Vibrio cholerae 01 and non- ( ) New Study
based on genetic and phenotypic traits. ( ) Continuation with change
( ) No change (do not fill out rest of form)

rcle 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:
(a) Nature and purposes of study Yes No NA
(b) Procedures to be followed including alternatives used Yes No NA
(c) Physical risks Yes No NA
(d) Sensitive questions Yes No NA
(e) Benefits to be derived Yes No NA
(f) Right to refuse to participate or to withdraw from study Yes No NA
(g) Confidential handling of data Yes No NA
(h) Compensation &/or treatment where there are risks or privacy is involved in any particular procedure Yes No NA

5. Will signed consent form be required:
(a) From subjects Yes No NA
(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.

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

Shah Md. Faruque
Principal Investigator 2/11/93

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APPLICATION FOR PROJECT GRANT

1.	PRINCIPAL INVESTIGATOR	:	Dr. Shah M. Faruque
2.	CO-INVESTIGATORS	:	Dr. A.K. Siddique Dr. M.J. Albert Mr. A.R.M.A. Alim Mr. Q.S. Ahmad
3.	CONSULTANT		Dr. R. Bradley Sack
4.	TITLE OF PROGRAMME	:	Characterization of epidemic strains of *Vibrio cholerae* 01 and non-01 based on genetic and phenotypic traits
5.	STARTING DATE	:	When funds are available
6.	DURATION	:	3 years from starting date
7.	TOTAL DIRECT COST	:	US$ 176,175
8.	FUNDING SOURCE	:	
9.	HEAD OF PROGRAMME	:	Dr. R. Bradley Sack Associate Director Laboratory Sciences Division

R. Bradley Sack

10. AIMS OF THE PROGRAMME

10.1. General aim

Differentiation of *Vibrio cholerae* isolates by genetic typing and phenotypic traits to identify and characterize epidemic strains in Bangladesh and in other geographic locations, to understand the geographical distribution and evolutionary relationships among clones of different epidemic strains.

10.2. Specific aims

a) To type *V. cholerae* O1 and non-O1 isolated during epidemic and inter-epidemic periods in Bangladesh and in other geographic locations based on the restriction patterns of the cholera toxin genes and the conserved ribosomal RNA genes.

b) To determine the association of different genetic types of *V. cholerae* with the presence of genes for the newly described Zonula Occludens toxin (ZOT), and the heat-stable toxin of non-O1 vibrios (NAG-ST).

c) To determine the association of different genetic types of *V. cholerae* with phenotypic traits such as (i) serotypes (ii) haemolysis (iii) haemagglutination, (iv) multilocus enzyme electrophoretic pattern (v) antibiotic resistance and (vi) plasmid contents.

d) To determine the relative abundance of different genetic types of *V. cholerae* in various regions of Bangladesh and possible contribution of these types in epidemics.

e) To determine the existence of different genetic types of *V. cholerae* over the last two decades in Bangladesh as an aid to study the evolution and genetic relatedness of different epidemic strains.

f) To determine the existence of these types of *V. cholerae* in other countries with endemic and epidemic cholera.

10.3. Significance

Characterization of epidemic strains of *V. cholerae* and the evolutionary patterns of different epidemic strains will aid to a better understanding of the pathogen, its relation to epidemics and survival during inter-epidemic

periods, and may help in devising means to control current epidemics and avert the possibility of future epidemics.

11. BACKGROUND

Cholera is one of the most devastating secretory diarrhoeas and is caused by *V. cholerae* which colonize the small intestine (1-3). Of all enteric pathogens that cause diarrhoea *V. cholerae* O1 induces the most rapidly fatal disease. Profuse diarrhoea is caused by a toxin, protein in nature that attaches to the specific receptors present on the enterocytes (2) and death occurs in 50-70% of untreated cases (4). Cholera is endemic in southern Asia and parts of Africa, where seasonal outbreaks occur widely and are particularly associated with poverty and poor sanitation. *V. cholerae* responsible for causing clinical disease are of two biotypes, Classical and El Tor, and two serotypes, Inaba and Ogawa (1). Clinical features and severity in both types of *V. cholerae* are similar, although the carrier state may be more prolonged in case of the El Tor biotype (5). Many aspects of the disease and the pathogen remain unknown, particularly its epidemiological characteristics, interepidemic reservoir, and some aspects of pathogenesis.

