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SECTION I - RESEARCH PROTOCOL

1) Title: Polyphasic characterization of Vibrio cholerae NAG and related organisms

2) Principal Investigator: William M. Spira, Ph.D.

3) Starting date: September, 1977

4) Completion date: August, 1980

5) Total direct cost: $ 169,100

6) Abstract summary: Isolates of Vibrio cholerae NAG and related organisms will be obtained from patients, aquatic sources and from established culture collections elsewhere in the world. Data on associated clinical picture, biological activity and virulence factors, and phenetic characters will be gathered, as appropriate, for each isolate. This data base will be subjected to numerical taxonomic analysis to identify and characterize clusters of potentially pathogenic strains. Representatives from each cluster will be used for further genetic analysis and in an attempt to establish a molecular genetic taxonomy of Vibrio spp. Identification schemes for diagnostic use will be developed from differential characters identified in the taxonomic analysis.

NAG vibrio enterotoxin(s) will be purified and compared for structural, immunological and functional similarity to choleragen and E. coli LT. Preliminary determination of differences in subunit structure and activity between these enterotoxins will be carried out. The existence and role of slime layer material as an adherence factor will be examined. The role of plasmids in pathogenicity and antibiotic resistance will be examined. Screening procedures for plasmids will be developed. Curing experiments and transfer experiments on pathogenic or resistant strains found to harbor plasmids will be carried out.

This research will establish a rational taxonomy for this group of organisms which will yield valuable insight into its potential for pathogenicity and provide procedures for identifying its pathogenic members. It also represents another approach to a unified body of knowledge concerning enterotoxins and adherence as virulence factors in Gram-negative pathogens.

7) Reviews:

a) Research Involving Human Use _____

b) Research Committee _____

c) Director _____

d) BMRC _____

e) Controller-Administrator _____

SECTION II - RESEARCH PLAN

A. INTRODUCTION

1. Objective: The long range goals of the research are to characterize and distinguish pathogenic and non-pathogenic members of the V. cholera-like group of organisms and establish their taxonomic position; to define the disease process associated with pathogenic strains and its relation to cholera; and to develop an identification scheme for clinical laboratory use.

2. Background: Vibrio-like organisms that have similar biochemical reactions to those of Vibrio cholerae but which fail to agglutinate in Gardner and Venkatraman's O-group I antiserum have been variously identified as non-agglutinating (NAG) vibrios, choleriform vibrios (Pollitzer, 1959) and non-cholera vibrios (NCV; McIntyre et al., 1965). In general, these have been considered aquatic organisms and, indeed, their survival outside the intestine is very much greater than that of V. cholerae. Yajnik and Prasad (1954) have suggested that some members of this set might be pathogenic for man. Their association with outbreaks of diarrheal disease in the past has also been discussed by Pollitzer. In 1965, McIntyre, et al. reported 19 cases in which these

Organisms were predominant in stool specimens and in which no other pathogen could be detected. They further noted that six patients had a four-fold or greater serum antibody titer rise against their isolate.

The clinical features of diarrhea caused by these organisms range from rice-water stools to a mild gastroenteritis. In most cases, the volume of stool output is less than that seen in cholera and the disease is self-limiting (Carpenter et al., 1965: Lindenbaum et al., 1965; McIntyre et al., 1965; and Aldova et al., 1968). Biochemical and clinical analysis of stool and serum from patients shows no absolute differences between these diarrheas and cholera except that the former are sometimes associated with a reddish-brown stool not commonly seen in patients with cholera. The fact that these infections do not consistently give rise to a detectable immunological response also differentiate them from cholera (Sack et al., 1965).

Much of the variation in clinical observations and patients' serological response probably reflects differences in the isolates studied. As will be discussed below, this set of organisms has been poorly characterized,

particularly with respect to its potential pathogenicity. In some cases, the organisms isolated may not have been the agents of the disease at all. Present work at CRL, for example, indicates that NAG vibrios or NCV are sometimes isolated from cases of enterotoxigenic Escherichia coli diarrhea, an illness not recognized in the studies reported above. Thus, while we know that this set contains some pathogenic members, their identity and the etiology of the disease with which they are associated is largely unknown.

The taxonomy of the so-called NAG organisms has been investigated by several laboratories in the past decade. McIntyre and Feeley (1965) were able to show on the basis of decarboxylase and dihydrolase reactions that 13 of 82 strains thought to be non-cholera vibrios were in fact Aeromonas. Sakazaki et al. (1967), Colwell (1970), Charterjee et al. (1970) and Sil et al. (1975) followed with detailed taxonomic analyses which have clearly established that the set contains at least four clusters of strains: isolates phenetically identical to V. cholerae, isolates within the genus Vibrio but significantly different from V. cholerae (including some halophilic strains

such as V. parahaemolyticus), Aeromonas and Plesiomonas species. Vibrio parahaemolyticus were unequivocally distinguished from V. cholera-like vibrios in these studies. Genetic analysis by Citarella and Colwell (1970) demonstrated that a high degree of relatedness exists between V. cholerae and the isolates phenetically identical to V. cholerae. This relatedness was not shared by V. parahaemolyticus.

Diagnostic criteria for identifying NAG vibrios or NCV currently include indophenol oxidase (pos), gram negative asporogenous rods motile by means of a single polar flagellum, catalase (pos), fermenatation of glucose without the production of gas, no NaCl requirement, lysine and ornithine decarboxylase (pos) and arginine dihydrolase (neg), sensitive to O/129 vibriostatic compound (Shewan et al., 1954). The "string" test of Smith (1958) for vibrios was found by Sil et al. (1975) to be variable and incapable of distinguishing Aeromonas and Plesiomonas species. Classification within the set of NAG organisms on the basis of Heiberg sugar fermentation (sucrose, mannose and arabinose; Heiberg, 1936) is routinely practiced at many laboratories, including CRL, but its diagnostic

significance is extremely doubtful. According to Chatterjee (1974), the V. cholera-like vibrios from human and non-human sources fall into groups I, II, and V. There is some indication that the first two groups are more frequently associated with human isolates while group V more often contains isolates from non-human sources. But the overlap within groups is too great to use Heiberg grouping as a means of distinguishing human source isolates. In our laboratory, isolates in groups V and VII most often prove to be V. parahaemolyticus. This study also found that group III isolates were Aeromonas species and group VI isolates were all Plesimonas shigelloides, but again there is overlap into other groups. CRL in the past year has experienced a large number of cases in which group III organisms were isolated. These have proved to be organisms intermediate between Vibrio and Aeromonas. Aeromonas hydrophila and Plesimonas shigelloides have been implicated in outbreaks of diarrhea elsewhere (Chatterjee and Neogy, 1972; Bhat et al., 1974), though the former is generally considered to be a soil or water organism.

The current diagnostic schemes are usually capable of identifying particular groups within the set of NAG organisms, but cannot distinguish pathogenic or even human-associated strains from those which are free-living. It is not even known if such a distinction can actually be made in reality. Serotyping of the NAG vibrios by Sakazaki et al. in 1970 and since continued has established 75 serogroups so far. There is no reported link between pathogenic characters and serotype. In the past, Bhattacharjee and Bose (1964) claimed that the passage of NAG vibrios through the rabbit intestine engendered a serological conversion to O-group I. This work highlighted a discussion then current as to whether NAG vibrios were simply V. cholerae which had lost their antigenic determinant through environmental stress. Work in other laboratories (Gangarosa and Mosley, 1974) failed to confirm this observation, however. On molecular genetic grounds, as well, the hypothesis is given little credence at present.

There is a need for further characterization of this group aimed specifically at distinguishing its pathogenic members so that their virulence can be evaluated and methods of

identifying them developed. This will involve taxonomic analysis which is sufficiently sensitive to distinguish clusters at least to the biotype level. A taxonomy will be required which can assimilate information about clinical, biological and phenetic characters along the lines of the "polyphasic" taxonomy of Colwell (1968). Using computer-based methods of cluster analysis, such a taxonomy should be capable of determining if pathogenic subgroups do, in fact, exist and of revealing distinguishing features which would be useful as diagnostic tools.

The current trend in Vibrio taxonomy is to place the V. cholerae-like NAG vibrios into that species (Sakazaki et al., 1967; Colwell, 1970). While this trend is opposed by some on the basis of its impact on international health regulations (Finklestein, 1973), it seems to us to be a desirable one. In keeping with its spirit, we will refer to all such strains as V. cholerae NAG in recognition of the fact that their failure to agglutinate in O-group I antiserum is, thus far, the sole consistent differential character to be demonstrated. All other members of the original set of NAG organisms (including Aeromonas and Plesiomonas species) will be classed simply as "related organisms."

A second approach to an understanding of the role of V. cholerae NAG and related organisms in human disease is by defining their mechanisms of virulence. Sakazaki et al. (1967) demonstrated that ten isolates phenetically identical to V. cholerae were able to produce choleraic diarrhea in suckling rabbits, while strains identified as Aeromonas were not. Dutta et al. (1963) had earlier demonstrated the same phenomenon and had also noted the fact that a striking enhancement of pathogenicity occurred after passage of isolates through the suckling rabbit intestine. Using eight strains, they showed that challenge of 10^9 unpassaged cells per 100g body weight induced, at most, a mild diarrhea in only 15% of the rabbits, resulting in no deaths. After passage, however, the same strains elicited choleraic diarrhea in 55% of the animals challenged and the mortality rate was 50%. The challenge level was 10^6 per 100g body weight. No difference between NAG isolates from human sources and from water were noted.

Zinnaka and Carpenter (1972) and Ohashi et al. (1972) studied V. cholerae NAG isolated from outbreaks of human diarrhea and found that some strains were capable of eliciting fluid accumulation or diarrhea in rabbit experimental

models. They also found that some of these strains produced a permeability factor similar to that of V. cholerae (Craig, 1965). Both activities could be demonstrated in cell-free culture filtrates, though the levels, as with whole cultures, were much lower than seen with V. cholerae. The most significant finding in both studies is that skin activity of these filtrates could be neutralized by antiserum prepared against choleragen or choleragenoid (see Finklestein 973). The neutralization titration pattern, in particular, contains highly interesting implications. The results of Ohashi et al. clearly show that the neutralization titation of NAG toxin by anti-choleragen antiserum passes through two distinct inflection points before complete neutralization is achieved. Only the first inflection point is reached in the titation with anti-choleragenoid antiserum. We now know that choleragen is a bipartite molecule consisting of A and B subunits (Cutrecasa: et al., 1973; Finklestein et al., 1974; Holmgren and Lonnoth, 1975) and that choleragenoid is in fact the B subunit. It thus appears likely that the NAG toxin of Ohashi et al. shared partial antigenic identity with the B subunit of choleragen and complete identity with the A subunit (at least with respect to

neutralization). This implies to some extent that this NAG toxin may also have been a bipartite molecule with characteristics similar to choleragen. The titrations carried out by Zinnaka and Carpenter (1972), while not as clear cut as those of Ohashi et al., also demonstrate the same phenomenon. The possibility that the NAG vibrio and cholera enterotoxin resemble each other closely is not at all unlikely. It has already been demonstrated that other Gram negative pathogens such as E. coli produce enterotoxin which is immunologically and functionally similar to cholera toxin (Kolata, 1975). Within genus or species similarities would be expected, a priori, to be at least as great.

Zinnaka et al. (1973) further observed that the NAG vibrio toxin they studied and V. cholerae enterotoxin eluted at the same point from Sephadex columns. Zinnaka and Fukuyoshi (1974) then compared the two toxins for serological identity and for electrophoretic mobility. Reactions of identity on gel diffusion plates took place in only some of the combinations of antigen and antibody. They also found that the electrophoretic mobility of the NAG toxin was greater than that of the cholera toxin.

Though these data indicate that some differences do exist between the NAG and cholera toxins, the extent and nature of these differences remain unclear.

Reports by other investigators concerning biological activity of V. cholerae NAG are difficult to interpret because of limited characterization of the isolates studied or because enterotoxic or skin reactive activity is accompanied by toxic manifestations of extracellular proteases. This appeared in the work of Ohashi et al. (1972) as "hemorrahagic principle" in the rabbit skin assay, and has also been reported by Karolcek et al. (1976). This "principle" was observed in most strains which possessed skin reactivity but each activity was shown to be present in some strains lacking the other. Attempts at CRL to demonstrate enterotoxigenic activity in V. cholerae NAG culture filtrates using the Chinese hamster ovary (CHO) cell assay (Guerrant et al., 1974) have shown only a cytotoxic effect, presumably due to proteases. Such an effect could easily mask any enterotoxic activity present in the crude filtrate used.

No work has been reported, as yet, concerning adhesive properties or chemotactic responses of V. cholerae NAG

as virulence factors. The work of Jones et al. (1976) and Allweiss et al. (1977) on the nature of these properties in V. cholerae indicate that they are important determinants in the pathogenicity of cholera organisms. One adhesive factor in V. choleae, a hemagglutinin, is primarily a protein existing as a slime layer on the surface of the cell (Lankford and Legsomburana, 1965). It is likely that this factor is, in part, responsible for adherence of cells to other tissue surfaces, as well.

Plasmids play an important role in the pathogenic process of the Gram negative organisms. Control of enterotoxin production in E. coli is due to a transmissible plasmid (So et al., 1974) as is the production of K88 antigen, an adherence factor. R factor conferring multiple antibiotic resistance to strains possessing them have become the most serious problem currently in the treatment of certain Gram negative diseases.

