Attachment 1. 9 Date _____

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Principal Investigator Dr. M.I. Huq
Application No. 81-011
Title of Study Characterisation of the Antibiotic Resistance in the Multiply ~~Resistant Vibrio cholerae, Related~~ Vibrios and Enterobacteriaceae.

Trainee Investigator (if any) _____
Supporting Agency (if Non-ICDDR,B) _____
Project status:
( ) New Study
( ) Continuation with change
( ) No change (do not fill out rest of form)

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

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

Study involves only stocked bacterial cultures.

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

Principal Investigator

Trainee

81-011
recd 16/3/81

SECTION I - RESEARCH PROTOCOL

(1) **Title:** Characterisation of the Antibiotic Resistance in the Multiply Resistant *Vibrio cholerae*, Related Vibrios and Enterobacteriaceae.

(2) **Principal Investigator:** Dr. M.I. Huq

Co-Investigators: Dr. A.R. Samadi
Dr. K. Wachsmuth

(3) **Starting Date:** March 15, 1981

(4) **Completion Date:** March 14, 1982

(5) **Total Direct Cost:** $ 19,022.00

(6) **Scientific Program Head:**

This protocol has been approved by the DTWG Working Group.

Signature of Scientific Program Head: Hamadi

Date: 4/3/81

(7) **Abstract Summary:**

The protocol essentials covers in detail the pilot study which was undertaken to characterise the multiply antibiotic resistant *V. cholerae* isolated from patients and environment. The study is aimed to characterise the R-plasmids responsible for this resistance in terms of their pattern in the bacterial genome and also the viability of these R-plasmids in the patients' gut, environment, surface water and in laboratory animals (monkey).

This will be a laboratory based study and does not involve any experimentation on the human subject excepting culturing a few stool samples from them. When human subjects will be used in the study, the consent form designed for the pilot study will be used.

(8) Reviews:

(a) Ethical Review Committee: _____

(b) Research Review Committee: _____

(c) Director: _____

(d) BMRC: _____

SECTION II

A. INTRODUCTION

1. Objective: (a) To study the resistance pattern of the V. cholerae isolates and to study its relationship to other gut flora isolated at the same time. (b) To study the plasmid responsible for the antibiotic resistance in V. cholerae and their morphological pattern in bacterial genome. (c) To study the viability of these R-plasmids in the patients gut, environment (water) and in laberatory animals. (d) Effect of Kappa phage present in the environment on the resistant plasmids and (e) To study the transfer pattern of the R-plasmids in the V. cholerae to recipient E. coli strains.

2. Background: Antibiotic resistant Vibrio cholerae has been reported in several different parts of the world (1, 2). In several cases these resistance were apparantly due to the antibiotic resistant plasmids. (R-plasmids). In December, 1979 while testing V. cholerae isolates for antibiotic sensitivity, we detected a few multiply resistant V. cholerae from Matlab. All these strains were resistant to Tetracycline, Ampicillin, Kanamycin, Streptomycin and Septrin. A retrospective search on the stock cultures of appreximately 400 strains isolated from Dacca and Matlab and strains isolated from different parts of Bangladesh revealed that the first case with multiple antibiotic resistant V. cholerae appeared in August 26, 1979 in Matlab. Since then the MARVC strains are being isolated in increasing numbers. As all these antibiotic resistance can be mediated

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by R-plasmids (3) we intended to study them in detail.

Plasmids are self-replicating extrachromosomal DNA elements of bacteria that represents a reasonably stable, but dispensable, gene pool in bacteria (4, 5). The best known plasmids, at least from the standpoint of human medicine, are those that encode for antimicrobial resistance, the R-plasmid (formerly designated R-factor) (5, 6) R-plasmid mediated resistance is generally due to the synthesis of proteins which may enzymatically destroy the drug, modify the antibiotic to an innocuous form or interact with the cell envelope to make it impermeable to the antibiotic (7). Plasmids may be conveniently classified into two major types: conjugative or non-conjugative. A plasmid is classed as conjugative (or a sex factor) if it is self transmissible from one cell to other (5, 8). These plasmids have the capability to mediate their own transfer by means of conjugal mating.

The stability of the R-plasmid were studied by Yokota et al (9) while studying the genetic behaviour of the R-plasmids in V. cholerae (3). The results obtained by them differed markedly from our preliminary studies which showed that these R-plasmids are more stable and remain in the V. cholerae strains so long they are viable in the gut or in surface water without losing any resistance determinant for any single antibiotic. On the other hand Anderson et al (10) in an experiment involving feeding E. coli K_{12} strains to human volunteers showed that strains lacking R-plasmids survived in preference to those carrying plasmids.

