Attachment 1: - Libform

Date 19-4-84

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Principal Investigator Dr. M.Q. Huq

Trainee Investigator (if any) 28

Application No. 84-017

Supporting Agency (if Non-ICDDR,B) _____

Title of Study Molecular Characterisation of multiple antibiotic resistant (R-plasmid) and virulence plasmid of Shigella sp. and R-plasmid of V. cholerae

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

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

1. Source of Population: NA
   (a) Ill subjects Yes No
   (b) Non-ill subjects Yes No
   (c) Minors or persons under guardianship Yes No
2. Does the study involve: NA
   (a) Physical risks to the subjects Yes No
   (b) Social Risks Yes No
   (c) Psychological risks to subjects Yes No
   (d) Discomfort to subjects Yes No
   (e) Invasion of privacy Yes No
   (f) Disclosure of information damaging to subject or others Yes No
3. Does the study involve: NA
   (a) Use of records, (hospital, medical, death, birth or other) Yes No
   (b) Use of fetal tissue or abortus Yes No
   (c) Use of organs or body fluids Yes No
4. Are subjects clearly informed about: NA
   (a) Nature and purposes of study Yes No
   (b) Procedures to be followed including alternatives used Yes No
   (c) Physical risks Yes No
   (d) Sensitive questions Yes No
   (e) Benefits to be derived Yes No
   (f) Right to refuse to participate or to withdraw from study Yes No
   (g) Confidential handling of data Yes No
   (h) Compensation &/or treatment where there are risks or privacy is involved in any particular procedure Yes No
5. Will signed consent form be required: NA
   (a) From subjects Yes No
   (b) From parent or guardian (if subjects are minors) Yes No
6. Will precautions be taken to protect anonymity of subjects NA Yes No
7. Check documents being submitted herewith to Committee: NA
   ___ Umbrella proposal - Initially submit an overview (all other requirements will be submitted with individual studies)
   ___ Protocol (Required)
   ___ Abstract Summary (Required)
   ___ Statement given or read to subjects on nature of study, risks, types of questions to be asked, and right to refuse to participate or withdraw (Required)
   ___ Informed consent form for subjects
   ___ Informed consent form for parent or guardian
   ___ Procedure for maintaining confidentiality
   ___ Questionnaire or interview schedule *

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

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

FOR – Khaleda Haider

Principal Investigator

Trainee

SECTION I - RESEARCH PROTOCOL

1. Title : Molecular characterization of multiple antibiotic resistant (R-plasmid) and virulence plasmids of *Shigella* sp. and R-plasmid of *V. cholerae*.

2. Principal Investigator : Dr. M.I. Huq

   Co-Investigators : Mrs. Khaleda Haider
   Dr. Goutam Podder
   Mr. Q.S. Ahmed

3. Starting Date : When approved by RRC/ERC and budget approved.

4. Completion Date : One and half year from the date of starting

5. Total Direct Cost : US$ 20,536.00

6. Scientific Programme Head :

This protocol has been approved by the Disease Transmission Working Group.

Signature of the Programme Head : K. M. S. Aziz

Date : 19/4/84

7. Abstract Summary:

The study is aimed to see antibiotic resistance pattern of *V. cholerae* 01, non 01 and V. parahaemolyticus and also of different *Shigella* species isolated from patients and their contacts and to characterise the R-plasmids responsible for the resistance in terms of the pattern in their bacterial genome.

Studies done by us have shown that during the last 4 years we have isolated drug resistant *V. cholerae* from patients having seven different

R-type at different times. Preliminary genetic studies have shown that all the resistance factors are encoded in one plasmid band in different molecular weight which could be transferred to a recipient E. coli strains. As V. cholerae bears only one plasmid band even having different resistant patterns, we would like to carry out restriction endonuclease digest experiment on a sample of these strains isolated from patients to look at the difference in their banding pattern. Multiply resistant Shigella strains on the other hand harbour 3-8 plasmid bands of which one of the middle order bands is suspected to be encoding the self transmissible resistance. The strains isolated from carriers contains lesser number of plasmid bands. We would like to transfer the R-plasmid in E. coli K12 and do the restriction endonuclease cleavage to study the difference in banding pattern among isolates of different sources.