11.1. *Vibrio cholerae* O1 and non-O1

In the past, a wide variety of Gram-negative, polar-flagellated rods were classified as belonging to the genus *Vibrio*. During the mid-sixties, however, some criteria had been established for the taxonomy of the genus *Vibrio* and the International Sub-committee on Taxonomy of *Vibrios* recommended a provisional definition according to which the majority of species previously classified as *Vibrios* are excluded from the genus. On the other hand, taxonomic studies on the related organisms have indicated a close relationship

between the three genera, *Vibrio*, *Aeromonas*, and *Plesiomonas* (6). On the basis of biochemical characteristics, it is possible to differentiate members of the genus *Vibrio* from those of allied genera. The current classification has been reviewed by West and Colwell (7). The genus *Vibrio* contains several bacterial species, of which *V. cholerae* and *V. parahaemolyticus* are the two most important pathogens of humans. Besides, there are many unknown *Vibrios* isolated from sea-water and sea-fish and may sometimes be confused with *Vibrios* that infect humans. *V. cholerae* is classified on the basis of its somatic antigens (O-antigens) (8,9) into 138 serovars or serotypes (T. Shimada and R. Sakajaki, personal communications). Until recently, the serovar O:1 was supposed to include all the strains responsible for epidemic and endemic cholera and has two subtypes, Ogawa and Inaba; a Hikojima subtype has been reported rarely. These subtypes can be further distinguished into two biotypes, Classical and El Tor. El Tor differs from Classical biotype in several respects. El Tor is resistant to Mukherjee's group IV phage and to polymixin B, it causes agglutination of chicken erythrocytes (10), it generally produces a haemolysin, and it is Voges-Proskauer positive (11).

There has been some controversy over the terminology of the "NCV" (non-cholera vibrio). These vibrios possess biochemical and morphological characteristics very similar to those of the cholera vibrio but are non-agglutinable with polyvalent O:1 antiserum. Such vibrios are agglutinable by their own antisera. Vibrios not agglutinating with O-subgroup 1 antiserum were later to be called "NAG" (non-agglutinable) vibrios. Both the terms, "NCV" and "NAV" are unsatisfactory but they are used for the sake of convenience. There are 137 known serotypes of non-01 vibrios. Non-01 serotypes of *Vibrio cholerae* have been mostly associated with sporadic cases of diarrhea and extraintesti-

nal infections (12) until a recent large cholera-like outbreak in Bangladesh which has been caused by a *Vibrio cholerae* non-01 strain (13).

11.2. Epidemic outbreak of cholera due to *Vibrio cholerae* non-01

In the middle of January 1993, an outbreak of acute watery diarrhoea resembling cholera was noted in southern Bangladesh which affected mostly adults. Through the middle of February, it was estimated that approximately 10,000 people had been affected with an estimated 500 deaths (A.K. Siddique: unpublished observation) and cases are still continuing to occur. Since the third week of January, an unusually large number of adult diarrhoeal cases clinically resembling cholera have also been seen at the clinical research centre of the ICDDR,B in Dhaka.

Rectal swabs cultured from the field outbreak, and from hospitalised patients, yielded mostly *V. cholerae* non-01. The bacterium responsible for the outbreak resembled *V. cholerae* 01 both by colony morphology on Monsur's medium (14) and biochemically, but did not agglutinate with *V. cholerae* 01 antisera. All strains were tested for reactivity with a monoclonal antibody specific for the 'A' factor of *V. cholerae* 01 by an indirect fluorescent antibody technique (15), and all were non-reactive. It was not immediately possible to determine the serotype of this strain.

All the strains tested were positive for cholera toxin (CT) production by Y1 adrenal cell assay and the toxin could be neutralised by rabbit polyclonal antiserum to CT (12). Primers specific for the CT operon from *V. cholerae* 01 amplified sequences corresponding to CT in these strains in the polymerase chain reaction (16). A selected subsample of strains induced fluid accumulation in the adult rabbit ileal loop assay (12) and induced profuse

watery diarrhoea in the reversible ileal tie adult rabbit diarrhoea (RITARD) model (17) similar to that due to *V. cholerae* 01. All strains were resistant to Mukherjee's phages specific for both biotypes of *V. cholerae* 01. These additional data suggest that the strains are not *V. cholerae* 01 (12).