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It has been observed by Falkow et al (11) that when R-plasmids do appear in E. coli that possesses good survival potential, the R-plasmids survives along with its host. Hence, the outcome of in vivo plasmid transfer is dependent upon the ability of the recipient bacterium to colonize and compete well with other E. coli strains when antibiotics are absent as well as on the transient ability of the microorganism to survive when antibiotics are present. Andersen et al (6) showed that once the essential pathogens stably acquire an R-plasmid, they will spread with or without antibiotics, often with grim results as has been illustrated by a recent outbreak of Sh. dysenteriae, (12).

As described earlier the resistant bacteria have the ability to transfer their antibiotic resistance to a recipient plasmid free E. coli K-12 strains with chromosomal resistance to Nalidixic acid, Streptomycin, or rifanifin. This process helps in classifying the resistant bacteria on the basis of the resistance determinants transferred to the recipient cells.

Agarose gel electrophoresis has been widely employed in the analysis of plasmid, viral, mitochondrial and bacterio-phage DNA and has recently been employed for the detection and preliminary characterisation of plasmid DNA present in clinical isolates and laboratory strains of Gram-negative bacteria (13). By using agarose gel technique it is not only possible to directly visualise plasmids DNA. The molecular mass of plasmid covalently closed circular (ccc) DNA can be estimated by their relative migration in an agarose gel when plasmid DNA of known molecular

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mass are included in the gel as standard. Myers etal (13) have shown that ccc plasmid DNA ranging in mass from 0.6 to 95X10^6 Daltons can be satisfactorily resolved by electrepheresis in 0.7 % agarose gels. It has been observed that the molecular mass estimates by the agarose gel method is in rather close agreement with more laborious procedure (i.e. electron microscopy or sedimentation studies).

One of the earliest and still one of the most convenient method of plasmid classification is called incompatibility grouping which acts as a good marker for the classification of the bacteria coding the plasmid which ultimately helps in the epidemiological investigation.

The results of the pilot study done under pilot protocol No. 80-011 clearly showed that all the isolates showed at least 4 different R-types. All our isolates were chloramphenicol sensitive which differs it from isolates from other parts of the world which were chloramphenicol resistant. All our isolates have molecular size of 98 megadalton which is same as other isolates from different parts of the world. The complete resistance spectrum of each strain was encoded by a plasmid of the C compatibility group. The transfer of the group C plasmids from the wild V. cholerae host strains was enhanced by incubating the making mixtures at 28°C rather than 37°C but in subsequent crosses from a K_{12} host, the effect of temperature was reversed and the plasmid transferred at higher frequency at 37°C than at 28°C (3).

3. Rationale: The recognition of plasmid-mediated antibiotic resistance is not only useful in the treatment of individual cases of cholera but can also be a powerful tool for Epidemiological studies. Vibrio cholerae is generally sensitive to tetracycline and this antibiotic in conjunction with oral rehydration in the accepted treatment for cholera at ICDDR,B. If the V. cholerae strain causing infection contains a plasmid coding for resistance to tetracycline the usual treatment of a patient is ineffective and the pressure for transfer of the plasmid to another bacterium is greatly increased. It is necessary to study the specific plasmids involved in such outbreak to determine the transmissible quality and to compare one plasmid to another. In this way one can determine the plasmid's potential to spread whether or not several plasmids are involved, indicating a single source or more, and determine other characteristics which might be carried on the R-plasmid under study. This knowledge could aid in determining both the source of resistance and the control of its spread among other bacterial strains, such as E. coli, Shigellae and others.

B. SPECIFIC AIMS

1. To study the transfer of the R-plasmids in vivo from Resistant E. coli to sensitive vibries and Resistant vibries to sensitive E. coli using rabbit and Monkey as animal model.

2. To determine if resistance to tetracycline is plasmid-mediated in the multiply-resistant strains of V. cholerae isolated in Bangladesh.

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3. To determine the transmissibility and stability of the resistances and of the plasmid by conjugation experiments and gel electrepheretic techniques.

4. To characterize the plasmid by molecular weight estimation and compatibility grouping to determine the origin and relationships among these plasmids and their occurrence among other bacterial strains.

5. To make well-founded statements regarding the incidence of this R-plasmid among vibries and E. coli, thereby contributing to both the treatment of cases and the control of infection do to multiply-resistant V. cholerae.