Despite similarities in molecular size and functional expression, very little information has been available concerning molecular relationship among the virulence plasmids. We would propose to isolate the virulence plasmid of different Shigella strains and do molecular comparison of the virulence plasmid by endonuclease digestion and DNA homology.

8. Review:

a. Research Involving Human Subjects: _____

b. Research Review Committee: _____

c. Director: _____

SECTION II - RESEARCH PLAN

A. INTRODUCTION

1. Objectives:

a. To isolate, purify and characterise the plasmid DNA from the multiple drug resistant and sensitive V. cholerae and Shigella isolates by using compatibility grouping, molecular digestion, and other methods.

b. To study the transfer pattern of the plasmids responsible for drug resistance and to do molecular comparison of virulence plasmid by endonuclease digestion and DNA homology.

2. Background:

Isolation of antibiotic resistance V. cholerae strains have been reported from different parts of the world (1,2). In several cases these resistances were apparently due to acquisition of R-plasmids. In December, 1979 while testing V. cholerae isolates for antibiotic sensitivity, a few multiply resistant V. cholerae (MARV) strains were detected in the Matlab field station. All of them were resistant to tetracycline, ampicillin, kanamycin, streptomycin and septrin. A retrospective search on the stock cultures of nearly 400 strains isolated in Dhaka, Matlab and other

parts of Bangladesh revealed that the first case with multiple antibiotic resistant V. cholerae appeared in August 26, 1979 in Matlab (3). Since then MARV strains are being isolated in increasing numbers. As all these antibiotic resistances were mediated through R-plasmids it is intended to study them in detail. Laboratory studies during the past several years have demonstrated that most of the naturally occurring strains of enterobacteriaceae contain plasmids of different molecualr weight (4,5). The plasmids are extrachromosomal genetic elements found in a variety of bacterial species. They are double stranded closed circular DNA melecules that range in size 1 kb. to greater than 200 kb. The molecular weight of the plasmids vary significantly even within a single bacterial species. Plasmids in the enterobacteria mediate the transfer of a variety of genetic determinants including those of drug resistance, virulence, production of antigens, production of colicines, production of enterotoxins and production of restriction and modification enzymes.

The term plasmid originally was used by Lederberg (6) to describe all extra chromosomal hereditary determinants. Plasmids are self replication extra-chromosomal DNA elements stable but dispensable gene pool in bacteria (7). The best known plasmids at least from the standpoint of human medicine, are those that encode for antimicrobial resistance, the R-plasmids (1) R-plasmid mediated resistance is generally due to the synthesis of protein which may enzymatically destroy the drug, modify the antibiotic to an inocuous form or interact with the cell envelope to

make it impermeable to the antibiotic (8). Plasmids are found in wide variety of bacteria, and it is as difficult as it is to generalise about the bacteria that harbour them. Over and above the R-factor and pathogenicity, plasmids are of equal importance for the study of the structure and function of DNA. Most recently plasmids have taken on paramount importance in recombinant DNA technology.

Many of the techniques currently employed for the isolation of plasmid DNA are based on its supercoiled covalently closed ciruclar configuration (9). All of the techniques require some means for gently lysing bacterial cells so that the plasmid DNA is preserved intact and can be physically separated from the more massive chromosomal DNA.

The initial characterisation of a bacterial plasmid is usually at the genetic level. If a bacterial trait is suspected to be plasmid mediated, gene transfer experiments will often document transmissibility of plasmid determinants independently of chromosomal determinants (10). Moreover the elimination of a genetic trait by exposure of a bacterial population to "curing agents" such as acridine orange or ethidium bromide may strongly suggest the presence of a plasmid. In most cases, however, it is essential to dicument that a plasmid is present and unequivocally associated with the genetic trait in question (11).