Vibrio species have also been shown to harbor plasmids, though not to the extent as in E. coli. Sex factors, which mediate transfer of chromosomal genes, have been demonstrated in V. cholerae (Bhaskaran, 1960; Parker and

Romig, 1972; Dutta et al., 1973). R factors have also been found in V. cholerae (Prescott et al., 1968; Hedges and Jacob, 1975) and can be transferred to V. cholerae from Shigella and E. coli in the laboratory (Kuwahara et al., 1963; Yokota, 1970). Both naturally occurring or artificially transferred R factors tend to be fairly unstable. Instability of antibiotic resistance in V. cholerae, possibly due to plasmid loss, was noted in a recent global surveillance carried out by W. H. O. (O'Grady, et al., 1976).

Recombination studies by Vasil et al. (1975) have shown that the genetic determinants for cholera toxin are located on the chromosome. It is entirely possible, however, that enterotoxin production by V. cholerae NAG is controlled by plasmid DNA as in E. coli. If enterotoxin or other virulence factors in V. cholerae NAG are determined by plasmids, the likelihood of their detection may be influenced to some extent by the media commonly used for the isolation of Vibrio species. All selective media incorporate bile salts, an anionic surfactant which may act as a curing agent for plasmids. Such an effect has been noted on plasmids harbored by E. coli (Tomoeda et al.,

1968; Inuzuka et al., 1972) and Staphylococcus aureus (Sonstein and Baldwin, 1972) when these organisms were exposed to sodium dodecyl sulfate, another anionic surfactant. Too little, however, is known at present about the plasmids of Vibrio species to judge this possibility.

3. Rationale: The rationale behind the proposed approach is that a thorough, polyphasic taxonomic analysis of V. cholerae NAG and related organisms, which incorporates an appreciation of the important recent work on virulence factors and plasmids, will reveal stable and significant clusters of pathogenic members. Identification of these pathogens and an understanding of their disease process will result in better diagnostic procedures for their detection, and, perhaps, better methods of controlling or preventing the diseases they cause. The study of virulence factors in this group of organisms will also shed light from a new angle on the pathogenicity of allied and more clinically significant Gram negative pathogens.

B. SPECIFIC AIMS

The specific aims of this research are to:

1. Isolate V. cholerae NAG and related organisms from hospitalized pateints and aquatic sources using

procedures which minimize the likelihood of plasmid loss; characterize these isolates on the basis of traditional diagnostic criteria; and characterize the clinical picture associated with each presumptive pathogenic isolate, including its occurrence in mixed infections.

2. Screen for the presence of antibiotic resistance among the V. cholerae NAG and related organisms associated with human disease.

3. Using laboratory model systems, compare the pathogenic potential of organisms isolated from cases of human disease with those which appear to be free-living aquatic organisms; compare freshly isolated strains to those maintained in stock cultures.

4. Examine enough phenetic characters for each isolate to give a representative sample of the properties of the organisms.

5. Form clusters of isolates on the basis of phenetic characters and pathogenicity-related properties; assess

genetic relationships among groups so formed and further characterize them using centrotype strains; establish which characters are most useful in distinguishing pathogenic strains and develop diagnostic schemes for their identification.

6. Purify and characterize enterotoxins produced by the organisms under investigation; determine the degree of serological relatedness and similarity in mechanism of activity among them, and between them and choleragen or E. coli LT; compare the subunit composition of NAG enterotoxin with that of choleragen.

7. Assessthe role of slime layer as an adherence factor; characterize material responsible for adherence; and characterize the role of chemotaxis in the adherence process.

8. Develop the agarose gel electrophoresis analysis for plasmid DNA and use it to screen for plasmids in V. cholerae NAG and related organisms.

9. Assess, through curing and transfer experiments, the role of plasmids in determining pathogenicity or antibiotic resistance.

10. Determine the stability of pathogenic characters and antibiotic resistance in V. cholerae NAG and related organisms, especially in relation to the effect of standard Vibrio isolation procedures on their retention.

C. METHODS OF PROCEDURE

1. Characterization of isolates and associated illness, and selection of isolates for further study.

a. Isolation of presumptive pathogens:

Initial stool specimens will be obtained by rectal catheter from patients immediately after admission and examined by darkfield microscopy (Benenson et al., 1964) for the presence of vibrios. Positive cultures will be tested for inhibition of motility by Gardner and Venkatraman (1935) O Group I antiserum. Patients with non-agglutinating vibrios will be admitted into the study.

Admission stool samples will be homogenized in sterile physiological saline pH8.5, and appropriate dilutions (based on vibrio concentration estimated drom microscopic examination) will be spread on modified gelatine agar [GA'; Bacteriological Analytical Manual for Foods (BAM), 4th ed., AOAC, 1967; modified by deleting sodium taurochlorate and including sodium carbonate, 1g/l]. Viable plate count will be determined. Plates containing 100-200 colonies will be replica plated to a second GA' plate and to antibiotic-containing media for the isolation of antibiotic resistant vibrios (see below). The replica GA' plate will be compared to the plates containing antibiotic and only non-resistant colonies will be picked. Predominant colony types and those showing gelatinase activity will be tested by the "string" test (Smith, 1970; Neogy and Mukerji, 1970), and the oxidase test (BAM) and for agglutination in Vibrio O-Group I or "rough" antisera

One of each type of VLO (Vibrio-like organism) colony (i.e., "string"--and/or oxidase--positive, non-agglutinating will be selected for confirmatory testing. These will be streaked for purity to another plate of GA'.

Stool samples will also be streaked directly onto MacConkey agar, SS agar and xylose-lysine-deoxycholate (XLD) agar for detection of other bacterial enteric pathogens. Salmonella or Shigella will be confirmed by routine biochemical and serological tests. Escherichia coli isolates, in addition to routine biochemical confirmation, will be tested for the production of heat-labile toxin (LT) using the Chinese Hamster Ovary (CHO) assay (Guerrant et al., 1974) and for heat-stable toxin (ST) by the infant mouse assay (Dean et al., 1972). Five colonies will be picked and tested individually in each assay. Non-toxigenic colonies obtained from patients whose admission stools contain polys and are negative for Salmonella or Shigella will be pooled and tested for invasiveness by the Sereny test (Sereny, 1955).

Admission stool samples will be tested microscopically for ova and parasites. A portion of stool will be frozen and examined subsequently for the presence of rota-virus using the ELISA procedure which will be developed at the CRL in the coming year.

b. Isolations from aquatic sources:

Aquatic specimens (including plants, phyto-plankton, zoo-plankton, and bivalve molluscs) and surface water samples will be collected by hand or net and stored in sterile plastic containers on ice until processed. Samples will be collected from a wide variety of water sources in the immediate vacinity of Dacca and Matlab. Sources will be chosen from those which have little or no use by humans. Fresh and salt-water fish will be purchased from local markets. The fecal contents of these specimens will be collected aseptically and examined for the presence of vibrios.

All solid specimens will be homogenized in sterile physiological saline, pH 8.5. A portion of each homogenized or water sample (1g or 10ml, respectively) will be diluted to 100ml with alkaline peptone water (BAM, 1976) for enrichment. Since vibrios will be difficult to detect on non-selective media, an initial screening for their presence will be made by streaking six hour enrichment cultures onto taurochlorate-tellurite-~~glycine~~ gelatin agar (TTGA; Monsur, 1961). Enrichment cultures will then be chilled to 4°C and held pending

the results on TTGA. Enrichment cultures from which vibrio-like colonies are recovered will be streaked onto GA'. Predominant or gelatinase-positive colonies isolated on GA' will be tested as given above. Replica plating will not be done. Fifty to 75 strains will be isolated from aquatic sources.

c. Confirmatory tests:

A well-isolated colony from each purity streak will be characterized by the following tests, taken, in part, from the work of Hugh and Sakazaki (1972) (unless specifically noted, all procedures will follow those of BAM):

1) Gram reaction morphology of 12-16h trypticase soy agar growth.
2) Flagellation (Leifson, 1951).
3) Reaction in Kligler Iron Agar (BBL, Cockeysville, MD).
4) Growth characteristics in Motility-Indole-Urea medium (Hormaeche and Peluffo, 1959).
5) Oxidation-fermentation test of glucose in Hugh-Leifson's (note gas production).
6) Fermentation of sucrose, mannose, arabinose or mannitol in bromcresol purple broth within 7 days incubation.

7) Lysine and ornithine decarboxylase and arginine dihydrolase activity.

8) Sodium chloride tolerance or requirement tested by growth within two days in halophilism shake culture test with 0,3,7, and 10% NaCl.

9) Growth in peptone water at 42C.

10) Sensitivity to 0/129 vibriostatic compound (2,4-diamino 6,7 diisopropyl pteridine) using discs containing 10 and 150 mcg (Shewan et al., 1954).

Media used 5-7 and 9-10, above, will be adjusted to 3.0% NaCl when testing isolates shown to be halophilic. All ambiguous or non-characteristic results will be repeated. At the time isolated colonies are picked from purity plates for testing, they will be inoculated onto heart infusion agar slants. Growth will be washed into 2x concentrated skim milk and lyophilized for stock culture. Reference stock cultures will be maintained in sealed ampoules. "Working" stocks will be maintained under oil on heart infusion agar slants at 4C. Fresh cultures will be prepared by streaking from the stock onto a GA purity plate and selecting a single colony for study.

d. Patients and clinical data-

Patients will be entered into the study after informed consent is obtained by a Bengali-speaking physician (consent form attached, Appendix A).

Male patients admitted to the CRL hospital during the period September, 1977 to September 1978 will be included in the study. Female patients will be studied only if there is an insufficient number of male patients. The decision to admit a patient to the study will be made by the principal investigator or a specifically designated representative.

Upon admission, the subjects will be questioned using the standard hospital history form about the presence and severity of diarrhea, vomiting, abdominal cramps; muscle cramps, chills and urine output during the previous 24 hour period. They will also be asked about previous cholera vaccination and if they have taken any antibiotic medication prior to coming to the hospital. Patients will receive routine care by staff physicians and nurses. All patients will receive a physical examination at the time of admission, which will include a measure of weight. Discharge height and weight will be recorded and used to assess nutritional status according to

international standard (Jelliffe, 1966).

The following laboratory measurements will be made on admission to the study:

1) Stool will be collected by rectal catheter or by manual collection of whole stool and analyzed:
   a) Methylene blue stain for fecal leucocytes (Harris, et al., 1972)
   b) Electrolytes: Na^+, K^+, Cl^-, osmolality
2) When possible, blood will be drawn on admission (10cc) and during convalescence (5cc) by trained medical personnel and divided for:
   a) (admission only) Plasma-specific gravity, Hct, protein, BUN, Na^+, K^+, Cl^- HCO_3^- and WBC with differential
   b) Serology-acute and convalescent sera will be tested for agglutinating titer against V. cholera biotype el tor and against the organism isolated from the patient (McIntyre et al., 1965). Remaining sera will be frozen for later use in characterizing enterotoxins produced by NAG vibrios and related organisms.

All clinical biochemistry tests will follow the standard procedures used at CRL. Blood will not be drawn when

the staff physician on duty determines that such action may be detrimental to the patient. If necessary, acute blood specimens may be collected in part on subsequent days or delayed for several days.

All patients will receive intravenous fluid replacement using the standard `Dacca` solution for the first four hours after admission. They will then be placed on standard oral therapy. Fluid requirement will be calculated and recorded at four hour intervals on the basis of fluid lost in stool, vomitus and urine in the previous four hour period. Additional drinking water will be allowed ad libitum after the electrolyte solution has been consumed. A standard diet will be given starting 24 hours after hospitalization. If a patient fails to keep up with stool loss by drinking, or is vomiting, a nasogastric tube will be used. If clinical assessment indicates dehydration at the end of a four hour interval or if the patient's plasma specific gravity exceeds 1.025, intravenous fluid will be readministered.

Patients will receive no antibiotic medication except those 1) with grossly bloody stool and/or temperature over 101.0F or 2) who are found to have a mixed infection with Salmonella or Shigella along with vibrio. In these

two cases, appropriate antibiotic therapy will be instituted.

All patients will be kept in the hospital for 24 hours after diarrhea has ceased. This will be defined as the absence of liquid stool for two consecutive 4 hr. intervals. Patients will be brought back to the hospital 10-15 days after admission to draw a convalescent blood sample.

Subject records for this study will be assigned the identification number of the bacterial isolates. This will be a purely arbitrary coding scheme with no patient identifier included. A separate sheet indicating the patient number of each case will be maintained separately by the principal investigator.

In estimating the number of patients who will be needed, we have considered the following data: In the period September, 1976 to July, 1977, 751 cases of diarrhea at CRL were associated with isolation of NAG vibrios and similar organisms, including 534 cases associated with Heiberg Group III VLO.

The distribution of the rest was:

Heiberg Group	I	27 cases
`	II	60 `
`	V	86 `
`	VII	44 `

In a previous study on NAG diarrhea at CRL, an increase in serum agglutinating titer against the homologous organism was observed in 1 in 3 cases (McIntyre et al., 1965). All but one of the isolates studied proved to be in Group II (McIntyre and Feeley, 1965). In our observation of the Group III cases this year, no titer rise was observed in any of the cases studied.