In the past, *V. cholerae* non-01 has never been associated with such a large outbreak of diarrhoea. *V. cholerae* non-01 are known to produce CT at a very low frequency (12) unlike the present outbreak, in which all tested strains produced the toxin. The higher attack rate of diarrhoea in adult populations is reminiscent of cholera epidemics in virgin populations (18). Similar cholera-like outbreaks have occurred recently in neighbouring India (19) and it is important to determine whether these are due to the same clone of *V. cholerae* non-01 as the one in Bangladesh. This "new" strain of *V. cholerae* needs to be monitored in the Indian subcontinent and adjacent regions for its possible pandemicity.

11.3. Surveillance of cholera due to *Vibrio cholerae* 01 in Bangladesh

Systematic surveillance of cholera has been carried out in two different regions of Bangladesh by the ICDDR,B and the former PSCRL. Dhaka, the capital of Bangladesh is surrounded by large urban and semi-rural population centres. Dhaka has an enormous influx of people from rural villages making the population extremely crowded. It has far outstripped the capacity for housing and sanitary facilities. The Clinical Research Center of the ICDDR,B has a surveillance system in which every 25th diarrhoeal patient reporting to the hospital is entered into a programme for in-depth clinical, microbiological and demographic investigation (20). Of 3550 diarrhoeal patients of different age groups studied by this surveillance system from December 1979 to November

1980, *V. cholerae* O1 was identified in 6% of patients. Between the ages of 5 and 14 years, *V. cholerae* infection was more frequent (11%). *V. cholerae* O1 showed a pronounced seasonality with a high frequency of infection just after the monsoon season (20).

Since 1963, the ICDDR,B has been operating a diarrhoea treatment centre in the Matlab sub-district, a rural area of Bangladesh. In May 1983, a surveillance system was set up to study a 20% systemic sample of all admissions. In a total of 2,635 patients studied Between May 1983 and April 1984, *V. cholerae* O1 was found to be the most common enteropathogen and was detected in 39% of the patients (21). The detection rate was highest in children between 5 to 9 years (60%), but the isolation rate was also high in other age groups from 3 years through the adulthood. Similar to the seasonality in Dhaka, *V. cholerae* O1 showed a pronounced seasonality in Matlab with a large peak after the monsoon (October to January inclusive).

In addition to the systematic surveillance mentioned above, ICDDR,B has sent out team of physicians under epidemic control programmes to rural districts of Bangladesh. Between September 1988 and October 1989, 6365 suspected cholera patients were treated in epidemic outbreaks in 24 rural districts of Bangladesh (22). A non-random sample of 2,386 rectal swabs, collected before any antibiotic therapy was given, were analyzed within 72 hr of collection by standard laboratory techniques. *V. cholerae* O1 was isolated from 951 (40%) of the patients and was the most frequently isolated enteric pathogen. Biotyping identified 167 (18%) classical isolates and 784 (82%) El Tor. Seven southern districts had a significantly higher proportion (79%) of classical than El Tor isolates whereas 17 northern and middle-belt districts nearly all isolates (99%) were of the El Tor biotype. Serotyping showed that 98% of the

classical biotypes were Ogawa, whereas 86% of El Tor isolates were of the Inaba serotype. Nearly all classical biotypes in the southern area of the country were resistant to tetracycline in contrast to El Tor biotypes which were sensitive to the drug (22, 23).

Data obtained from epidemic interventions in nearly 400 rural sub-districts by ICDDR,B medical teams between 1985 and 1989 and in 1991 showed that *V. cholerae* O1 was the most frequently (40%) isolated enteropathogens during the epidemics (24). Only 24% of laboratoy confirmed cholera patients were below 5 years of age, and children below 2 years of age accounted for only 10% of the total. In September 1991, an outbreak of cholera that started in the north-western region spread to most of northern Bangladesh. The 1991 epidemic was estimated to have produced between 210,000 and 235,000 cases and over 8,000 deaths (24).

Cholera surveillance in Bangladesh has so far shown that *V. cholerae* O1 continues to be a significant cause of infection and morbidity, the frequency of infection varies from year to year in different regions, and a striking difference in the geographical distribution of biotypes exists (20-24). In addition to the epidemic cholera caused by *V. cholerae* O1, the recent epidemic caused by a non-O1 strain adds a new dimension to the already existing problem.