Agarose gel electrophoresis has been widely employed in the analysis of plasmids, viral, mitochondrial DNA and has recently been employed for the detection and primary characterisation of plasmid DNA present in the clinical isolates and laboratory strains of gram negative bacteria (12). The technique is simple, rapid to perform and capable of resolving mixtures of DNA fragments that cannot be separated adequately by other sizing procedures, such as density gradient centrifugation. Furthermore the location of DNA within the gel can be determined directly. Band of DNA in the gel are stained with low concentration of fluorescent intercalating dye ethidium bromide. As little as 1 ng of DNA can then be detected by direct examination of the gel in ultra-violet light. An adjnuct ot gel electrophoresis, Polyacrylamide gel electrophoresis has also been wisely used for analysing DNA fragments of bacteria.

Main use of this procedure were to analyse and prepare fragments of DNA less than 1 kb. in length. They may be cast in a variety of polyacrylamide concentration ranging from 3.5% to 20%, depending on the sizes of fragments of interest.

The banding pattern can also be studied thoroughly by digesting the DNA with specific restriction endonucleases. Restriction endonucleases catalyse double stranded, staggered cleavages at specific recognition sites in DNA (12,13). Plasmid DNA (and all form of DNA for that matter) after endonuclease treatment have characteristic fragment patterns which depend upon the number and specing of the specific recognition

sites within the genome. The DNA fragments can be separated by electrophoresis in agarose gels. Comparison of the patterns obtained for different plasmids can be used to determine qualitatively their degree of relatedness (14). The DNA fragments can also be transferred from the agarose gel to a strip of cellulose nitrate. After hybridization with a radioactive DNA probe, the fragments containing sequences homologous to these present in the probe can be detected as sharp bands by autoradiography of the cellulose nitrate strips (15).

The results of the previous study done with MARV and antibiotic resistant Shigella strains have shown that during the last 4 years 6 different R-types have appeared at different times in the V. cholerae isolates (16). The molecular weight of the plasmids carrying the antibiotic resistance factor was found to be ranging from 98-140 megadalton and was encoded in the plasmid of compatibility group C. Drug resistant Shigella strains also showed from 3-8 bands of which a middle order band of molecular weight 68-78 has been found to carry the antibiotic resistance factor (17). The strains from clinical cases has also been found to carry more plasmid bands than those isolates from carriers, though one 140 megadaltons plasmid has been isolated from each of the above strains. In cases of Shigella, we also found same banding patterns in different strains having different antibiotic resistance pattern. We would like to study the R-plasmids of different strains of V. cholerae and Shigella isolated from clinical cases and environment in detail after restriction endonuclease digestion;

in prior step of which we will have to make large amount of plasmid DNA from the specific bacteria under test.

It has been reported recently that in Shigella 120 to 140 Mdal plasmids are necessary for the expression of virulence in S. sonnei (18) S. flexneri (19) and enteroinvasive E. coli (20). Among different species, these virulence plasmids showed unrelated endonuclease cleavage patterns, whereas hybridization experiments showed that homologous sequences were present throughout the molecules (21). To monitor these plasmid transfer, the 120 and 140 Mdal plasmid of Shigella was tagged with the Kanamycin resistant transposon Tn5. This tagged plasmid was not self-transmissible, but was mobilized by one of several different conjugative plasmids into avirulent Shigella strain which had lost the comparable large plasmid (18,19). Transconjugants which received the tagged plasmid regained virulence.

Isolation of DNA fragments is a pre-requisite for mapping of genetic material with restriction endonucleases, DNA hybridization studies and recombinant DNA techniques. This can be done by recovering the DNA from gels in good yield and free of contaminants which interfere with further processing. Over the past few years a number of techniques for purifying DNA from agarose have been described (22,23,24). GRVITZ (1980) (25) described a method by which bearly complete recovery of the DNA is obtained and the DNA can be used as a substrate for further cleavage, and hybridization. Sucrose gradient ultracentrifugation can also be used to separate and purify plasmid DNA (26).

3. Rationale:

The recognition of plasmid mediated antibiotic resistance is not only useful in the treatment of individual cases of cholera and Shigellosis but also a powerful tool for epidemiological studies. It is therefore necessary to study the specific plasmids involved in that outbreak in detail in order to determine the transmissible quality and to compare one plasmid with other. In this way one can determine the plasmids potential to spread, whether or not several plasmids are involved indicating a single or more source and determine other characteristics which might be carried on the R-plasmid under study. The knowledge could aid in determining both the sources of emergence of resistance and the control of its spread among strains of its own species as well as other bacteria like E. coli. By studying the endonuclease pattern and hybridization experiment one can determine the molecular relationship among tbe virulence plasmid in different shigella strains.