C. METHODS AND PROCEDURES

The pilot study covered all the preliminary studies done on the in Vitretransfer of R-factor from Resistant V. cholerae to sensitive E. coli and back transfer to sensitive vibrio. In this protocol we will look at the possible in vivo transfer of R-factor from vibrio to E. coli and E. coli to vibrio using rabbit and monkey as animal model and also purify and characterise the plasmid from the resistant strains of V. cholerae and E. coli and other gram-negative isolates.

In vivo transfer of R-factor from resistant V. cholerae to sensitive E. coli and vice versa will be done by inoculating the 10^8-10^{10} bacterial growth of recipient and donor in the loop of the animal transfer, if occurs, will show up in first 24 hours and the bacterial isolates (conjugate will be tried from R-plasmid transfer. Different inoculum size will be tested in

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both rabbit and monkey. When necessary prior feeding with Sodium bicarbonate will be done to break the acidity barrier when the animal will be challenged orally.

Purification and Characterisation 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, nitrocellulose adsorption and sedimentation by alkaline sucrose gradient centrifugation, all depends on certain characteristic unique to ccc-DNA. To obtain a first enrichment 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 a 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

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labelled DNA through a sucrose gradient. Recently, agarose gel electrophoresis has been widely employed in the analysis of restriction endonuclease generated fragments of plasmid and viral DNA. A simple adaptation of these electrophoretic methods for the identification and characterisation of plasmid DNA has recently been published by Meyers et al, 1976. This method is suitable for the detection and preliminary characterisation of one plasmid DNA present in clinical isolates and laberatery strains of gram-negative and gram-positive organisms. This procedure requires a vertical gel slab apparatus a regulated power supply and a short wave UV light source. Electrepheresis 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.

D. SIGNIFICANCE

With the emergence of multiply antibiotic resistant Shigella species, Escherichia coli and very lately V. cholerae it has become necessary to study the transfer of drug resistance which is plasmid mediated. This study will allow us to enumerate and characterise the specific plasmid present in the gut flora (either pathogenic or nonpathogenic) and to study their migration or transfer from one genera or species. Plasmid characterisation thus can act as a suitable tool to construct an epidemiological mapping to see the migration of the plasmids. This will also help us finding

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the source of drug resistance which in turn will help in eventually controlling the transfer of R-plasmid to sensitive bacteria.

E. FACILITIES REQUIRED

Now laboratory set up will be required to install the newly procured equipment. A small room measuring 10'X10' has already been earmarked for this study.

About 12 rabbits and 12 monkey will be needed to do the transfer experiment. No other logistic support will be needed. All the specialised equipment required for the study has been bought and have reached the laboratory.

F. COLLABORATIVE ARRANGEMENTS

This work will be carried out in close collaboration with Dr. Kaye Wachsmuth of the Enteric Branch, Centre for Disease Control, Atlanta. Dr. Stanley Falkow will be providing consultative services whenever necessary. Dr. Kaye Wachsmuth has already arrived Dacca and is setting up the procedures.

REFERENCES

1. Knwahara S, Yokota T, Kano T: Recent trend of drug resistance Plasmids (R) in Vibrio cholerae. Proceedings of the 15th Joint Conference on cholera sponsored by the US-Japan Co-operative Medical Science Program, NIAID, NIH: DHEW, Bathesda, Maryland July 23-25, 1979, PP 237-241.

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J. Bacteriol. 127:1529, 1976.

SECTION III - BUDGET

A. DETAILED BUDGET

1. PERSONNEL SERVICES

Name	Position	% of effort or number of days	Annual Salary	Project requirement Taka	Dollar
M.I. Huq	Branch Head	15 %	$ 30000		4500
Dr. A.R.Samadi	Program Head	5 %	$ 36000		1800
Dr.K.Wachsmuth	Visiting Investigator for 2 months	100 %	-	-	-
Q.S. Ahmed	Sr.Res.Officer	25 %	$ 39192	10398	-
New	Res. Officer	100 %	$ 24200	24200	-
Z.A. Khan	Lab.Attendant	50 %	$ 14796	7398	-
				41996	6300

2. SUPPLIES AND MATERIALS

Media		300
Chemicals & Laboratory supplies		1200
Office supplies	3000	
Miscellaneous	3000	

3. EQUIPMENT 800

Some equipment necessary for the study has been acquired through another protocol.