11.4. Epidemiology of *Vibrio cholerae* O1 infection in Bangladesh

Surveillance of cholera in Dhaka and Matlab treatment centres have shown that until 1970, more than 90% of cholera in Bangladesh was caused by the classical Inaba serotype and by 1972 85% of all cases were Ogawa classical (25). Since

the present seventh cholera pandemic started in 1961. El Tor cholerae has spread extensively from its home in Indonesia throughout Asia, the West coast of Africa, parts of Europe, and recently through South America (26-29). During the last 30 years various changes have been noticed in the property of vibrios including the appearance and disappearance of drug resistant strains (30), the replacement of Classical Vibrios by El Tor in Bangladesh and a sudden reappearance of classical Vibrios after 10 years (31).

El Tor *Vibrio* appeared in Bangladesh in the middle of 1973 (25) and since then this biotype has completely replaced the classical biotype as surveyed in Dhaka and Matlab. However, in 1982 when an outbreak of cholerae due to the classical biotype occurred it initiated the controversy surrounding the disappearance of classical *V. cholerae* in Bangladesh. In a recent study (22), the authors presented circumstantial evidence suggesting that the classical biotype would have always existed in the southern parts and El Tor biotype in the middle and northern parts of Bangladesh because of the differing favourable environmental conditions. The apparent replacement of classical *vibrios* by the El Tor in Bangladesh could, therefore, be due to an artifact of sampling. Studies have also found that the frequency and distribution of Inaba and Ogawa serotypes varies in a cyclical fashion, and is thought to be due to the immune status of the population.

In 1979, an outbreak of cholera due to multiple drug resistant *V. cholerae* O1 occurred in Matlab (30,32). Screening of 262 isolates stocked between June 1979 and February 1980 showed that 44 (16.7%) of the isolates were resistant to five antibiotics: tetracycline, ampicillin, kanamycin, streptomycin, trimethoprim-sulfamethoxazole, and another 10 isolates were resistant to any four of these antibiotics including tetracycline. An antibiotic resistance

plasmid was identified in these isolates and the plasmid was transferable to an *E. coli* K-12 recipient by simple conjugation. Epidemiological assessment of the outbreak suggested that the outbreak began from the introduction into the area of a single multiple drug resistant strain of *V. cholerae* O1 (31). However, screening of 91 strains isolated from cholera patients in January 1986 in Dhaka showed that none of these isolates was resistant to tetracycline, streptomycin, chloramphenicol, amoxicillin or nalidixic acid (33). The reemergence of tetracycline resistance was observed during the 1991 epidemic (24) and most recent data on antibiotic resistance show that 70% of strains isolated are resistant to tetracycline, often in addition to other antibiotics.

11.5. Identification and typing of *Vibrio cholerae* strains

Epidemiological studies on *V. cholerae* O1 have been hampered by the lack of suitable typing systems. Limited phage typing studies have been carried out in the past, but this has proven to be unsatisfactory due to inability of phages to finely discriminate between strains and the difficulty of making these methods widely available (34). Plasmids are known to carry genes conferring resistance to various antibiotics. Analysis of plasmid profiles has also been shown to be a powerful tool in epidemiological studies of infectious diseases caused by various microorganisms (28). However, plasmid profile analysis cannot be very effective in the case of *V. cholerae*, because *V. cholerae* O1 strains rarely carry plasmids. Chromosomal banding patterns after restriction endonuclease digestion have been analyzed for strain discrimination, but the fragments generated are too numerous by this method for a practical and useful comparison among strains (34,35). Hence, new typing methods as described below are needed to effectively differentiate between different strains of

V. cholerae. However, a combination of different typing methods can obviously be more useful in defining different epidemic strains.