B. SPECIFIC AIMS

1. To isolate bacterial plasmids in a large scale and to purify them.

2. To characterise the isolated plasmids responsible for drug resistance by molecular weight estimation, compatibility grouping, endonuclease cleavage and transfer pattern in order to determine the relationship among these plasmids and their occurrance among their bacterial strains.

3. To study the invasive plasmids by fragmentation with restriction endonucleases followed by gel electrophoresis and $^{32}_{p}$ labelled hybridization on Nitrocellulose filter.

C. METHODS AND PROCEDURES:

All clinical and environmental strains of V. cholerae as well as clinical strains of Shigella from Dhaka Hospital and field specimens will be tested for sensitivity to different antibiotics by using standard Kirby Bauer method (27). V. cholerae and Shigella strains are routinely tested for sensitivity in the diagnostic unit from where the resistance pattern will be collected and analysed in a monthly basis to see the changing pattern of antibiotic resistance. The multiple antibiotic resistant strains of V. cholerae and Shigellae will be picked up and studied in detail for plasmid characterization and transmissibility.

During the year of 1983 we isolated about 1600 Shigella strains out of which approximately 200 strains are multiple antibiotic resistant (resistant to 3 and more antibiotics). In last year, 15 resistant V. cholerae strains have been isolated and in this year we have so fare isolated 3 resistant V. cholerae strains. Based on this previous data we presume to isolate more multiple antibiotic resistant V. cholerae and Shigellae strains during the year, 1984 which we shall include into this study.

Purification and characterization of plasmid DNA:

Most of the techniques currently employed for the isolation of plasmid deoxyribonucleic acid (DNA) are based on its super coiled covalently closed circular (ccc) configuration. Ethidium bromide-Cesium chloride density gradient centrifugation, nitrocellulos absorption and sedimentation by alkaline sucrose gradient centrifugation, all depends on certain characteristic unique to ccc-DNA. To obtain a large pool of plasmid DNA other methods rely on the preferential sedimentation of the high molecular weight chromosomal cellular DNA in the presence of sodium Lauryl sulfate and high concentration of salt. Generally cells

are lysed by using the Brij lysis technique or modification thereof. Recently several modification to this method have been introduced such as the use of the non-ionic detergent Triton X-100 instead of Brij. All these methods with little modification in some cases permit quick isolation and characterisation of plasmids of different sizes, from different bacterial species.

Plasmid DNA may be visualised in a Cesium chloride-ethidium bromide gradient by use of a long wave (4000 A^{o}) ultraviolet light but if one wishes to determine the number and size of plasmid species it is necessary to either examine the plasmid DNA under the electron microscope or sediment labelled DNA through a sucrose gradient. Recently, agarose gel electrophoresis has benn widely employed in the enalysis of restriction endonuclease generated fragments of plasmid and viral DNA. A simple adaptation of these electrophoretic methods for the identification and characterization of plasmid DNA has recently been published by Kado et al, 1981 (28). This method is suitable for the detection and preliminary characterization of ccc plasmid DNA present in clinical isolates and laboratory strains of gram-negative and gram-positive organisms. The procedure requires a vertical gel slab apparatus, a regulated power supply and a short wave UV light source. Electrophoresis is carried out at room temperature at 60 mA, 120 volts for 2 hours. By measuring the distance travelled by these plasmid markers a graph can be constructed. Interpolation of the values of distance travelled obtained for the unknowns permit the calculation of their molecular weights.