We wish to isolate at least three representatives of each organism type which elicits a serum titer rise. Assuming that a titer rise will occur in every third patient infected with an `immunogenic` organism type, we must include at least 18 patients from whom paired sera is obtained to have a 90% probability of obtaining three sero-conversions. Organism types for which no sero-conversion is demonstrated in the first 18 cases will be assumed to be `non-immunogenic`. This will establish an arbitrary breakpoint at a 98% probability of detecting sero-conversion in illnesses in which the rate of conversion is 1 out of 5 cases or greater.

These figures assume that the cases include only those with unmixed infection. Cases of mixed infection will be included in the study as a separate category. Up to five cases of each organism type will be studied.

Since we will follow traditional diagnostic criteria during the course of these isolations, organism types will be defined primarily on the basis of Heiberg grouping. We expect a maximum of six classes of organism to be included for study (HG, I, II, III, V, VII and possibly other VLO). We can classify isolates in 48h. A final decision to keep a subject in the study will then be made. The total case requirement is estimated to be 150-200 for the period Sept. '77 to Sept. '78.

e) Selection of isolates for further study-

In addition to organisms isolated in this study, representatives of NAG vibrios and similar organisms will be sought from established culture collections elsewhere in the world. Strains from human cases of diarrhea will be sought from Dr. R. Sakazaki, NIH, Japan; Dr. Barua, WHO; Dr. J.V. Lee, Public Health Laboratories, U.K., and Dr. G.K. Morris, CDC. Preference will be given to isolates for which some clinical data on associated illness is available. Aquatic isolates will be obtained from the same sources and from Dr. R. Colwell, University of

Maryland, U.S.A. Past human and aquatic sources maintained in the CRL collection will also be included in the study. All strains obtained in this fashion will be subjected to confirmatory testing and stock culturing in the same manner as the fresh isolates.

The collection of isolates being built up will encompass six classes with regard to source.

A-Patients with a 4-fold titer rise or greater to single pathogen

B-Patients with less than 4-fold titer rise to single pathogen

C-Patients with mixed infection

D-Aquatic sources

E-Human-associated isolates from stock cultures

F-Water-associated isolates from stock cultures

The total number of isolates in each class will be approximately: A-30; B-100; C-50; D-50; E-100; F-100. A `core set` of isolates will be selected from these for studies on pathogenicity and taxonomy. The remaining strains will be used as a pool, if necessary, for testing specific points raised in these studies.

All isolates included in the core set will be gram-negative, asporogenous rods which are oxidase-positive, motile with a single polar flagellum, and which ferment

glucose without the production of gas. Strains will be scored on the following characteristics: gelatinase; string test reaction; indole; urease; fermentation of sucrose, mannose, arabinose and mannitol; lysine and ornithine decarboxylase; arginine dihydrolase; NaCl requirement; growth in 7 and 10% NaCl; growth at 42C; and sensitivity to 10 and 150 mcg discs of O/129. Within each class, clusters will be formed among isolates sharing all 17 characters. While, in theory, this could result in a large number of groups being formed, past data indicate that about 6-8 major clusters can be expected in each class of isolates. Up to 3 isolates representing each major cluster will be selected for the core set. Selection of representatives from available strains will be made so as to include the widest variety of source characteristics. Minor clusters of one or a few strains will be represented by a single isolate. Sorting of isolates into clusters will be facilitated by the use of punched cards and mechanical card sorting. It is expected that the core set will eventually contain approximately 150-180 representative isolates. To this will be added any antibiotic-resistant VLO discovered by the screening procedure discussed below and a small number of control strains (e.g. well characterized V cholerae biotypes el tor, cholerae, proteus and metchnikovii; as well as V. parahaemolyticus

and Aeromonas formicans.) The total number of isolates should be approximately 200.

The core set will be established by June-July, 1978. We expect that the isolates in classes E and F will be available for cluster formation by January 1978 and characterization of their biological activity in laboratory models will begin at that time.

2. Isolation of antibiotic resistant presumptive pathogens.

The initial GA' spread plate cultures from patients with non-agglutinating vibrios will be replica plated on GA' plates containing antibiotics to which resistance by V. cholerae has been demonstrated. These antibiotics and the approximate minimal inhibitory concentration (MIC) for susceptible organisms are: tetracycline, 4mcg/ml; chloramphenicol, 125mcg/ml; sulfisoxazole, 4mcg/ml; streptomycin, 6mcg/ml; streptomycin, 6mcg/ml; polymixin B, 50U/ml; cephalothin, 10mcg/ml (Kuwahara et al., 1967; Prescott et al., 1968; Lennette et al., 1974; O'Grady et al., 1976). Antibiotics which are poorly soluble in water will be first dissolved in an appropriate solvent (see O'Grady et al., 1976). Plates containing antibiotics will be kept no longer than 4 days.

Since vibrio plasmids have been reported to be extremely unstable (Yokota et al., 1972; Hedges and Jacob, 1975; O'Grady et al., 1976) attempts to isolate antibiotic resistant strains by replica plating will be accompanied by direct plating of stool on antibiotic-containing media. This back-up procedure will ensure that no resistant strains are missed due to a low frequency in the population and it will also indicate whether resistance plasmids are being lost due to the initial plating on media without antibiotics, as suggested by O'Grady et al.(1976).

Resistant colonies will be tested for `string` formation, oxidase and agglutination with O-group I antisera. The resistance pattern of colonies on replica plates will be determined by comparison. The pattern of those isolated from direct streak plates will be determined by subsequent multipoint inoculation of antibiotic containing GA'. One isolate of each VLO colony-type and resistance pattern will be streaked for purity, subjected to confirmatory tests and stock-cultured. All media used for carrying cultures destined to be stocked will contain the antibiotic on which the strain was isolated. When possible, cultures prepared from these stocks for future studies will also be carried in antibiotic-containing media. It is expected that the number of distinct antibiotic resistant isolates will be few enough that all

strains can be included directly in the core set. If this is not the case, selection will follow the formation of clusters and selection process described previously.

All isolates in the core set will be tested for antibiotic resistance using the standard Kirby-Baur disc diffusion method (Lennette et al., 1974). Mueller-Hinton agar plates will be flooded with an appropriate dilution of an overnight trypticase soy broth culture. Standard filter paper discs containing the agents under test will be applied to the surface and the plates incubated overnight. The disc contents (mcg) will be: ampicillin-10; chloramphenicol-30; furozolidine-300; sulfisoxazole-2.0; streptomycin-10; gentamycin-10; tetracycline-30; polymixin B-50U; cephalothin-30; kanamycin-30; and septrin-25. Known sensitive and resistant control strains will be included. Zone diameters will be measured with calipers in two directions at right angles and the readings averaged.

3. Screening isolates for biological activity in laboratory model systems-

The core set of isolates plus appropriate control strains will be screened for biological activity in the following laboratory model systems:

a. Infant rabbit-experimental infection will be induced in 10-day-old New Zealand White rabbits using the technique of Dutta and Habbu (1955). Erlenmeyer flasks containing trypticase soy broth +0.6% yeast extract (TSBYE; in a 1:5 medium to flask volume ratio) will receive a 1% (v/v) inoculum from overnight cultures of the test isolates started from stock. Cultures will be incubated at 32C for 6h on a reciprocal shaker at 120 cpm. Each culture will be diluted with an equal volume of fresh medium and 1.0ml will be injected at laparotomy into the small intestine of two rabbits under anaesthesia. After surgery, animals will be maintained by gastric feeding of 5.0% glucose + 0.45% NaCl initially, followed by whole milk. The time of onset of diarrhea and the time of death will be noted. All animals will be sacrificed when death is imminent or at 96hr in other cases. Autopsies will be performed and fluid accumulation in the small intestine and colon noted. Cultures on GA' will be prepared from the intestinal contents of every animal. An oxidase positive colony demonstrating the same gelatinase and `string` reactions as the original isolate will be picked and maintained on heart infusion agar slants. Pairs of rabbits will again be challenged with TSBYE cultures of these passaged strains for each isolate which failed to elicit diarrhea in the first series. This is in line with the findings

of Dutta et al. (1963) that passage increases the frequency of diarrheagenic activity among vibrio isolates.

b. Virulence test- The virulence of each strain will be determined in mice of 18-20g body weight. TSBYE subcultures prepared and diluted as above will be used. One-half ml of culture will be injected intraperitoneally into each of 3 mice. The animals will be observed for 72 hours and the occurrence of illness or death noted. Filter-sterilized undiluted culture fluid prepared from 18 hour cultures of all virulent strains will then be tested as above for the presence of extracellular toxin. Avirulent isolates will be retested using subcultures from passaged strains.

c. Rabbit ileal loop test- The ligated ileal loop model (De and Chatterjee, 1953) will be used to test for extracellular enterotoxic activity. TSBYE subcultures from stock will be used for strains which elicited diarrhea in the first series of infant rabbit tests; subcultures of passaged strains will also be used for the rest. Filter-sterilized culture fluids prepared from 18 hour shake flask cultures incubated as above will be injected into the ligated ileal loop in each of two rabbits for each isolate. One rabbit will be examined after 6 hours, the other after 18 hours for the presence

of fluid. The ratio of the volume of accumulated fluid to the length of the ligated segment will be recorded. The test will be repeated for all isolates which were diarrheagenic in the infant rabbit model but which fail to elicit fluid accumulation in the ligated loop.

The culture filtrates for this assay will also be tested for activity in the:

d. Chinese Hamster Ovary (CHO) assay (Guerrant et al., 1974).

e. Suckling mouse assay (Dean et al., 1972; Gianella, 1976); and

f. Rabbit vascular permeability factor assay (Craig, 1965).

The first two of these assays are run on a routine basis at CRL; the third is also a routine assay, though one not used consistently at present. The presence of hemorrhagic factor (Ohashi et al., 1972) in the latter assay will also be noted. A high level of expertise exists for all three assays, and there is no need for any modification of the procedures being followed currently.

g. Sereny test: Isolates from patients with dysentery-like symptoms and whose stool yields no Shigella or invasive E. coli will be tested for invasiveness in the Sereny test (Sereny, 1955).

h. Adherence assays: The potential for isolates to adhere to the intestinal mucosa will be assessed using three in vitro model systems which are known to be active receptors for V. cholerae--isolated rabbit brush border cells (Jones et al., 1976), human group O erythrocytes (Jones et al., 1976), and intact rabbit mucosal surfaces (Freter and Jones, 1976).

Brush border cells will be obtained from rabbit small intestine using the exact procedure of Jones et al. (1976). Essentially, epithelial cells will be released by physical manipulation after exposure to EDTA, then disrupted in an Elvejehm tissue grinder. Brush borders will be isolated and purified by alternate centrifiguation and washing with 5mm EDTA. Adhesion will be tested by exposing brush borders to bacterial suspensions in a modified Krebs-Ringer solution at a ratio of 1 brush border to 100 cells. Adherence of cells to brush borders will be determined by phase-contrast microscopy.

Hemagglutination assay will also follow that described in the above paper. Tests will be run in microtiter plates with 25 ml of bacterial suspension in modified Krebs-Ringer solution and an equal volume of 1% suspension of human group erythrocytes. Bacterial suspensions will be prepared from 16-18 hour TSB cultures. Erythrocytes will be obtained from the same group O donor (the principal investigator) throughout the study and will be used within 24 hours of preparation.

Tests for bacterial adhesion to intact rabbit mucosal tissue will follow the procedure detailed in Freter and Jones (1976). Identical-size slices of rabbit ileum bathed in modified Krebs-Ringer solution will be exposed to suspensions of the isolate under test (at a concentration of 1-5 x 10^5/ml). After exposure for 20 minutes at 37°C in an atmosphere of pure O_2 and being shaken gently, the slices will be washed twice by dipping them into sterile saline. Slices of ileum in 25 ml of cold TSB will be homogenized in a Virtis 45 homogenizer as will a portion of bathing fluid. Viable counts on homogenates will be made by spread plating appropriate dilutions (in sterile saline) on duplicate plates of TTGA.

All isolates will be run in duplicate in each of the tests covered in d-h above and triplicate determinations will be made in each test.

4. Characterizing the Phenotype of Isolates:

a. General methods: Fresh cultures of isolates will be prepared from "working" stock cultures by subculture onto GA' purity plates. Isolated colonies will be picked, inoculated into flasks of TSB (1:5 v/v) and incubated at 30°C on a reciprocal shaker (120 cpm) for 18 hours. Unless otherwise specified, standardized inocula will refer to identical volumes taken from these flasks.

Tests will be inoculated by the Lidwell phage-typing apparatus (Parker, 1972); Lovelace multipoint inoculator (Lovelace and Colwell, 1968) for Repli-dishes (Sneath and Steven, 1967); or a drop from a Pasteur pipette. Unless otherwise stated, tests will be carried out at 30±2°C.

Viable plate count determinations will be made using the microtiter dilution method of Fung and Kraft (1968)

followed by the spot plate enumeration technique of McKinney et al. (1959). TSA + 0.5% glucose plates will be used throughout.

b. Physiological tests: Temperature range of growth will be determined in tubes of TSB held in covered water baths. All tubes will be equilibrated at the test temperature then inoculated by Pasteur pipette. Each isolate will be tested at : 5, 15, 20, 30, 37, 40, 42, and 44±0.2°C. Visible turbidity after 24, 48, and 168 hours will be recorded.