11.6. Ribotyping of *Vibrio cholerae*

Recent developments in DNA analysis techniques have introduced several typing methods dependent on genotypes rather than phenotypes. One of these techniques is the use of rRNA gene probes to study restriction fragment length polymorphisms (RFLPs) of conserved rRNA genes in different strains, and the typing system is known as ribotyping (36). The probing of chromosomal DNA with *E. coli* rRNA probe provide a widely applicable system to investigate the molecular epidemiology of bacteria. In preliminary studies at the ICDDR,B we have used ribotyping to study clonal relationships among isolates of *V. cholerae* O1 (29). Ribotyping of classical *V. cholerae* O1 isolates in our laboratory also provided genetic data to indicate that the classical biotype of *V. cholerae* O1 was never completely replaced by the El Tor biotype in Bangladesh (37) which has confirmed previous assumptions based on surveillance data (22,24).

11.7. Restriction analysis of cholera toxin (CT) genes

Strains of *V. cholerae* O1 can also be differentiated based on the restriction fragment length polymorphisms in the cholera toxin genes (38). This approach to differentiation has been applied to a limited extent on isolates from Hong Kong (39), South America (40) and the US gulf coast (41). These studies demonstrated that a single clone of enterotoxigenic *V. cholerae* O1 was resident in the US gulf coast and that a second reservoir of cholera toxin genes existed in *V. cholerae* non-O1 strains in Louisiana. These studies also

showed that strains from the epidemic in Peru are not closely related to the previously recognized gulf coast clone (40). Our preliminary data on the restriction site polymorphisms in the CT genes also demonstrated the usefulness of this approach (unpublished observation) and the method is being applied to analyze *V. cholerae* O1 isolates from various geographical locations including Bangladesh, India, Tanzania, Nigeria, Peru and Ecuador.

11.8. Multilocus enzyme electrophoresis (MEE)

Multilocus enzyme electrophoresis, has been used to estimate the genetic diversity in natural populations of a variety of species of bacteria (42) including *Escherichia coli*, *Shigella* spp., *Salmonella* spp., and recently *Vibrio cholerae* O1 (43, 44). MEE has been applied to study the possible evolutionary relationships between toxigenic and non-toxigenic *V. cholerae* O1 strains isolated in the Western Hemisphere between 1973 and 1990 (43). MEE has also been used to study the molecular epidemiology of recent cholera epidemic in Latin America (44). This research has provided data for useful marker systems for epidemiology and to study evolutionary relationships among strains of toxigenic *V. cholerae*.

12. PLAN OF STUDY

ICDDR,B has a large collection of isolates of *V. cholerae* O1 in lyophilized form from endemic and epidemic cases of cholera since 1965. A selected number of strains from this collection and isolates collected from the recent cholera surveillance and epidemic control programmes in different rural districts of Bangladesh will be included in the study. These isolates will include 20 classical *Vibrio cholerae* isolates from each year between 1965 and

1972, 20 El Tor isolates from each year between 1973 and 1981, and 10 isolates each of classical (if available) and El Tor *Vibrios* isolated each year between 1983 and 1992. Approximately 300 isolates obtained from the epidemic control programmes in different rural districts and 200 isolates from the recent cholera outbreak due to non-O1 *Vibrio cholerae* will also be studied. In addition, we have a collection of 200 strains from different geographic locations in Asia, Africa and the Americas; these will also be included in the study. All these isolates will be classified based on restriction site polymorphisms in the cholera toxin and conserved rRNA genes using one or more suitable restriction enzymes. The strains will also be characterized in terms of biotypes and serotypes, and will be analyzed for the presence of plasmids, drug sensitivity patterns, haemolysin and toxin production. Details of the methods for these analysis are described in the following sections.

12.1. METHODS

The identity of all isolates obtained from the culture collection will be reconfirmed biochemically and serologically by standard methods recommended by WHO (31). Biotype will as determined by sensitivity to polymyxin B (15 µg/ml). Haemolytic properties will be examined by sheep blood-agar plate cultures and haemagglutinating properties will be examined by the slide agglutination method using chicken erythrocytes.

12.1.1. Analysis of restriction site polymorphisms

High molecular weight chromosomal DNA will be isolated from strains grown overnight in T1N1 broth as described by Stull *et al* (36). Approximately 5 µg of purified whole cell DNA will be digested with appropriate restriction endonucleases as instructed by the manufacturer (Bethesda Research

Laboratories) using 5 units of enzyme per µg of DNA. The digested DNAs will be electrophoresed in 0.8% agarose gels in Tris-borate-EDTA buffer by a standard method (45), and transferred onto nylon membranes (Hybond-N, Amersham) by Southern blotting (46).