4. PATIENT HOSPITALISATION

None

5. OUTPATIENT CARE

None

6. ICDDR,B TRANSPORT

1000 miles of automobile transport @ 3.50/mile Tk.3500.00

7. TRAVEL AND TRANSPORATION OF PERSON

One return Air ticket for one person DAC/ATL/DAC $ 2200.00

8. TRANSPORTATION OF THINGS

None

19. RENT COMMUNICATION AND UTIDITIES

None

10. PRINTING AND REPRODUCTION

Xerox	Tk.3000.00
Others	None
Publication	Tk.3000.00

11. OTHER CONTRACTUAL SERVICES

None

12. CONSTRUCTION, RENOVATION AND ALTERATION

None

BUDGET SUMMARY

		Taka	Doller
1.	Personnel	41,996.00	6,300.00
2.	Supplies	6,000.00	1,500.00
3.	Equipment	-	-
4.	Hospitalisation	-	-
5.	Out patient care	-	-
6.	ICDDR,B transport	3,500.00	-
7.	Travel	-	2,200.00
8.	Transport of things	-	-
9.	Rent/Communication	-	-
10.	Printing/Publication	6,000.00	-
11.	Contractual services	-	-
12.	Construction	-	-
	Sub-total Tk.	57,496.00	10,800.00
	= US $	3,833.00	

Total US $ = 3,833.00 + 10,800.00

= $ 14,633.00

30 % Overhead $ 4,389.00

Grand Total $ 19,022.00

Attachment 1a

Abstract Summary

The study in summary will cover the characterisation of the multiply antibiotic resistant V. cholerae and other enteric bacteria isolated from patients and environment. This will be completely a laboratory study with an aim to characterise the bacterial genome by various modern Microbiological Techniques which will give us a lead to the phenomenon of transfer of drug resistance within V. cholera and other enteria bacteria.

The study will not involve any human subject and as such there is no potential risk or anything of concern to the human subject.

SECTION III - BUDGET

A. DETAILED BUDGET

1 PERSONNEL SERVICES

NAME	POSITION	% OR NO. OF DAYS	ANNUAL SALARY	PROJECT REQUIREMENT TAKA	DOLLAR
Dr. M.I. Huq	Branch Head	15%	$ 53,740	-	8,061
Dr. A.R. Samadi	Programme Head	5%	$ 66,500	-	3,325
Dr. K. Wachsmuth	Visitg. Investi. for 2 months	100%	-	-	-
Mrs. Khaleda Haider	Sr. Res. Officer	40%	Tk. 50,860	20,344	-
Mr. A. Alim	Sr. Res. Officer	40%	Tk. 75,980	30,392	-
				50,736	11,386

2. SUPPLIES AND MATERIALS

Media		300
Chemicals & Laboratory supplies		1,200
Office Supplies	3,000	
Miscellaneous	3,000	
	6,000	1,500

3. EQUIPMENT

Some equipment necessary for the study has been acquired through another protocol.

4. PATIENT HOSPITALIZATION - None

5. OUTPATIENT CARE - None

6. ICDDR,B TRANSPORT

1000 miles of automobile transport @ Tk. 3.50/mile	3,500

7. TRAVEL AND TRANSPORTATION OF PERSONS

One return Air Ticket for one person DAC/ATLANTA/DAC	2,200

8. TRANSPORTATION OF THINGS - None

		TAKA	DOLLAR
9.	RENT, COMMUNICATION AND UTILITIES		750
10.	PRINTING AND REPRODUCTION		
	Xerox	3,000	
	Others	-	
	Publication	3,000	
		6,000	
11.	OTHER CONTRACTUAL SERVICES - None		
12.	CONSTRUCTION, RENOVATION AND ALTERATION None		

BUDGET SUMMARY

		TAKA	DOLLAR
1.	Personnel Services -	50,736.00	11,386.00
2.	Supplies and Materials -	6,000.00	1,500.00
3.	Equipment -	-	-
4.	Patient Hospitalization -	-	-
5.	Outpatient Care -	-	-
6.	ICDDR,B Transport -	3,500.00	-
7.	Travel and Transporation of Persons -	-	2,200.00
8.	Transportation of Things -	-	-
9.	Rent, Communication and Utilities -	-	750.00
10.	Printing and Reproduction -	6,000.00	-
11.	Other Contractual Services -	-	-
12.	Construction, Renovation and and Alteration -	-	-
	Sub-Total :	66,236.00	15,836.00

Total : 3,311.00 + 15,836.00

Grand Total : US$ 19,187.00 (Personnel
US$ 13,922.00 and Others
US$ 5,265.00)

Conversion Rate US$ 1.00 = Tk. 20.00