Salt tolerance will be tested in tubes of TSB containing: 0, 3, 5, 7, 9, or 11% NaCl. Visible turbidity after 24, 48, or 168 hours will be recorded.

The pH range of growth will be tested in 0.05M phosphate-buffered TSB adjusted (by the addition of 0.1M NaCH or 0.1M HCl) to 4.0, 4.5, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, or 11.0±0.1 pH unit. The pH will be checked after autoclaving and readjusted if necessary.

The growth rate constant and yield of each isolate under nutrient limiting conditions will be determined. Flasks of TSB +0.2% yeast extract in 1:10, 1:100, and 1:1000

dilution will be used. Preliminary study of six strains will establish the relationship between turbidity and viable plate count in each medium. Test flasks will be inoculated with twice-washed suspensions (in saline) of TSB-grown cells. A starting concentration of $1\text{-}2\times10^6$/ml will be established. Growth will be monitored spectrophotometrically. A sample will be taken for viable plate count at the time culture turbidity plateaus. The overall growth rate, expressed as generation time, will be calculated.

Rate of growth in partially inhibiting concentrations of organic and inorganic acids will be determined using hydrochloric, sulfuric, acetic or lactic acids. Preliminary tests will be carried out with six strains to determine the highest concentration of each acid which supports the growth of all strains. Tests will be run in flasks of acidified TSB shaken at 120 cpm. Inoculum will be taken directly from TSB cultures. A starting concentration of $5\text{-}10\times10^6$/ml will be established. Growth will be monitored and the generation time will be calculated as above.

c. Resistance tests: Resistance to organic and inorganic acids will be assessed by observing the rate at which viability is lost during exposure. Preliminary tests will be carried out on six isolates to determine the lowest concentration of acetic, lactic, citric, hydrochloric or sulfuric acid which is lethal for all strains. All isolates will then be screened for rate of destruction at these concentrations. Tests will be run in flasks of physiological saline acidified with the appropriate acid. TSB-grown cells will be washed twice and suspended in saline, then inoculated to give a concentration of $1\text{-}2 \times 10^7$/ml. Flasks will be shaken at 60 cpm on a reciprocal shaker. Samples will be taken at 0, 0.25, 0.5, 1, 2, 4, and 8 hours and plated. The shape of the destruction curve will be classified and the overall decimal reduction rate calculated.

The rate of destruction at 50, 56, or $60 \pm 0.2^\circ$C will be determined in covered water baths. Flasks of physiological saline will be equilibrated at the test temperature before being inoculated to a concentration of $1\text{-}2 \times 10^7$/ml. Samples will be taken at 0, 10, 30, and 60 minutes and viable cells enumerated. The shape

of the destruction curve will be classified and the decimal reduction rate calculated.

The ability of the isolates to resist various concentrations of ox bile will be determined. Plates of TSA + 0.5% glucose containing 10, 20, 30, or 40% (w/v) ox bile will be inoculated by multipoint inoculator. The presence of growth after 24 and 48 hours will be recorded.

The inhibitory effects of neutral red, crystal violet and malachite green will be determined. Preliminary tests will be carried out as above to determine the range of dye concentrations which are inhibitory. All isolates will be tested at various concentrations within these ranges. Plates of TSA + 0.5% glucose incorporating the various concentrations of each dye will be inoculated by multipoint inoculators. The presence of growth after 24 and 48 hours will be recorded.

The effect of UV irradiation on viability will be determined. TSB-grown cells will be harvested and resuspended in physiological saline at a final cell density of $4\text{-}5 \times 10^4$/ml. One-tenth ml of this suspension will be spread evenly over the surface of 20 plates of TSA +5%

glucose. Duplicate plates will be irradiated by a UV light source of 254 nm under conditions which expose the surface of the plate to 40 mcW/cm^2/sec. Pairs of plates will be exposed for 1 to 256 seconds by doubling intervals. A control pair of plates will not be exposed. All plates will be incubated at 37°C in the dark immediately after exposure. Colonies will be counted after 24 hours and the decimal reduction time calculated.

d. Nutritional studies:

Utilization of various compounds as the sole carbon and energy source will be determined in Simmons ammonium agar base (Sakazaki et al., 1967). Utilization of nitrogenous compounds will be tested in the same medium with the ammonium salt omitted (Colwell, 1970). The ability of isolates to utilize succinic, malic, citric, or pyruvic acid; and B-alanine or L-phenylalane will be tested. TSB-grown cells will be washed twice and resuspended in the basal salt medium at a final concentration of 1-2x10^6/ml. This suspension will be used to inoculate (1% v/v) test media containing 0.25% w/v of organic acid or 0.1% w/v nitrogenous compound. Growth within 14 days will be noted.

Acid production from organic compounds will be determined using BCP agar (bromcresol purple broth +1.5% agar; Leininger, 1976) in Repli-plates. Isolates will be tested in media containing (1% w/v) arabinose, cellobiose, lactose, ONPG, mannose, sucrose, sorbitol, inositol, mannitol, inulin, salicin, xylose, d-tartrate, citrate, or mucate. The production of acid within seven days will be recorded.

e. Biochemical tests: Acetoin production from glucose in Sasagawa-Ikemura's VP agar (Sakazaki et al., 1967) will be determined by Barritt's method. Isolates will be inoculated by multipoint inoculator onto agar contained in Repli-dishes.

Acetion production from 2, 3-butanediol will be tested in two day old Repli-plate cultures using the medium of Schubert and Kexel (1964). Barrett's method of detection will be used.

The ability of isolates to hydrolyse aesculin to aesculetin and glucose will be tested by the method of Holding and Collier (1971).

The ability of isolates to deaminate acetamide, decompose tyrosine to melanin and decompose glycerol to dihydroxyacetone will be tested by the methods of Colwell (1964).

Reactions in litmus milk will be tested according to the method of Holding and Collier (1971).

f. Extracellular products: Amylase activity will be tested in TSA plates containing 0.2% w/v soluble starch. Isolates will be spot inoculated and incubated. Hydrolysis of starch will be detected by flooding the plates with a dilute iodine solution.

Hydrolysis of cellulose or chitin will be tested by the method of Holding and Collee (1971). The insoluble polymers will be incorporated into TSA and hydrolysis will be shown by clearing of the medium around each colony. Plates will be examined daily for up to 5 days for evidence of hydrolysis.

Pectinolytic activity will be demonstrated by the method of Holding and Collee (1971). A pectate gel will be prepared by layering a solution of pectin (pH 9.4) over a layer of mineral salts agar with added $CaCl_2$

at pH 5.0 in Repli-dishes. The gel will be examined for up to 5 days for evidence of liquefication.

Deoxyribonuclease activity will be tested by the plate method using DNase test medium (Speck, 1976). Plates will be inoculated and, after incubation, flooded with 1N HCl. Deoxyribonuclease activity is indicated by clearing around colonies.

Hyaluronidase activity will be tested by a modification of the ACRA test (Holding and Collee, 1971); penicillinase activity will be tested by the method of Moffett and Colwell, 1968; and neuraminadase will be detected by the method of Holding and Collee (1971).

g. Serological characterization: All isolates in the core set will be serotyped according to the standard reference systems established in the laboratories of Dr. R. Sakazaki, National Institute of Health, Japan, and Dr. H. L. Smith, Jefferson Medical College, Philadelphia, PA, USA.

Acid agglutination will be tested by mixing a saline suspension of TSB-grown cells (approximately 10^7/ml)

with an equal volume of citrate-phosphate buffer or lactic acid. pH values to be tested will range from 2.5 to 6.0 by increments of 0.5 pH units. After mixing, tubes will be incubated at 37°C for 18 hours. Agglutination at each pH will be recorded.

Isolates will be tested for agglutination by rabbit antiserum prepared against V. cholerae H antigen. Antiserum preparation and testing will be carried out according to the method of Sakazaki et al., (1970).

5. Polyphasic Taxonomy of V. cholerae NAG and Related Organisms: Results from phenetic tests, biological assays and clinical observations will be scored for analysis by computer. A two-state coding system will be employed throughout. When more than two grades of reaction are distinguishable, multistate additive coding will be used. Prior to inclusion, biological and clinical characters will be tested statistically (coefficients of correlation) and by observation to eliminate those which overlap. Clinical data will also be analyzed for the single and interactive effects of age, nutritional status, prior treatment and demonstrable pathogens on the clinical picture. This will involve four factor analysis of variance by computer. The clusters of clinical characters formed by the procedures discussed below will be used to define the clinical picture for this analysis.

Cluster analysis of characters in the three classes (phenetic, biological, clinical) will be carried out using the CLUSTAN 1B suite of programs at the University of Surrey (See Everett, 1974). Similarity coefficients which include (S_{SM}) and exclude (S_J) negative matches (Sneath and Sokal, 1973) will be calculated. Coefficients of vigor and pattern (Sneath, 1968) will be calculated for all matches, Cluster analysis will be carried out by the hierarchial techniques available with the CLUSTAN package. Clusters formed by each procedure will be cross-checked against those formed by other techniques. The stability and usefulness of clusters obtained will be tested by reanalyzing randomly selected subsets of entities or variables and comparing the results to the original. An ordination technique utilizing principal components analysis is also available with CLUSTAN and will be used to generate two-or-three dimensional plots of the data as an alternate means of recognizing clusters. The natural distribution of isolates from human or non-human sources will be observed in all sets of clusters formed. In particular, the degree to which human-associated or pathogenic isolates tend to form homogenous groups will be strongly emphasized. Once stable taxa have been defined by cluster analysis, schemes for the identification of clinically-important organisms will be developed. The primary consideration for differential weighting of characters will be their constancy within taxa. Among

characters of equal constancy, selection will be based on the ease and rapidity with which the test for each character can be conducted. If necessary, a probability-based diagnostic table will be generated using the data base available to calculate the likelihood of a correct or incorrect identification (Dybowski and Franklin, 1968).

Genetic characterization of clusters will be carried out on the isolate from each cluster which is the most typical actual strain (centrotype; Sneath, 1972). These will be determined by evaluating the taxonomic distance of each strain in a cluster from the `hypothetical mean organism` calculated for that cluster.

DNA will be extracted from TSB-grown cultures using Citarella and Colwell's (1970) modification of the method of Marmur (1961). Guanine plus cytosine (GC) content will be determined by dye buoyant density centrifugation using the method of Wohlhieter et al. (1964). DNA-DNA reassociation between centrotypes will be examined in the laboratory of Dr. R. Colwell, Univ. of Maryland, as part of an effort to develop a molecular genetic taxonomy of Vibrio species.

Proteases and alkaline phosphatases of centrotypes will be partially purified and compared for serological identity

by the methods of Hsieh and Liu (1970) as a further check on cluster stability and significance.

6. Characterization of enterotoxins:

Enterotoxin producing isolates will be selected so as to include at least one representative for each pattern of biological activity observed in the screening process.

Purified choleragen will be obtained from Shwartz-Mann (Rockville, MD). Choleragenoid will be prepared by formalizing choleragen in the presence of glycine using the method of Ohtomo et al. (1973), followed by chromatography on phosphocellulose (Mekalanos et al., 1977). Standard anti-choleragen antiserum will be requested from Dr. Carl Miller, NIH. It is hoped that E. coli LT can be obtained from sources outside CRL. If necessary, LT will be produced and purified according to the procedure of Evans et al. (1976). This will involve polymixin-release of toxin followed by purification on the affinity gel Affi-Gel 202 (Biorad Laboratories, Rockville Center, NY).

Biological assays of enterotoxic preparations will be carried out using the vascular permeability assay of Craig (1965) and the rabbit ileal loop titration of Kasai and Burrows (1966). We anticipate that these tests will be

generally applicable to all toxins studied. Should other assays, such as CHO or suckling mouse, prove acceptable, appropriate substitutions will be made.

Inactivation of biological activity of enterotoxin by gangliosides will be determined using ganglioside mixture Type II (Sigma) and gangliosides G_{T_1}, $G_{D_{1a}}$, G_{M1}, and G_{M2} (Supelco Inc.). G_{M1} and G_{M2} will be treated before use with V. cholerae neuraminadase (Calbiochem, B grade) using the method of Takeda et al. (1976). Differential inhibitory effects of G_{M1} on the activity of NAG enterotoxin, choleragen and LT will be compared, if indicated, by the procedure of Holmgren (1973).

Preliminary experiments will be carried out in shake flask culture to determine the best conditions for enterotoxin production, Syncase (Finklestein et al., 1970) and TRY (Richardson and Noftle, 1970) media will be tested with and without supplementation by yeast extract dialysate or selected amino acids. The effect of temperature, pH and shaking speed will also be observed. A full-scale search for optimum conditions for toxigenesis is not planned; investigation will stop when we establish a procedure which yields sufficient toxin for the proposed investigation.

Rabbit antiserum to crude toxic filtrates, purified NAG enterotoxin, choleragen and choleragenoid will be prepared as follows: An emulsion of 50mcg of protein dissolved in 0.25ml of 0.1M Tris, pH 7.0 plus 0.25ml of Freunds complete adjuvant will be injected intramuscularly into the left hind leg of a rabbit. A week later, an identical preparation will be injected into the right leg. After four more weeks, the same amount of toxin in incomplete adjuvant will be injected. The animal will be bled one week later. All protein measurements will be made by the method of Lowry et al. (1951) using lyophilized crude bovine serum albumin as standard.