The rRNA gene probe is a 7.5 kb *Bam*HI fragment of pKK3535 (47) which is a pBR322 derived plasmid containing an *E. coli* ribosomal RNA operon consisting of one copy each of the genes coding for 5S RNA, 16S RNA, 23S RNA, and $tRNA^{Glu}$. The DNA probe for cholera toxin genes consists of a 554 bp *Xba*I-*Cla*I fragment encoding 94% of the A-subunit of cholera toxin cloned into pBR325 through *Eco*RI linkers. The recombinant plasmid pCVD27 has been kindly provided to us as transformed culture by Dr. James B Kaper, Centre for Vaccine Development, Baltimore, MD. The recombinant plasmids will be prepared, digested with appropriate restriction enzymes, and the inserts will be separated by electroelution from agarose gel as described by Maniatis *et al* (45).

The probe DNAs will be labelled by random priming (48) using [α-^{32}P]-deoxycytidine triphosphate (3000 Ci/mmol, Amersham International plc., U.K.) and a random primers DNA labelling system (Bethesda Research Laboratory). Southern blots will be hybridized and washed under stringent conditions as described previously (49). Autoradiographs will be developed from the hybridized filters using Kodak X-Omat AR film (Kodak Rochester, NY), with intensifying screens at -70^{o}C for 24 hr.

12.1.2. Plasmid analysis

Plasmids will be prepared by the alkaline lysis method of Birnboim and Doly (50) from a 1.5 ml overnight culture of the bacteria in Luria-Bertini (LB)

broth. The extracted plasmid DNA will be electrophoresed in a 0.7% agarose gel by standard methods (45). The gels will be stained in 0.5 µg/ml aqueous ethidium bromide for 20-30 min and photographed on type 57 Polaroid film using short wave ultraviolet light. Plasmid size will be extrapolated from a plot of band migration distance versus log of molecular size (base pairs) using supercoiled DNA standards of known size.

12.1.3. Antimicrobial susceptibility tests

The antimicrobial susceptibility tests will be done by the method of Bauer *et al.* (51) using commercially available disks (BBL Microbiology Systems) at the following antibiotic concentrations (µg/disc): ampicillin, 10; chloramphenicol, 30; streptomycin, 10; tetracycline, 30; trimethoprim-sulfamethoxazole, 1.25 and 23.75 respectively; kanamycin, 30; gentamycin. 10; and nalidixic acid, 30.

12.1.4. Multilocus enzyme electrophoresis (MEE)

Isolates will be assayed for allelic variation in 12 enzyme loci. Electrophoretic typing of the 12 enzymes including threonine dehydrogenase (THD), isocitrate dehydrogenase (IDH), adenylate kinase (ADK), malate dehydrogenase (MDH), glucose-6-phosphate dehydrogenase (G6P), malic enzyme (ME), glutamic pyruvic transaminase (GPT), Glutamic-oxalacetic transaminase (GOT), leucine aminopeptidase (LAP), phosphoglucose isomerase (PGI), alanine dehydrogenase (ALD), L-lactate dehydrogenase (ALD), and nucleoside phosphorylase (NSP) as described by Selander *et al* (42).

13. DATA ANALYSIS

Genetic and phenotypic data obtained from the study will be analyzed using computer programmes as described previously (42). Genetic distance between pairs of isolates will be calculated as the proportion of loci at which dissimilar alleles occur, i.e. the proportion of mismatches and this will be ex pressed by a coefficient as described by Nei (52). Dendograms will be generated using the data to determine divergence and evolutionary relatedness among different clones of *V. cholerae*.

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14. WORK PLAN FOR 3 YEARS

(a)	Standardization of techniques and training of technicians	...	6 months
(b)	Selection of strains and testing for phenotypic properties	...	6 months
(c)	Selection of appropriate restriction enzymes for genetic typing	...	3 months
(d)	Genetic typing by restriction patterns of rRNA genes	...	9 months
(e)	Multilocus enzyme analysis		6 months
(f)	Analysis of data and writing of reports	...	6 months

15. TASKS OF INVESTIGATORS

Investigator		Task
Dr. S.M. Faruque	:	Standardization of techniques, analysis of data and writing up reports.
Dr. R.B. Sack Dr. M.J. Albert	:	Provide strategic and academic feedback, help in interpretation of results, and in designing future studies.
Dr. A.K. Siddique	:	Select isolates from epidemic surveillance and environmental samples for analysis, Interprete and process epidemiological data.
Dr. S.M. Faruque Mr. Q.S. Ahmad Mr. A.R.M.A. Alim Mr. S. K. Roy		Characterization of *V. cholerae* strains based on genetic and phenotypic properties.