Density gradient centrifugation with Ethidium bromide:

Plasmid DNA molecules extracted from bacterialcells have been characterized as double stranded covalently closed, circular and with no free end of

rotation. Ethidium bromide is one in a series of phenathridium dyes that bind to DNA and ribonucleic acid and inhibit nucleic acid function (7, 29). Cells are lysed and the lysates are added to Cesium Chloride gradients containing ethidium bromide. The DNA is sedimented to equilibrium by high speed centrifugation. Ethidium bromide intercelates into the DNA thus reducing its density (30). In this method the linear DNA and open circular molecule does not show the same physical constraints that are imposed upon a ccc molecule of plasmid DNA. Consequently the former types of DNA can bind significantly more ethidium bromide molecules and are rendered less dense than the latter type of DNA. This difference in binding permits the separation of the different forms of plasmid DNA. In gradients of Cesium chloride, these forms can be visualised as discrete bands when the gradients is illuminated with UV light due to the fluorescence of the ethidium bromide inter-calated in the DNA.

Restriction endonuclease cleavage of plasmid DNA:

Restriction endonucleases fragments the DNA in different patterns depending on the number and spacing of the specific recognition sites within the genome. Different enzymes are needed to cleave different bacterial DNA. The procedure for endonuclease cleavage includes, a) treating the purified plasmid DNA in a buffer with the required enzyme, followed by incubation at 37° (for 60 minutes. The reaction stopped by adding either solution of EDTA or a mixture of containing bromophenol blue, SDS and glycerol. The mixture is electrophoresed and the banding pattern is compared after staining with ethidium bromide (31).

Tests for transferrability of drug resistance:

The conjugal crosses are ideally set up with one lactose fermenter and the non-lactose fermenter. Thus by plating on a MacConkey Agar, the parent can easily be differentiated on the basis of lactose fermentation. In our case the recipient E. coli K_{12} lac^+ N_x^r strain carriers also the chromosomal resistance to halidixic acid (4).

Broth cultures of donor and recipient strains are grown in nutrient broth with shaking at 37°C to an exponential phase (4-5 hours). The cultures are mixed in equal volume, usually one ml. and incubated overnight. Crosses can be set up at both 28°C and 37°C because transfer frequencies vary between compatibility groups. After incubation, serial decimal dilutions are prepared in phosphate buffer and 0.01 ml volumes plated on MacConkey agar with suitable selective antibiotic in duplicate. The mating mixture dilutions are also plated on MacConkey agar without any selective agent to obtain colony counts for each parent. The plates are incubated overnight at 28°C or 37°C and colonies counted. Transfer frequency is expressed as the proportion of resistant progeny per recipient cell.

Mobilization of non auto-transferring plasmids: (32)

Where no direct transfer would be detected, strains will be examined for resistance mobilization in a triparental cross. Equal volumes of a late exponential culture of the donor strain carrying a standard transfer factor, and the wild strain, will be mixed and incubated at the appropriate temperature for at least 2 hours. The plasmid free recipient will then

be added and the mixture will be incubated overnight. The cross will then be plated on selective media with counter selection against both the donor and wild strain. Resistant recipient colonies are then picked and R-typed as above.

Isolation of virulence plasmid (26):

(1) Virulence plasmid of Shigella strains will be isolated by sucrose density gradient in which the plasmid DNA extract willbe spinned in 5-20% sucrose gradient at 35,000 rpm for 1 hour in nitrocellulose ultracentrifuge tube. Virulence plasmid band at the bottom, visualized by UV light will be collected by piercing the nitrocellulose tube and will be followed by dialysis overnight with TES buffer.

(2) Recovery of plasmid DNA from agarose gels (25):

After running electrophoresis the 140 Mdal plasmid will be localized by using a UV lamp. Using a sharp scalpel the gel slice containing the 140 Mdal plasmid band will be cut out. The gel will be photographed after cutting out the band in order to keep a record of which band was eluted. A dialysis bag will be filled with 0,5 x TBE buffer and the gel will be placed inside the dialysis bag. The bag will be clipped very carefully so that there is no leakage and avoid trapping air bubbles. The bag will then be immersed in a shallow layer of 0,5 x TBE in an electrophoresis tank, 100 V current for 1 hr, passing through the bag will electroelute the DNA out of the gel onto the inner wall of the dialysis bag. The polarity of the gel will be reveresed for 5 second to release the DNA from the wall of the

dialysis bag. All the buffer will be collected from the bag very carefully and the bag will be washed out with a small quantity of 0.5 x TBE buffer. The gel slice will be stained with Etbr again to check that all the DNA is eluted.