Serological relationships among the NAG enterotoxins and between them and choleragen, choleragenoid and LT will be assessed by immunodiffusion and neutralization tests. I_gG from antisera will be purified by ammonium sulfate precipitation, washed over an Amicon PM-10 membrane, then adjusted to 100mg Lowry protein/ml in 0.9% saline preserved with 0.01% merthiolate. Antigenic cross-reactivity among the preparations will be demonstrated using standard Ouchterlony immunodiffusion of appropriate dilutions of toxins and antisera in 0.05M sodium phosphate, 0.9% NaCl (PBS), pH7.0 (Ouchterlony, 1968). Neutralization tests for vascular permeability activity will be carried out as given by Zinnaka and Carpenter (1972) except that toxins

to be tested will be adjusted to eight blueing doses (BD) per 0.1ml (Craig, 1966) rather than two. The same procedure, with appropriate modifications in dilution of toxins and antisera, will be used for testing neutralization of ileal loop activity. All dilutions for neutralization tests will be carried out in borate-gelatine-buffered saline. Neutralization by antisera prepared against homologous and heterologous NAG enterotoxin, and against choleragen and choleragenoid will be tested to establish whether the observations of Zinnaka and Carpenter (1972) and Ohashi *et al*. (1972) can be repeated with our preparations.

We expect that the enterotoxins under study will demonstrate at least partial antigenic identity with choleragen. We propose to purify NAG enterotoxin(s) by taking advantage of this identity and using affinity chromatography with anti-choleragen. This procedure has already been shown to be an efficient means of purifying *E. coli* LT (Dafni and Robbins, 1976). Separation will take place on a Pharmacia K15/30 column containing approximately 17 ml (bed volume). Of CN Br- activated Sepharose 4B to which is coupled rabbit anti-choleragen I_gG. The specifity of I_gG for NAG enterotoxin in the presence of other culture proteins will be tested by immunoelectrophoresis. If necessary, monospecific antibody will be prepared by affinity chromatography

using purified choleragen (Ohtomo et al., 1976). Coupling will be carried out using the procedure suggested by the manufacturer. Enterotoxic protein fractions obtained by ammonium sulfate precipitation will be applied to the column dissolved in 0.2M Tris/Hcl buffer, pH8.0 containing 0.5M NaCl. (Solution in the buffer will be accomplished by washing over an Amicon PM-10 membrane.) The column will be washed with two bed volumes of buffer. Toxin will then be eluted by lowering pH and increasing ionic strength (e.g. 0.2M glycine/HCl, pH 2.8 containing 1.0MNaCl). Optimum conditions for purification of toxin will have to be determined empirically. Non-bound protein and eluted toxin will be reestablished in 0.05M PBS, pH 7.0 by washing over UM-10 membranes. We anticipate that conditions needed to elute enterotoxin from its complex with antibody might also lead to dissociation of the toxin molecule (LoSpalluto and Finkelstein, 1972). If NAG enterotoxin is similar to choleragen in physical structure, reassociation should be possible but may yield material which is not identical to the original. A selective loss in rabbit skin activity in reassociated choleragen has been observed by Battacharjee and Mosley (1973). The problem, if it occurs, may be overcome by maintaining high concentrations (>1mg/ml) of toxin during the reassociation process (van Heyningen, 1976) and by gradual adjustment from dissociating conditions to those permitting reassociation.

Enterotoxic factors which fail to bind to anti-choleragen antibody will be purified using traditional methods of molecular sieve and ion-exchange chromatography. The method of Finkelstein and LoSpalluto (1970) will be used, if possible, because of its extremely gentle treatment of the toxins being purified. These procedures will also be used to further purify, if necessary, the toxin recovered after affinity chromatography.

Purity of toxins at each stage in purification will be monitored by immuno-diffusion and immunoelectrophoresis and by disc electrophoresis in polyacrylamide gels (PAGE; Davis, 1964). Rates of recovery will be determined by titration of biological activity and by determinationn of antigen concentration using rocket immunoelectrophoresis (Axelson et al., 1974).

Enterotoxins purified to homogenity will be characterized by isoelectric focussing in polyacrylamide gels using the procedure of Catsimpoulis in Brewer et al. (1974). Isoelectric points will be compared to those of choleragen. Antigenic relationships between NAG vibrio enterotoxins, choleragen, choleragenoid and LT will again be determined using purified preparations. The specific biological activity of each enterotoxin will be determined as well

as its sensitivity to antisera (both prepared and from patients) and gangliosides.

We assume that the most likely structure of NAG enterotoxin is a bipartite molecule consisting of A and B subunits analagous to choleragen and LT. To test this possibility and to compare the biological characteristics of NAG enterotoxin subunits with those of choleragen, the following series of experiments will be carried out.

Subunits of NAG enterotoxins will be characterized by SDS-polyacrylamide gel-electrophoresis (SDS-PAGE) using a modification of the method described by Weber and Osborn (1969). Samples of toxin will be treated with 1% SDS in 0.05M phosphate buffer with 8M urea (pH 7.2) at 45C for 50 min. Protein will be applied to gels and electrophoresis will be carried out in 0.1M NaH_2PO_4 -NaOH containing 0.1% SDS, pH7.2. The molecular weight of each subunit will be estimated by comparison to marker proteins of known molecular weight, including the subunits of choleragen.

Separation of subunits will be carried out by gel filtration on Sephadex G-75 equilibrated with either 0.1M glycine-HCl buffer (pH3.2) containing 6M urea or 0.1M proprionic acid buffer, pH4.0 (Ohtomo et al., 1976). NAG vibrio enterotoxin

or choleragen, dissolved in 0.1M Tris buffered saline, pH 8.0, will be treated with an equal volume of equilibration buffer at 37C for 1 hour then applied to the column. Fractions will be washed and concentrated over a UM-10 membrane using 0.1M Tris - 0.05M NaCl, pH8.0 buffer.

Reduction of subunits with 2-mercaptoethanol dithiothreitol and alkylation with iodoacetamide will also be carried out as described by Ohtomo *et al*. The toxicity of subunits will be determined in bioassays. Subunits will be characterized by immunodiffusion analysis and SDS-PAGE. Their relationship to bands seen with whole enterotoxin will be established. Subunits of NAG enterotoxin will also be compared with subunits of choleragen for identity. Reassociation between homologous subunits and between subunits from NAG enterotoxins and those from choleragen will be encouraged by direct mixing or by mixing under dissociating conditions followed by equilibration to conditions under which reassociation can occur. Reassociation will be monitored by chromatography in Sephadex G-75. Materials which successfully reassociate will be tested for alterations in biological activity and for sensitivity to antisera and gangliosides. They will also be subjected to Ouchterlony immunodiffusion to confirm the original or hybrid antigenic pattern.

7. Characterization of adherence factor(s):

The role of capsular material or slime layer in the adhesion of cells to gut mucosa will be studied using the adherence models previously described: brush border cells, slices of rabbit ileum, and hemagglutination. Hemagglutination activity will be used to monitor the extraction and partial purification of adherence factor(s). Strains to be studied will be selected from both adherent and non-adherent groups of isolates. Control strains will include both classical and el tor biotypes of V. cholerae.

Phase-contrast microscopy and dilute India ink wet mounts (Lankford and Legsomburana, 1965) will be used to screen for the presence of capules or slime layer. Production of cell-bound and free hemagglutinin and the physical appearance of cells will be determined for cultures grown in TSB and nutrient broth and on TSA plates with and without added glucose. The effect of temperature (25, 30, 37C), pH (6.0, 7.0, 8.0) and aeration (in broth cultures) on hemagglutinin production will be assessed.

Extraction of cell bound hemagglutinin will be based on the assumption that these are predominantly protein in character (Lankford and Legsomburana, 1965). Appropriate procedures

must be determined empirically, but will include acetate, citrate or mild urea extraction based on the procedures of Sutherland and Wilkinson (1971). If necessary, concomitant mild phenol or alkali treatment will be used to aid in recovery of hemagglutinin. Extraction will be monitored by determining recovery of hemagglutinin as well as residual hemagglutinating activity on cells after tretment. The effect of extraction on cell viability will be assessed using standard viable cell counts.

Extracted bound-hemagglutinin as well as soluble hemagglutinin found in sterile culture filtrates will be partially purified by ammonium sulfate precipitation and reprecipitation. Salt will be removed by washing over a PM-10 membrane. Hemagglutinin dissolved in 0.02M PBS, pH8.0 will be further purified by chromatography on Sephadex G-100. Fractions will be monitored for hemagglutinating activity, Lowry protein and carbohydrate (anthrone reagent; Herbert et al., 1971). Hemagglutinin-containing fractions will be pooled, concentrated over a PM-10 membrane and stored at -70C. The specific hemagglutinin titer will be determined.

Antisera to hemagglutinin will be prepared in rabbits by the method described previously. Neutralizing titer against the hemagglutinating activity of viable cells will be determined by the method of Jones and Freter (1976). In

similar fashion, the neutralizing effect of antisera on adhesion of cells to brush borders or slices of rabbit ileum will be assessed.

The ability of hemagglutinin to inhibit the binding of cells to mucosal tissue will be examined by incubating brush borders and slices of ileum with graded concentrations of hemagglutinin prior to exposing them to cell suspensions. Neutralization by antisera and inhibition by hemagglutinin will first be determined for the homologous strain. We will then examine cross-reactivity with other strains, including control strains. Antigenic relationships between hemagglutinins will be examined by immunodiffusion analysis in Ouchterlony plates.

Inhibition of adherence by pepsin digests of rabbit intestinal mucosa (PMS; Freter and Jones, 1976) and L-fucose and D-mannose (Jones and Freter, 1976) will be tested in the brush border and intestinal slice models using the exact procedures indicated in the references.

The chemotactic response of isolates to pieces of rabbit small intestine will be observed under a darkfield microscope using the technique of Allweiss et al. (1977). The effect of PMS on positive chemotactic responses and on bacterial

association with intestinal tissue will be observed using the method described in the same paper. In a similar manner, the effect of partially purified hemagglutinin will also be examined.

8. Modification of the agarose gel electrophoresis plasmid analysis.

The agarose gel electrophoretic method for the identification and characterization of plasmid DNA is a three part process: 1) preparation of crude bacterial lysates for electrophoresis; 2) agarose gel electrophoresis in vertical slab gel apparatus and 3) visualization of bands by exposure to black light and recording of results with special photographic equipment (Meyers et al., 1976). Most problems encountered by laboratories which have established this procedure involve setting up the vertical slab gel apparatus or the photographic equipment for data recording. Meyers et al. (1976), however, reported the successful use of horizontal slab gel electrophoresis especially for agarose gels of less than 0.7%. They also reported that their original procedure for visualizing bands required only a hand-held black light and millimeter ruler to record the results. We propose to use these equally sensitive, if less sophisticated, methods in developing

this plasmid assay at CRL.

Crude lysates will be prepared from overnight brain-heart infusion broth cultures using the (SDS)-salt preparation method described by Guerry et al. (1973). The Brij 58 lysis technique (Clewell and Helinski, 1969) will be tested as well for comparison. Agarose gel electrophoresis will be carried out as described by Meyers et al. (1976) except that a horozontal gel slab will be used. Bands of plasmid DNA will be visualized using a UV light (365 nm) transilluminator and their distance from the origin measured with a vernier calipers. E. coli K-12 strains containing plasmids of known molecular mass obtained from Dr. R. Colwell will be used as controls and markers. Detection of plasmids in Vibrio isolates will be compared to results in Dr. Colwell's laboratory for the same strains.

9. Assessing the role of plasmids in pathogenicity and antibiotic resistance-

Isolates in the core set will be sent to Dr. R. Colwell of the University of Maryland along with the results of biological assays and tests for antibiotic resistance. Isolates will be screened for the presence of plasmids using the agarose gel electrophoretic technique and dye buoyant density centrifugation. Pathogenic or antibiotic

resistant isolates containing plasmids will be further examined to determine whether the genes for resistance are extrachromosomal. These will be done through curing experiments and transfer experiments. Details of proposed methods are given in Dr. Colwell's grant application to the NIH, on file with the principal investigator.

10. Determining the stability of pathogenic characters or antibiotic resistance.

A significant difference between the pathogenic characters and antibiotic resistance of freshly isolated Vibrio species and those obtained from stock cultures may reflect the loss of plasmids with time or as a result of exposure to curing agents during culture. If such a difference is noted, we propose to study the effect of bile salt incorporated in the standard selective media for Vibrio species on the stability of pathogenic characters or antibiotic resistance. We will also observe the stability of these characters in stock culture over a one-year period.

Strains for study will be selected from among the fresh cultures isolated on nonselective media. The plasmid content of each will be determined using agarose gel electrophoresis. Isolates will be subcultured repeatedly

on GA', TTGA and thiosulfate-citrate-bile salts (TCBS) agar. Subculturing will be carried out at 24 hr intervals from `sweeps` taken from the area of confluent growth. Retention of resistance to antibiotic(s) will be tested by spot inoculating from ten isolated colonies onto GA' plates containing antibiotic. Tests for pathogenic characters will be limited to the best (i.e. simplest, least expensive and most rapid) assay for enterotoxic activity and for adhesive ability. These may vary between strains and will have to be selected empirically. For rapid assays such as CHO or hemagglutination, up to 10 colonies will be tested per subculture. For cumbersome assays such as the ileal loop test, only two colonies will be picked. Isolates from subcultures which have lost resistance or pathogenicity, and those which have retained them after 5 sub-cultures will be analyzed for plasmid complement. These will be compared to the complement in the original isolates.