16. BUDGET FOR THREE YEARS

Description	Yearly budget (in US$) 1st year	2nd year	3rd year	Total
1. Personnel				
Name & Level (% of time)				
Dr. S.M. Faruque NO-C	8,211 (60%)	13,548 (90%)	14,903 (90%)	36.662
Mr. A.R.M.A. Alim NO-B	5,303 (50%)	10,500 (90%)	11,550 (90%)	27.353
Mr. Sanjit K. Roy GS-5	2,992 (100%)	3,291 (100%)	3,620 (100%)	9,903
Mr. M.H. Tariq GS-2	2,664 (100%)	2,930 (100%)	3,223 (100%)	8,817
Mr. Q.S. Ahmad	2.595 (20%)	2,855 (20%)	3.140 (20%)	8.590
Mr.K.M.Belayet Hossain	2,265 (40%)	2,492 (40%)	2.742 (40%)	7.499
Secretarial Service	600 (20%)	660 (20%)	726 (20%)	1,986
TOTAL	24,630	36,276	39,904	100.810
2. Travel (local)	200	200	200	600
3. Supplies and Materials				
Glasswares	1,000	1,000	1,000	3,000
Chemicals and media	4,000	4,000	4,000	12,000
Office supplies	1,500	1,500	1,500	4,500
Stock items	3,000	3,000	3,000	9,000
Non-stock items	6,467	6,613	6,774	19,854
TOTAL:	15,967	16,113	16,274	48,354

3. Other contractual

Repair and maintenance	1,000	1,000	1,000	3,000
Rent, Comm.. Utilit.	1,000	1,000	1,000	3,000
Printing & Publication	800	800	800	2,400
TOTAL	2,800	2,800	2,800	8,400

4. Interdepartmental

Xerox & mimiography	1,000	1,000	1,000	3,000
Medical illustration	500	500	500	1,500
Maintenance	1,000	1,000	1,000	3,000
Staff Clinic	300	300	300	900
Bioengineering	100	100	100	300
Library Service charges	100	100	100	300
TOTAL	3,000	3,000	3,000	9,000
TOTAL OPERATING COST	46,597	58,389	62,189	167,164

5. Capital equipment

Freezer	1,500	-	-	1,500
Shaking water bath	2,500	-	-	2,500
Eppendorf centrifuge	5,000	-	-	5,000
TOTAL	9,000	-	-	9,000
TOTAL DIRECT COST	55,597	58,389	62,189	176,175

Add: Overhead 31% on Direct cost	54,615
TOTAL PROJECT COST	230,790

Budget has been reviewed by Budget Office
and found Okay.

Shaming Hon 31/10/93

Head, Budgetary Accounting.

Reviewer # 1

Title: Characterization of epidemic strains of V. cholerae 01 and non-01 based on genetic and phenotypic traits.

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 [X]

b) with qualification [ ]

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

I do not support the application [ ]

Name of Refere

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: Characterization of epidemic strains of Vibrio cholerae...

PI: DR. Shah M. Faruque

Reviewer:

The specific aims of this proposal include the characterization of 500 Vibrio erae strains by a variety of methods including RFLP patterns of cholera toxin ribosomal RNA genes, phenotypic characteristics (e.g., serotype, biotype,antibiotic stance, plasmid content). These data will then be used to determine the relative abun- e of various strains world wide, thus determining possible patterns of spread regional specificities that may be environmentally linked. Overll the proposal mbitious in size but well thought out. These data have not been systematically ected for V. cholerae strains worldwide before so the authors might indeed generate ry important data base when they are finished.