DNA in buffer and the agarose strip was placed in a ependorf tube containing glass wool at the bottom. This tube has a hole at the bottom and will be placed on the top of another ependorf tube. Two tube together will be spunined for few seconds. To remove protein and other contaminants phenol, chloroform and ether extraction will be done. In the last step we will concentrate the DNA by ethanol precipitation and will be ready for enzyme digestion.

Incompatibility grouping of plasmids (33):

Incompatibility of two plasmids is shown by their inability to coexist stably in the same cell line. Distinguishable variants of a single plasmid are incompatible with one another and with their parent plasmid. Naturally, occurring plasmids, in all bacterial genera where they have examined, show compatibility and incompatibility relationships. Thus in a bacterial genus a classification may be made by testing pairs of plasmids, in a chosen host strain, for their ability to coexist (17).

Compatibility between two plasmids is demonstrated by introducing one plasmid into a strain carrying the other ande xamining recipient colonies for stability of the two plasmids. In general, if the plasmids belong to the same compatibility group, the resident plasmid will be displaced, where both plasmids are present, the clones are tested for segregation after growth at 37°C for 6 and 24 hours. The strains are then plated on nutrient agar and on the following day replica plated onto selective media compatible plasmids will be segregate at the same rate as their spontaneous loss from control strains carrying only one plasmid. If no segregation occurs the strains are tested for independent transfer of the two plasmids, since the resistance markers may have recombined or transposed onto the same plasmid.

Nitrocellulose filter hybridisation:

This will be performed under stringent conditions according to Southern's procedure with modification (21). $ECOR_1$ digested DNA will be depurinated by immersing 0.7% agarose gel in 0.25 M HCl for 10 minutes and denatured by two 15 minutes i-mersions in 1.5 M NaCl, 0.5 N NaOH, and neutralized by two 30 minutes immersion in 3 M NaCl, 0.5 M tris HCl, pH 7.0. The DNA was then transferred overnight onto a nitrocellulose filter along a 20 x 2 x SSC gradient (1 x SSC: 0.15 M NaCl 0.015 M Sodium Citrate. The filter will then be finsed in 2 x SSC for 20 minutes, air dried

and balled for 3 hours at 80°C. Plasmid DNA from different entero-invasive strains will be radiolabelled by nick translation using $^{32}_{p}$ deoxyribonucleotides achieving a specific activity of 2.5 x 10^7 cpm/ug DNA. The nitrocellulose filter will be prehybridised by immersion in the prehybridization mix for at least 4 hours. Hybridization will be done in a sealed plastic bag at 42°C for 17 hours in 10 ml of the hybridization solution. The filter will then be washed 3 times with 250 ml of 2 X SSC; 0.1% SDS for 5 minutes and twice with 250 ml of 0.1 X SSC, 0.1% SDS at 50°C for 30 minutes, air dried and exposed overnight to a "Kodak" X AR_5 film at -80°C.

Virulence assay:

Virulence of the cultures will be determined by Sereny test and tissue culture assay by using Hela Cell lines. The Sereny test as described by Sereny(34,35) will be done by instillating the bacterial growth in the eyes of Hela Cells will be inoculated with the bacteria and incubated for 90 minutes at 37°C. The procedure used for maintenance, culture and infection of cells done as described by Hale and Formal (36).

The whole work proposal will mainly cover the following steps:

a. Isolation and characterisation of different species of *V. cholerae* and *Shigella* isolated from cases of diarrhoea/dysentery and also from environment.

b. Large scale isolation of plasmid DNA from the isolated strains.

c. Purification and characterisation of CCC DNA by centtrifugation by Cesium chloride - ethidium bromide gradients and analysis of plasmids in respect od DNA content, molecular size and band appearance.

d. Digesting DNA with restriction endonucleases followed by agarose gel and polyacrylamide gel electrophoresis for the isolation and characterisation of the plasmid fragments.