Duplicate cultures of each isolate will also be made on heart-infusion-agar slants. These will be maintained at room temperature for a period of one-year. Subcultures onto GA' will be made after 1, 3, 6 and 12 months. Colonies will be tested for resistance or pathogenicity as given above.

11. Projected schedule of research:

It is anticipated that the isolation and preliminary characterization of strains from patients, aquatic sources, and stock cultures will be completed by July, 1978. Screening of antibiotic resistant isolates will finish at the same time. Characterization of biological activity in laboratory models can begin in January 1978 and should be completed by the end of the year. As they are obtained and characterized, strains will be sent to Dr. Colwell for genetic analysis and plasmid characterization. This work will continue at the University of Maryland for a three-year period. Development of the agarose gel electrophoresis analysis for plasmids will take place during the period Januar-June 1978. By this time, results at CRL can be compared to those obtained in Dr. Colwell's laboratory. In January, 1979, the research effort will be split. Phenetic characterization of strains will be carried out by Mr. Daniel at the University of Surrey, England. This, we expect, will occupy most of the year. The proposed studies on virulence factors and plasmids will be carried out during the same time period and extending to June 1980. Beginning at about January 1980, we anticipate that we will require a two-month period at the University of Surrey in order to carry out the computer-based analysis of the phenetic, clinical and biological data. The period from May 1980 through August 1980 will be reserved for analysis and writing of papers.

D. Significance:

The nature of the pathogenic organisms similar to V. cholerae has never been studied definitively. Nor has the disease process associated with these organisms been characterized. The direct importance of the proposed work is that the potential of these organisms as human pathogens in Bangladesh and elsewhere will be defined precisely. At the same time we will have developed diagnostic schemes for identifying them in a rational and consistent fashion so that further epidemiological or clinical studies can be carried out. Analysis of the survival-associated characters of V. cholerae NAG and related organisms may shed new light on factors which affect the maintenance and transmission of other water-borne diseases, especially cholera.

Since the V. cholerae NAG are so similar to V. cholerae yet produce a significantly different disease picture, the study of virulence factors is particularly important as a means of furthering the development of a unified body of knowledge concerning the enterotoxins and adherence patterns of Gram negative bacteria. It is not too much to expect that this research will serve as an additional way to an understanding of virulence in some of the more active Gram negative pathogens.

Finally, this work will help to clarify the taxonomic classification of Vibrio and vibrio-like organisms and lead to more natural and useful groupings in the future.

E. Facilities Required

1. Office space: Approximately 100 sq. ft. will be needed for the principal investigator and two other persons (shared time). The space currently used in Rm. 111 will be adequate for the time being. Desk space will also be needed for the clerk-typist required for part-time work under this protocol. Space will be required for three years.

2. Laboratory space: Approximately 250 sq. ft. of laboratory space will be required with 50 running feet of bench space in one room. Access to the microscope bay and tissue culture room will be required in the second and third year.

3. Hospital resources: Approximately 700 inpatient days will be required during the first year. There is no required special location for these patients.

4. Animal Resources: The following animals will be required:

Rabbit, New Zealand White	3kg	25 maintained from Jul78-Dec79
	8-10d	15 per wk for Jan-Dec 78
		10 per mo. for Jan79-May80
	1-2 kg	7 per week for Jan-Dec79
		4 per week for Jan79-May80
White mice	18-20g	30 per week for Jan-Dec78
		30 per mo for Jan79-May80
	2-3 d	40 per week for Jan-Dec78
		40 per mo for Jan79-May80
Guinea pig	adult	50 per year for 1978

5. Logistical support:

	Year 1	2	3
Automobile transportation in Dacca	2500mi	500mi	500mi
Return trips to Matlab	10	--	--
Speedboat transport in Matlab	10hr	--	--

6. Major items of equipment:

a. On-hand

Refrigerator, general purpose (2)
Freezer, -70C Revco (1)
Centrifuge, refrigerated Sorvall RC-2B (1)
Air conditioners (2)
Shaker, water bath (2)
Incubator, 37C, walk-in (1)
pH meter, Corning Model 7 (1)
Balance, top-loading, Mettler (1)
Microscopes: phase-contrast, darkfield (2)
Calculator, TI SR-56 (1)
Shaker, large (1)
Stationary water bath (2)

b. Access needed

Camera, Nikon
Cold room - will require approximately one-half the floor space in ground floor cold room for period Jan 1979 - May 1980.
Spectrophotometers
Immunoelectrophoresis punch set

c. To be purchased

Colony counter
Electrophoresis apparatus and accessories
Fraction collectors and monitor for column shromatography
Ultrafiltration equipment
Virtis homogenizer

7. Specialized requirements: None

F. Collaborative arrangements

One of the ivestigators, Mr. R. Daniel, is the recipient of a Wellcome fellowship. The taxonomic analysis incorporated into this protocol represents his proposed doctoral research project. He will also participate in the initial phase of this research, the clinical and biological characterization of isolates, which will be done at CRL. Most of the phenetic characterization and computer-based numerical taxonomy will then be carried out by him at the University of Surrey, England. His sponsor in England will be Dr. J.E. Smith, Professor, University of Surrey. All of Mr. Daniel's travel and personal expenses while in Dacca will be carried by his fellowship. CRL will provide laboratory facilities, equipment and supplies while he is here. It is expected that the principal investigator will travel to the U. of Surrey early in 1980 in order to participate in the data analysis scheduled at that time.

It is anticipated that Dr. R. Colwell, Professor of Microbiology at the University of Maryland will carry out molecular genetic analysis on the isolates under study. This will involve primarily the identification and characterization of plasmids followed by curing experiments and transfer experiments to assess their role in antibiotic resistance and pathogenicity. Dr. Colwell will also study DNA-DNA

reassociation using centrotype strains as part of an effort to develop a molecular genetic basis for Vibrio taxonomy. All aspects of this work are covered by an application for funding at the NIH. Thus far, no word has been received as to whether this application has been approved, so these arrangements must be considered tentative. If approval is not given, the portion of the research dealing with plasmids will be proposed in modified and reduced form as a CRL project to be taken up at a later date. The collaborative arrangement for this work calls for shared costs of shipping data and specimens and provides overseas travel funds for CRL personnel out of Dr. Colwell's grant.

LITERATURE CITED

Aldova, E., K. Lazonikova, E. Stepankova, and J. Lietova. 1968. Isolation of non-agglutinable vibrics from an enteritis outbreak in Czechoslovakia. J. Inf. Dis. 118:25-31.

Axelson, N.H., J. Kroll and B. Weeke. 1973. A Manual of quantitative immunoelectrophoresis. Universetats vorlaget, Oslo.

Battacharjee, A.K. and W.H. Mosley. 1973. Selective destruction of skin permeability activity in cholera toxin: Effect of acid on cholera enterotoxin. Infect. Immunity 8:133-136.

Benenson, A.S., M.R. Islam and W.B. Greenough III. 1964. Rapid identification of Vibrio cholerae by darkfield microscopy. Bull. W.H.O. 30:827-831.

Bhaskaran, K. 1960. Recombination between mutant stocks of Vibrio cholerae, strain 162. J. Gen. Microbiol. 23:47-54.

Bhat, P., S. Shanthakumair and D. Rajan. 1975. The characterization and significance of Plesiomonas shigelloides and Aeromonas hydrophila isolated from an epidemic of diarrhea. Indian J. Med. Res. 62:1051-1060.

Bhattacharji, L.M. and B. Bose. 1964. Field and laboratory studies on the transformation of the V. cholerae in the maintenance of cholera endemicity: A preliminary report. Indian J. Med. Res. 52:771-776.

Brewer, J.M., A.J. Pesce and R.B. Ashworth. 1974. Experimental techniques in biochemistry. Prentice-Hall, Inc. Englewood Cliffs, NJ

Carpenter, C.C., P.P. Mitra and R.B. Sack. 1965. True cholera versus non-choleraic diarrhea: A clinical and behavioral comparison. Bull. Calcutta School Trop. Med. 13:8-10.

Chatterjee, B.D. 1974. Present status of Heiberg groups for classifying cholera-like organisms. Indian J. Med. Res. 62:479-483.

Chatterjee, B.D. and K.N. Neogy. 1972. Studies on Aeromonas and Plesiomonas species isolated from cases of choleraic diarrhea. Indian J. Med. Res. 60:520-524.

Citarella, R.V. and R.R. Colwell. 1970. Polyphasic taxonomy of the genus Vibrio: Polynucleotide sequence relationships among selected Vibrio species. J. Bacteriol. 104:434-442.

Clewell, D.B. and D.R. Helinski. 1969. Supercoiled circular DNA-protein complex in Escherichia coli: purification and induced conversion to an open circular form. Proc. Nat. Acad. Sci. U.S.A. 62:1159-1166.

Colwell, R.R. 1964. A study of features used in the diagnosis of Pseudomonas aeruginosa. J. Gen. Microbiol. 37:181-194.

Colwell, R.R. 1968. Polyphasic taxonomy of bacteria. Symposium on Taxonomic Studies of Microorganisms by Instrumental Analysis of their Components and Metabolites. International Conference of Culture Collections, Tokyo, Japan. ICRO/UNESCO. University Park Press, Baltimore, Md.

Collwell, R.R. 1970. Polyphasic taxonomy of the Genus Vibrio: Numerical taxonomy of Vibrio cholerae, Vibrio parahaemolyticus and related Vibrio spp. J. Bacteriol. 104:410-433.

Craig, J.P. 1966. Preparation of the vascular permeability factor of Vibrio cholerae. J. Bacteriol. 92:793-798.

Craig, J.P. 1965. A permeability factor (toxin) found in cholera stools and culture filtrates and its neutralization by convalescent cholera sera. Nature (Lond.) 207:614-616.

Cuatrecasas, P., I. Parikh and M.D. Hollenberg. 1973. Affinity chromatography and structural analysis of Vibrio cholerae enterotoxin. Biochemistry 12:4253-4264.

Dafni, Z. and J.B. Robbins. 1976. Purification of heat-labile enterotoxin from Escherichia coli 078:H11 by affinity chromatography with antiserum to Vibrio cholerae toxin. J. Infect. Dis. 133 (Suppl.) S138-S141.

Datta, A., C.D. Parker, J.A. Wohlheiter and L.S. Bacon. 1972. Isolation and characterization of the fertility factor P of Vibrio cholerae. J. Bacteriol. 113:763-771.

Davis, B.J. 1964. Disc Electrophoresis. II. Method and application to human serum proteins. N.Y. Acad. Annals 121:404-424.

De, S.N. and D.N. Chatterjee. 1953. An experimental study of action of Vibrio cholerae on the intestinal mucous membrane. J. Path. Bact. 66:559-562.

Dean, A.G., Y-C. Ching, R.G. Williams and L.B. Harden. 1972. Test for Escherichia coli enterotoxin using infant mice: application in a study of diarrhea in children in Honolulu. J. Inf. Dis. 125:407-411.

Dybowski, W. and D.A. Franklin. 1968. Conditional probability and the identification of bacteria: a pilot study. J. Gen. Microbiol. 54:215-229.

Dutta, N.K. and M.K. Habbu. 1955. Experimental cholera in infant rabbits: A method for chemotherapeutic investigation. Brit. J. Pharmacol. and Chemother. 10:153-159.

Dutta, N.K., M.V. Panse and H.I. Jhala. 1963. Choleragenic property of certain strains of el tor, non-agglutinable and water vibrios confirmed experimentally. Brit. Med. J. 1:1200-1203.

Evans, D.J., D.G. Evans, S.H. Richardson and S.L Gorbach. 1976. Purification of the polymixin-released, heat-labile enterotoxin of Escherichia coli. J. Infect. Dis. 133(Suppl):S97-S107.

Everitt, B. 1974. Cluster analysis. Heinemann Educational Books, Ltd., London.

Finklestein, R.A. M. Boesman, S.H. Neoh, M.K. LaRue and R. Delaney. 1974. Dissociation and recombination of the subunits of the cholera enterotoxin. J. Immunol. 107: 1043-1051

Finklestein, R.A., K. Fujita and J.J. LoSpalluto. 1971. Procholeragenoid: an aggregated intermediate in the formation of choleragenoid. J. Immunol. 107:1043-1051.

Finklestein, R.A., H.T. Norris and N.K. Dutta. 1964. Pathogenesis of experimental cholera in infant rabbits. I. Observations on the intra-intestinal infection and experimental cholera produced with cell-free products. J. Infect. Dis. 114:203-216.

Freter, R. and G.W. Jones. 1976. Adhesive properties of Vibrio cholerae: Nature of the interaction with intact mucosal surfaces. Infect. Immunity 14:246-256.

Fung, D.Y.C. and A.A. Kraft. 1968. Microtiter method for the evaluation of viable cells in bacterial cultures. Appl. Microbiol. 16:1036-1039.

Gangarosa, E.J. and W.H. Mosley. 1974. Epidemiology and surveillence of cholera. In Cholera. ed. Barua, D. and W. Burrows, W.B. Saunders Co., Philadelphia, Pa.