The weakest portion of the proposal has to do with the lack of specifically uding non-01 strains in this analysis. I assume with the outbreak of 0139, there be investigators that are planning a similar study with non-01 as a focus point. ot, the proposal could be greatly strengthened by decreasing the number of 01 ins analyzed to about 300 and adding 200 non-01 strains to the analysis. This tion might help elucidate the origin of 0139. The study could also be improved he addition of probes for additional virulence genes such as tcp, rfa, vst, msh, For example, RFLP variations have been reported that differentiate classical El Tor strains with a tcp probe. If the authors found strains that were clearly biotype by one set of tests but were the other by tcp probe RFLP, then this would ide evidence that recombination was generating variation that might soon complicate itional biotype designations.

inally I am a little concerned about the authors interpretation of results which ot spelled out very well. For example, noting the pattern of antibiotic resistance t lead to the conclusion that some strains are related when in fact they are not but y share the same plasmid. I trust that the investigators will be aware of such ntial for mistakes but some discussion of the interpretation of results would have desirable.

End of Review

Reviewer # 2

Title: Characterization of epidemic strains of V. cholerae 01 and non-01 based on genetic and phenotypic traits.

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			

CONCLUSIONS

I support the application:

a) without qualification [ ]

b) with qualification [X]

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

I do not support the application [ ]

Name of Referee:

Signatur
Position

Institution:

Title: Characterisation of epidemic strains of *Vibrio cholerae* O1 and non-O1 based on genetic and phenotypic traits

PI: Dr. Shah M. Faruque

The major strength of this proposal is that it concerns an extremely important problem, namely, the emergence of an epidemic strain of *V. cholerae* non-O1 in Bangladesh and India. Given the seriousness of this problem, it is necessary to gather as much data as possible about the new strains of *V. cholerae* non-O1 so that insights may be gained as to the origin and potential spread of this epidemic clone.

In this proposal, the P.I. and colleagues propose to gather a variety of data on these strains and they appear to be qualified to conduct these experiments. However, the major flaw in this proposal is that it is not at all clear how the data will be analyzed. Section 13 states that the restriction fragment length polymorphisms and rRNA gene data will be analyzed as described by Wachsmuth et al. (ref. 47) to determine divergence and evolutionary relatedness among different clones of *V. cholerae*. However, this analysis is impossible given the proposed data to be collected. Wachsmuth et al. gathered phenotypic data on *V. cholerae* strains using a multilocus enzyme electrophoresis technique which yields data on the amino acid variation among a variety of bacterial enzymes. Such data can be quantitatively analyzed to give genetic distance among clusters of strains. This type of analysis cannot be carried out on the type of data that will be gathered in this study, namely, restriction fragment length polymorphisms and rRNA gene polymorphisms. Wachsmuth et al. also gathered restriction fragment length polymorphism and rRNA gene polymorphism data but did not analyze it to determine divergence and evolutionary relatedness. Thus, although the data to be gathered in this study will tell if individual strains are from the same genetic clone or not, it will not determine evolutionary relatedness.

INTERNATIONAL CENTRE FOR
DIARRHOEAL DISEASE
RESEARCH, BANGLADESH

Memorandum

To : Chairman
Research Review Committee

From : Shah M. Faruque Shah Md. Faruque

November 1, 1993

Subject : Investigators' response to reviewers' comments on the protocol entitled "Characterization of epidemic strains of *V. cholerae* 01 and non-01 based on genetic and phenotypic traits"

The above protocol has been reviewed by two external reviewers. We have carefully considered the reviewers' comments and have made modifications in the protocol in accordance with the reviewers' suggestions. The following is our response to the reviewers' comments:

1) We agree with reviewer #1 that with the outbreak of cholera due to *V. cholerae* 0139, it is important to include more strains of 0139 in the study. In accordance with the reviewer's suggestion we have decided to reduce the number of strains of 01 vibrios to 300 and increase the number of 0139 vibrios to 200. Modification has been made in the protocol accordingly.

2) We have considered the 2nd reviewer's concern on the data analysis section. In response, we have included the multilocus enzyme electrophoresis as described by Wachsmuth *et al.* (J Infect Dis 1993; 167:621-626) in our study. The data will be processed using available computer programmes as previously done (reference cited in the text). We have modified the data analysis section accordingly.

The comments of the reviewers are overall in favour of the study and both reviewers have agreed on the potential value and importance of the data that may be generated from the study.

The investigators will be happy to incorporate possible further suggestions, if any, made by the Research Review Committee.

Thank you.

SMF:mh/F3:RRC.MEM