D. SIGNIFICANCE:

With the emergence of multiply antibiotic resistant V. cholerae and also increasing resistance of Shigella species to available antimicrobial agents it has become necessary to study the transfer of drug resistance factors from bacterial genome to other. This study will also allow us to study the different specific plasmid responsible for different factors and their migration or transfer from one genus or species to the other thus acting as a suitable tool to construct an epidemiological mapping to see the migration of the plasmids. This will also help us finding the source of emergence of drug resistance which eventually will help in controlling the transfer of R-plasmids. The endonuclease restriction pattern comparison of virulence plasmids might be used as an epidemiological tool of Shigella infection. Moreover, a high homology background among all virulence plasmids would make any one of them suitable for use as a diagnostic $^{32}_{p}$ labelled probe for detecting enteroinvasive strains present in faeces.

E. FACILITIES REQUIRED:

No new laboratory set up will be needed. The existing room for doing the plasmid study will be enough for carrying out the laboratory studies. Animal models will be used which will be done at the Animal House with no alteration of the existing facilities.

F. COLLABORATIVE ARRANGEMENTS:

This work will be carried out in close collaboration with Dr James Kaper of the Center for Vaccine Development, Prof. R.R. Colwell of the University of Maryland and Dr D.J. Kopeckno of WRAIR, Bethesda, Maryland. Necessary training support and chemicals and equipments supplies for the project has already been assured.

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SECTION III - BUDGET

A. DETAILED BUDGET

1. PERSONNEL SERVICES

Name	Position	% time alloted		Annual Salary	Project Requirements Taka	Dollar
Dr. M.I. Huq	P. Investigator	15%	US$	53,740	-	8,061.00
Dr. K. Haider	Co-Investigator	30%	TK.	73,190	21,957.00	-
Mr. Q.S. Ahmed	Co-Investigator	25%	Tk.	87,290	21,882.00	-
Dr. G. Podder	Co-Investigator	25%	Tk.	61,910	15,477.00	-
Mr. Abdul Huq	Sr. Lab. Tech.	25%	Tk.	51,530	12,882.00	-
Mr. A. Rahman	Sr. Lab. Attn.	25%	Tk.	31,860	7,965.00	-
			Sub-Total:		80,103.00	8,061.00

2. SUPPLIES AND MATERIALS:

Chemicals and laboratory supplies	-		1,500.00
Media			800.00
Office supplies		5,000.00	-
Lab. animals		5,000.00	-
Polaroid Film		-	800.00
	Sub-Total:	10,000.00	3,100.00

3. EQUIPMENT

Horigontal slab electrophoresis equipment	1,600.00

4. PATIENT HOSPITALIZATION - None

5. OUTPATIENT CARE - None

6. ICDDR,B TRANSPORT

1000 miles automobile transport and 15 hours speedboat drive.	9,000.00

7. TRAVEL AND TRANSPORTATION OF PERSONS

One return air-fare Maryland/Dhaka/Maryland	-	2,200.00

B. BUDGET SUMMARY

		Taka	US$
1.	Personnel Services	80,103.00	8,061.00
2.	Supplies and Materials	10,000.00	3,100.00
3.	Equipment	-	1,600.00
4.	Patient Hospitalization	-	-
5.	Outpatient Care	-	-
6.	ICDDR,B Transport	9,000.00	-
7.	Travel of persons	-	2,200.00
8.	Transportation of things	-	-
9.	Rent, Communication and Utilities	-	1,050.00
10.	Printing and Reproduction	9,500.00	-
11.	Other Contractual Services	-	-
12.	Construction, Renovation and Alteration	-	-
	Total:	1,08,603.00	16,011.00
	Equivalent to US$	4,525.00	
	Grand Total US$	20,536.00	
	Salaries US$	11,400.00	
	Operational cost US$	9,136.00	

Conversion rate US$ 1.00 = Tk. 24.00

	Taka	Dollar
8. TRANSPORTATION OF THINGS: - None		
9. RENT, COMMUNICATION AND UTILITIES :		
Guest House charge 30 days @ US$ 35.00	-	1,050.00
10. PRINTING AND REPRODUCTION		
Xerox	4,000.00	-
Publication	5,500.00	-
Sub-Total:	9,500.00	-
11. OTHER CONTRACTUAL SERVICES - None		
12. CONSTRUCTION, RENOVATION AND ALTERATION - None		