Gardner, A.D. and K.V. Venkatraman. 1935. The antigen of the cholera group of vibrios J. Hyg. 35:262-282.

Gianella, R. 1976. Suckling mouse model for detection of heat-stable Escherichia coli enterotoxin: Characteristics of the model. Infect. Immunity 14:95-99.

Guerrant, R.L., L.L. Brinton, T.C. Schnaitman and L.I. Gilman. 1974. Cyclic adenosine monophosphate and alteration of Chinese hamster ovary cell morphology: a rapid, sensitive, in vitro assay forthe enterotoxin of Vibrio cholerae and Escherichia coli. Infect. Immunity 10:320-322.

Guerry, P., D.J. LaBlanc and S. Falkow. 1973. General method for the isolation of plasmid deoxyribonucleic acid. J. Bacteriol. 116:1064-1066.

Harris, J.C., H.L.Dupont and R.B.Hornick. 1972. Fecal leucocytes in diarrheal illness. Am. Int. Med. 76:697-703.

Hedges, R.W. and A. E. Jacob. 1975. A 98 megadalton R factor of compatability group C in a Vibrio cholerae El tor isolate from southern U.S.S.R. J. Gen. Microbiol. 89:383-386.

Heiberg, B. 1936. The biochemical reaction of vibrios. J. Hyg. (Lond.) 36:114-117.

Herbert, D.. P.J. Phipps and R.E. Strange. 1971. Chemical analysis of microbial cells. In Methods in Microbiology Vol. 5B. ed. J.R..Norris and D.W. Fibbons. Academic Press, London and New York.

Holding, A.J. and J. G. Collee. 1971. Routine biochemical tests. In Methods in Microbiology. Vol 6A. ed. J.R. Norris and D.W. Fibbons. Academic Press. London and New York.

Holmgren, J. 1973. Comparison of the tissue receptors for Vibrio cholerae and Escherichia coli by means of ganglioside and natural cholera toxoid. Infect. Immunity. 8:851-859.

Holmgren, J. and I. Lönnroth. 1975. Oligomeric structure of cholera toxin: characterization of the H and L subunits. J. Gen. Microbiol. 86:49-65.

Hormaeche, E. and C.A. Peluffo. 1959. Laboratory diagnosis of Shigella Salmonella infections. Bull. W.H.O. 21:244-277.

Hsieh, H-c., and P.V. Liu. 1970. Serological identities of proteases and alkaline phosphatases of the so-called non-agglutinable (NAG) vibrios and those of Vibrio cholerae. J. Infect. Dis. 121:251-259.

Hugh, R. and R. Sakazaki. 1962. Minimal numbers of characters for the identification of Vibrio species, Vibrio cholerae and Vibrio para-haemolyticus. Public Health Lab. 30:133-137.

Inuzaka, N., S. Nakamura, M. Inuzaka, and M. Tomoeda. 1969. Specific action of SDS on the sex factor of E. coli K-12 Hfr strains. J. Bacteriol. 100:822-835.

Jelliffe, D.B. 1966. The assessment of nutritional status of the community. W.H.O. Monograph Series no. 53.

Jones, G.W., G.D. Abrams and R. Freter. 1976. Adhesive properties of Vibrio cholerae: Adhesion of isolated rabbit brush border membranes and hemagglutinating activity. Infect. Immunity.14:232-239.

Jones, G.W. and R Freter. 1976. Adhesive properties of Vibrio cholerae: Nature of the interaction with isolated rabbit brush border membranes and human erythrocytes. Infect. Immunity 14:240-245.

Karolcek, J., M. Draskovicova and I. Cizvar. 1976. Toxin properties of filtrates of Nag vibrio cultures. (In Czech) Co. Epid. 23:227-235. (Excerpta Medica Microbiol. 29:129).

Kasai, G.J. and W. Burrows. 1966. The titration of cholera toxin and antitoxin in the rabbit ileal loop. J. Infect. Dis. 116:606-614.

Kolata, G.B. 1975. Intracellular bacterial toxins: Origins and effects. Science 190:969-970.

Kuwahara, S., T. Akiba, K. Koyana and T. Asai. 1963. Transmission of multiple drug-resistance from Shigella flexneri to Vibrio comma. Japan J. Microbiol. 7:61-67.

Kuwahara, S., S. Goto, M. Kimura and N. Abe. 1967. Drug sensitivity of El tor vibrio strains isolated in the Philippines in 1964 and 1965. Bull. W.H.O. 37:763-771.

Lankford, C.E. and V. Legsomburana. 1965. Virulence factors of choleragenic vibrios. In Proceedings of the Cholera Research Symposium, January 24-29, 1965, Honolulu, Hawaii: U.S. Public Health Science Publications No. 1238, U.S. Govt. Printing Office, Washington, D.C. pp. 109-120.

Leifson, E. 1951. Staining, shape and arrangement of bacterial flagella. J. Bacteriol. 62:377-389.

Leininger, H.V. 1976. Equipment, media, reagents, routine tests and stains. In M. L. Speck (ed.) Compendium of methods for the microbiological examination of foods. APHA.

Lennette, E.H., E.H. Spaulding and J.H. Truant. eds. Manual of Clinical Microbiology. 2nd ed. 1974. Amer. Soc. Microbiology. Washington, D.C.

Lewis, A.C. and S.H. Richardson. 1973. Properties of cholera enterotoxin from chemically defined media. Proc. of the Ninth Joint Conference of the U.S.-Japan Cooperative Medical Science Program. pp 82-92.

Lindenbaum, J.W., W.B. Greenough III, A.S. Benenson, R.O. Oseasohn, S. Rizvi, and A. Saad. 1965. Nonvibrio cholera. Lancet 1:1081-1083.

LoSpalluto, J.J. and R. A. Finklestein. 1972. Chemical and physical properties of cholera exo-enterotoxin (choleragen) and its spontaneously formed toxoid (choleragenoid) Biochem. Biophys. Acta 257:158-166.

Lowry, O.H., N.J. Rosebough, A.L. Farr and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

Lovelace, T.E. and R.R. Colwell. 1968. A multipoint inoculator for Petri dishes. Appl. Microbiol. 16:944-945.

McIntyre, O.R., and J.C. Feeley. 1965. Characteristics of non-cholera vibrios isolated from cases of human diarrhea. Bull. W.H.O. 32:627-632.

McIntyre, O.R., J.C. Feeley, W.B. Greenough III, A.S. Benenson, S.I. Hassan and A. Saad. 1965. Diarrhea caused by non-cholera vibrios. Am. J. Trop. Med. Hyg. 14:412-418.

McKinney, R.E., H.E. Langley and H.D. Tomlinson. 1959. Survival of Salmonella typhosa during anaerobic digestion. Sewage and Ind. Waste 30:1469.

Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. 5:109-118.

Mekalanos, J.J., R.J. Collier and W.R. Romig. 1977. Simple method of purifying choleragenoid, the natural toxoid of Vibrio cholerae. Infect. Immunity 16 789-795.

Meyer, J.A., S. Sanchez, L.P. Elwell and S. Falkow., 1976. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. 127:1529-1537.

Moffett, M., and R.R. Colwell. 1968. Adansonian analysis of the Rhizobiaceae. J. Gen. Microbiol. 51:245-266.

Monsur, K.A. 1961. A highly selective gelatin-taurocholate tellurite medium for the isolation of Vibrio cholerae Trans. Royal Soc. Trop. Med. Hyg. 55:440-442.

Neogy, K.M. and A.C. Mukerji. 1970. A study of the"string" test in Vibrio Identification. Bull. W.H.O. 42:638-641.

O'Grady, F., M.J. Lewis and N.J. Pearson. 1976. Global surveillence of antibiotic sensitivity of Vibrio cholerae. Bull. W.H.O. 54:181-186.

Ohashi, M., T. Shimada and H. Fukumi. 1972. In vitro production of enterotoxin and hemorrhagic principle by Vibrio cholerae, NAG. Jap. J. Med. Sci. Biol. 25:179-194.

Ohtomo, N., T. Murooka, H. Inoue, H. Sasaoka and H. Takahashi. 1973. Preparation of cholera toxin and immunization studies with cholera toxoid. In Proceedings of the Ninth Joint Conference, U.S. Japan Cooperative Medical Science Program, Cholera Panel, Grand Canyon, Arizona, October 1-3, 1973. U.S. Dept of State Publ. No. 8762. pp 132-142.

Ohtomo, N., T. Murooka, A.T. Tashiro, Y. Zinnaka and K. Amako. 1976. Size and structure of the cholera toxin molecule and its subunits. J. Infect. Dis. 133 (Suppl):S31-S40.

Ouchterlony, O. 1968. Handbook of immunodiffusion and immunoelectrophoresis. Ann Arbor Science Publishers, Ann Arbor, Mich.

Parker, C. and W.R. Romig. 1972. Self transfer and genetic recombination mediated by P, the sex factor of Vibrio cholerae. J. Bacteriol. 112: 707-714.

Parker, M.T. 1972. Phage typing of Staphylococcus aureus. In Methods in Microbiology Vol. 7B. ed. J.R. Norris and D.W. Ribbons. Academic Press, London and New York.

Prescott, L.M., A. Datta and G.C. Datta. 1968. Patterns of in vitro sensitivity in Calcutta strains of Vibrio cholerae. Bull. W.H.O. 39:967-970.

Prescott, L.M., A. Datta and G.C. Datta. 1968a. R factors in Calcutta strains of Vibrio cholerae and members of the enterobacteriaceae. Bull. W.H.O. 39:971-973.

Pollitzer, R. 1959. Cholera. World Health Organization Monograph No. 43, Geneva

Richardson, S.H. and K.A. Noftle. 1970. Purification and preparation of permeability-cholera toxin from complex and synthetic media. J. Infect. Dis. 121(Suppl):S73-S79.

Sack, R.B., D. Barua, R. Saxena and C.C.J. Carpenter. 1966. Vibriocidal and agglutinating antibody patterns in cholera patients. J. Infect. Dis. 116:630-640.

Sakazaki, R.G., C.Z. Gomez and M. Sebald. 1967. Taxonomical studies of the so-called NAG vibrios. Japan. J. Med. Sci. Biol. 20:265-280.

Sakazaki, R., K. Tamura, C.Z. Gomez and R. Sen. 1970. Serological studies on the cholera group of vibrios. Japan. J. Med. Sci. Biol. 23:13-22.

Sasagawa, I. and K. Ikamura. 1966. On the V.P. test by a new medium. Modern Media 12:129-135

Schubert, R.H.W. and G. Kexel. 1964. Der Ausfall der Butandiol hydrogenase-reaktion bei einigen Pseudomonodaceen und Vibrionen. Zbl. Bakteriol. Parasitenk. Abt. Orig. 194:130-132.

Sereny, B. 1955. Experimental shigella conjunctivitis: a preliminary report. Acta Microbiol. Acad. Sci. Hung. 2:293-296.

Shewan, J.M., W. Hodgkiss and J. Liston. 1954. A method for the rapid differentiation of certain non-pathogenic asporogenous bacilli. Nature 123:208-209.

Smith, H.L. 1970. A presumptive test for vibrios: the `string` test. Bull. W.H.O. 42:817-818.

Sneath, P.H.A. 1968. Vigour and pattern in taxonomy. J. Gen. Microbiol. 54:1-11.

Sneath, P.H.A.. 1972. Computer taxonomy. In ed. J.R. Norris and D.W. Ribbons. Methods in Microbiology. Vol 7A. Academic Press, London and New York.

Sneath, P.H.A. and R.R. Sokal. 1973. Numerical taxonomy. W.H. Freeman and Co., San Francisco, CA.

So, M., J.F. Crandall, J.H. Ciosa and S. Falkow. 1974. Extrachromosomal determinants which contribute to bacterial pathogenicity. In Microbiology-1974. ed. D.S. Schlessinger. Amer. Soc. Microbiology, Washington, D.C.

Sneath, P.H.A. and M.A. Steven. 1967. A divided Petri dish for use with multipoint inoculators. Appl. Bacteriol. 30:493-497.

Sonstein, S.A. and J.N. Baldwin. 1972. Loss of penicillinase plasmid on the treatment of Staphylococcus aureus with SDS. J. Bacteriol. 109:262-265.

Takeda, Y., T. Takeda, T. Honda, T. Miwatani. 1976. Inactivation of the thermostable direct hemolysin of Vibrio parahaemolyticus by ganglioside G_{T1}. Infect. Immunity 14:1-5.

Tomoeda, M., M. Inuzaka, N. Kubo and S. Nakamura. 1968. Effective elimination of drug resistance and sex factors in Escherichia coli by sodium dodecyl sulfate. J. Bacteriol. 95:1078-1089.

van Heyningen, S. 1976. The subunits of cholera toxin: structure, stoichiometry, and function. J. Infect. Dis. 133 (Suppl.):S5-S13.

Vercellana, G. 1926. La differenziazone del colera dai simili mediante un saggio di agglutinazione aspecifica. Pathologica, 18:418 (Quoted in Trop. Dis. Bull. 1927. 24:27).

Weber, K. and M. Osborn. 1969. The reliability of molecular weight determination by sodium dodecyl sulfate polyacrylamide gel electrophoresis. J. Biol. Chem. 244:4406-4412.

Wohlheiter, J.A., S. Falkow, R.V. Citarella and L.S. Baron. 1964. Characterization of DNA from a Proteus strain harboring an episome. J. Mol. Biol. 9:576-588.

Yajnik, B.S. and B.G. Prasad. 1954. A note on vibrios isolated in Kumbh Fair, Allahbad. Indian Med. Gazette 89:341-349.

Yokota, T. 1970. Thermosensitive and non-thermosensitive R factors in Enterobacteriaceae and Vibrio comma. In Progress in Antimicrobial and Anticancer Chemotherapy (Proc. of the 6th International Congress on Chemotherapy). Vol. 2. U. of Tokyo Press. Tokyo.

Yokota, T., T. Kasuga, M. Kaneko, and S. Kuwahara. 1972. Genetic behavior of R factors in Vibrio cholerae. J. Bacteriol. 69:440-442.

Zinnaka, Y. and C.C.J. Carpenter, Jr. 1972. An enterotoxin produced by noncholera vibrios. Johns Hopkins Med. J. 131:403-411.

Zinnaka, Y. and S. Fukuyoshi. 1974. Further obeervations on the NAG vibrio toxin. In Proceedings of the Ninth Joint Cholera Research Conference, U.S.-Japan Cooperative Medical Science Program, Grand Canyon, Arizona, October 1973. Dept. of State Publication No. 8672.

Zinnaka, Y., S. Fukuyoshi and Y.O. Yakamura. 1973. Some observations on the NAG vibrio toxin. In Proceedings of the Eighth Joint Cholera Research Conference, U.S.-Japan Cooperative Medical Science Program, Tokyo, August 1972.

SECTION III - BUDGET

A. Detailed budget

1. Personnel services

Name	Position	Pct. Effort No. days	Salary	Total Cost Taka	Dollars
Dr. W. Spira	Investigator	50 pct	D39732		19,866
Mr. R. Daniel	Pre-Doc. Fellow	100 pct	D 2150*		
Mr. Y.A. Saeed	Sr. Res. Asst.	50 pct	Tk39564	19,782	
Mr. Kibriya	Sr. Res. Asst.	15 pct	Tk35820	5,373	
Mr. Q.S. Ahmed	Res. Asst.	260 day	Tk22044	22,044	
Mr. M.A. Mutaleb	Res. Tech.	260 day	Tk16284	16,284	
Mr. A. Rahman	Sr. Lab Attndt.	130 day	Tk 9696	4,848	
Hospital duty physicians (3 at 5 pct. each)		15 pct	Tk35000	5,250	
Nurses (3 at 5 pct each)		15 pct	Tk14000	2,100	
Pathology	Sr. Lab. Tech	15 day	Tk12000	692	
Dr. Al-Mahmood	Veterinarian	30 pct	Tk40200	12,060	
Dr. Quader	Veterinarian	30 pct	Tk33387	10,016	
Mr. Safiullah	Handler	52 day	Tk15689	3,138	
Mr. R. Lashker	Caretaker	52 day	Tk 9905	1,981	
Mr. Hudda	Res. Tech	30 day	Tk20244	2,336	
To be appt.	Epidem. FA	65 day	Tk11000	2,750	
Statistics Br.	Key punch oper.	2 day	Tk13000	100	
Biochem. Br.	Sr. Lab Tech.	65 day	Tk12000	3,000	
Media Unit	Lab Tech	65 day	Tk11000	2,750	
Dish washing	Lab Attndt.	65 day	Tk 8000	2,000	
To be appt.	Clerk-typist	65 day	Tk11000	2,750	
Overtime for Q.A. Ahmed		15 day		1,270	
Overtime for M.A. Mutaleb		15 day		940	
				121,464	19,866

*Wellcome fellowship

2. Supplies and materials

Item	Taka	Dollars
Media		690.
Chemicals and biologicals		990.
Laboratory supplies-general		580.
Surgical supplies		560.
Medical supplies		530.
Chromatography supplies		282.
Electrophoresis and serology supplies		986.
Animals	53,450	
Glass and plasticware		850.
Biochemical and other tests (Unit cost)	3,650	
Membrane filtration materials		834.
Office supplies and stationery	1,150	245.
Miscellaneous	3,000	200.
	61,250	6,747

3. Equipment

Item	Unit Cost Dollars	No. Req.	Total Cost Dollars
1 Blendor, 2 speed commercial	100	1	100.
Mini sample containers, stainless steel	48	4	192.
2 Colony counter, Quebec	450	1	450.
3 Homogenizer, Virtis Model 45, Mini	650	1	650.
Accessories	-	-	100.
4 Transilluminator, UV	100	1	100.
5 Compressed gas tanks and regulators		2ea.	260.
6 Ultrafiltration equipment - Amicon			
TCF10 High performance UF system with SS reservoir and single pass option	1704	1	1704.
Standard UF cell, Model 12	160	1	160.
Standard UF cell, Model 52	185.	1	185.
Magnetic stirring table	97	2	194.
Reservoir, 800 ml.	130	1	130.
Accessories	-	-	150.
7 Membrane filtration equipment	-	-	365.
8 Chromatography equipment			
LKB Redi-rac fraction collector	850	2	1700.
Vario Perpex II pump	695	2	1390.

	Unit Cost Dollars	No. Req.	Total Cost Dollars
UV monitor - Uvicord III	4300	1	4300.
Potentiometric recorder, X-Y	2000	1	2000.
Ultrograd Gradient Mixer	4250	1	4250.
Accessories	-	-	440.
9 Electrophoresis equipment			
Pharmacia electrophoresis apparatus	785.	1	785.
Electrophoresis power supply	1150	1	1150.
Electrophoretic destainer	490	1	490.
Destainer power supply	450	1	450.
Accessories	-	-	240.
			21,933

Justification

Items 1-7 are either not currently available at CRL, are needed to replace non-working equipment or are needed to augment the equipment on hand. All items are for primary use under this protocol.

The large allowance for chromatography and electrophoresis equipment is being requested to cover the purchase of integrated analytical systems of high quality which will be versatile enough to be of use in many areas of research. The equipment now on hand is not adequate for analytical characterization using many of the newly developed techniques. These purchase requests have been fully co-ordinated with Dr. Seaton to ensure they will meet the most rigorous requirements of the Biochemistry laboratory.

4. Patient hospitalization

700 inpatient-days X Tk 135	Tk 94,500

5. Outpatient care

200 outpatient follow-ups X Tk 50	Tk 10,000

6. CRL transport

3500 miles at Tk 1.40 per mile	Tk 4,900
30 boat hours at Tk 98.84 per hour	Tk 2,965
	Tk 7,865

7. Travel and transport of persons

None

8. Transportation of things

(6,747 plus 21,933) X 0.25 — 7170 dollars

9. Rent, communication and utilities

	Taka	Dollars
Postage		30.
Shipping of specimens		150.
Long distance phone calls and cables	1500	
	1500	180.

10. Printing and reproduction

Xerox	3000	
Others	2500	
Publication costs		500.
		500.

11. Other contractual services

Payments to patients - 200 at Tk 15 ea — Tk 3000

12. Construction

Construction of stands for equipment, boxes, shelves, etc. — Tk 3000

B. Budget summary

Category	Year 1 Taka	Year 1 Dollars	Year 2 Taka	Year 2 Dollars	Year 3 Taka	Year 3 Dollars
Personnel	121,464	19,866	124,635[1]	20,359[2]	115,117[2]	21,902[2]
Supplies	61,250	6,747	36,230	8,286[3]	16,910	6,342
Equipment	------	21,933	------	1,500	------	1,500
Hospitalization	94,500	------	------	-----	------	-----
Outpatients	10,000	------	------	-----	------	-----
CRL transport	7,865	------	1,000	-----	1,000	-----
Travel-persons	------	------	------	1,900[4]	------	4,600[5]
Transportation-things	------	7,170	------	3,032	------	2,233
Rent-Communications	1,500	180	2,000	50	2,000	50
Printing-Reproduction	5,500	500	4,000	800	4,000	1,200
Contractual services	3,000	------	------	-----	------	-----
Construction	3,000	------	3,000	-----	3,000	------
	309,079	56,396	144,865	36,427	142,027	37,827

Grand Total: 169,100

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[1]Does not include physicians, nurses, biochemistry and pathology personnel, Epidem. FA or key punch operator. Dr. Ahmed included at 12 pct of time and statistics branch personnel added for 110 days. Includes 5pct increase in all salaries.

2Includes 5 pct increase in all salaries

3Increase due to purchase of electrophoretic and chromatography consumable items

4Conference travel

5Conference travel and trip to University of Surrey

Appendix A. Information on the use of human volunteers.

Abstract Summary:

I. The primary intent of the study is to distinguishand characterize those members of a diffuse group of bacteria which are, in fact, human pathogens. This will necessarily involvethe isolation of these organisms from infected human subjects. The study requires little more than standard hospital treatment of the patient except that the subjects will be requested to remain in hospital one day longer than they might if they were not in the study and that a blood specimen will be taken during the acute phase of the illness and during convalescence. Subjects' stool will be examined more thoroughly than usual for biochemical and clinical characteristics and for the presence of pathogens. Subjects' clinical data and patient history will be included when data analysis is performed.

It will be necessary to include children in the study, because they represent many of the cases associated with these organisms and there may be significant differences in the characteristics of the organisms or disease process involved.

2. This study presents very minimal risk to subjects in these areas. The nature of the laboratory analysis is such that potentially sensitive results (i.e., likely to cause

embarrassment) may occasionally be generated. A very small physical risk always accompanies the drawing of blood and must also be noted. For patients with unmixed NAG vibrio infection, antibiotics will not be given. The experience in the past is that diarrhea associated with these organisms improve so quickly that antibiotic treatment is of no demonstrable value. The greatest potential risk would be for those subjects suffering a mixed infection with Shigella spp. The procedures discussed below, however, are such that subjects face no greater risk than non-subject patients.

3. The risk potential in taking blood samples will be minimized as much as possible by having only properly trained medical personnel carry it out. Bleeding will be done only if the physician on duty certifies that it would present no undue risk to the subject. Sample volume will be reduced or the sample will not be taken if the duty physician recommends. Patients with simple diarrhea and no detectable Shigella or Salmonella will receive no antibiotics. This represents no real risk. If patients are admitted to the study with simple diarrhea and are found by laboratory analysis to harbor Salmonella or Shigella, appropriate antibiotic therapy will be started. A non-subject patient in these circumstances would have been started on tetracycline, then switched to ampicillin. Patients who are admitted to the study with dysentery or who later develop dysentery

will be started on ampicillin immediately.

4. Subject data will be filed under an arbitrary code number assigned to the organism isolated from the subject's stool. No patient identifiers will be included in these data. A sheet which relates code number to patient number will be maintained separately in the principal investigator's locked personal file. The signed consent form will be kept with the subject's hospital records, which are maintained separately. All data will eventually be combined and reduced in analysis to a point that information about a single patient will be impossible to extract.

5. A routine stool sample for diagnostic purposes will be obtained from patients admitted to the CRL hospital. If the sample contains the organisms of interest, the patient will be asked to enter the study. At this time, an explanation will be read to the subject or to a minor subject's parent or guardian and signed consent obtained.

6. Only the routine patient history and examination will be given as to all patients. Data will be taken from these records. No other personal data will be required.

7. Subjects will directly receive the benefit of immediate and intensive clinical, biochemical and microbiological

evaluation of their illness. This will be carried out rapidly enough that any clinically significant findings can be reported to the attending physician who can then take appropriate action. The potential benefits of this level of support far outweigh the small risks incurred. The benefit to the community lies in better diagnostic procedures for the illness caused by these organisms and in better treatment or prevention of disease once the nature of these pathogens and their disease process is understood.

8. This study requires the use of hospital records and samples of subjects' blood and stool.

INFORMATION TO PATIENT AND CONSENT FORM

(To be presented and read in Bengali)

The diarrhea you have may be caused by a bacteria called a vibrio. We are studying this bacteria to learn what kind of disease it causes and how it makes people sick. We also want to find betters ways of identifying this organism. We hope this study will help in treating and preventing the disease in the future.

We are asking you to join our study because we have found these vibrios in your stool. If you agree to join, we will take another sample of your stool now with a rectal catheter and do some more laboratory tests on it. We will also take a small amount of blood from your arm for laboratory tests. This may cause some discomfort. While you are in the hospital, you will be treated in the same way as other patients, but you will receive no antibiotics. This is necessary for the study. If we find other disease bacteria in your stool or if you develop dysentery, we will start giving you antibiotics. If your tests show anything that will help your treatment the physician will be notified immediately. We ask that you stay in that you stay in the hospital for one day after your diarrhea stops. After fifteen days, you will have to return to the CRL for a short time so we can take another sample of your blood. You will be paid at the time you come for your transportation cost and for time you loee from work. We will use information from the tests we do and also from the hospital records for our study. All the information we gather about you will be kept strictly confidential.

You are not required to join the study. If you do not, you will be treated as a regular patient. If you choose to join the study, you may stop at any time. You may ask any questions you wish before you join the study or at any time after.

Consent: I have read or heard the information given above and I am willing to help you by joining this study or by allowing my child bo join.

_____ _____

Signature of patient Date

_____ _____

Signature of parent or guardian Date

_____ _____

Signature of principal investigator Date