ETHICAL REVIEW COMMITTEE, ICDDR,B. 2 7/8/90

cipal Investigator Dr. Khaleda Haider Trainee Investigator (if any) _____

ication No. # 90-014 Supporting Agency (if Non-ICDDR,B) _____

e of Study : Genetic analysis and notypic correlation of the Plas- universally present in strains . dysenteriae type 1.

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

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

Source of Population: NA

(a) Ill subjects Yes No
(b) Non-ill subjects Yes No
(c) Minors or persons under guardianship Yes No

Does the study involve:

(a) Physical risks to the subjects Yes No
(b) Social Risks Yes No
(c) Psychological risks to subjects Yes No
(d) Discomfort to subjects Yes No
(e) Invasion of privacy Yes No
(f) Disclosure of information damaging to subject or others Yes No

Does the study involve:

(a) Use of records, (hospital, medical, death, birth or other) Yes No
(b) Use of fetal tissue or abortus Yes No
(c) Use of organs or body fluids Yes No

Are subjects clearly informed about: 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 Yes No
7. Check documents being submitted herewith to Committee:
   ___ Umbrella proposal - Initially submit an overview (all other requirements will be submitted with individual studies).
   ___ Protocol (Required)
   ___ Abstract Summary (Required)
   ___ Statement given or read to subjects on nature of study, risks, types of questions to be asked, and right to refuse to participate or withdraw (Required)
   ___ Informed consent form for subjects
   ___ Informed consent form for parent or guardian
   ___ Procedure for maintaining confidentiality
   ___ Questionnaire or interview schedule *

* If the final instrument is not completed prior to review, the following information should be included in the abstract summary:

1. A description of the areas to be covered in the questionnaire or interview which could be considered either sensitive or which would constitute an invasion of privacy.
2. Examples of the type of specific questions to be asked in the sensitiv areas.
3. An indication as to when the questionnaire will be presented to the Cttee. for review.

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

Khaleda Haider
Principal Investigator

Trainee

A - 034371

90-014
7/8/90

APPLICATION FOR PROJECT GRANT

1.	INVESTIGATORS	:	Dr. K. Haider Dr. Shah M. Faruque Ms. S. Nahar
2.	TITLE OF PROJECT	:	Genetic analysis and phenotypic correlation of the plasmids universally present in strains of *S. dysenteriae* type 1.
3.	STARTING DATE	:	When money becomes available
4.	COMPLETION DATE	:	3 years from starting date
5	TOTAL FUND REQUESTED	:	US$ 69,950
6.	FUNDING SOURCE	:	
7.	HEAD OF PROGRAMME	:	Dr. S. Tzipori Associate Director Laboratory Sciences Division

8. AIMS OF PROJECT :

a) General aim

The study will help to clarify the role of unique plasmids in *Shigella dysenteriae* type 1 by investigating the association of plasmids with the OMP and/or LPS profiles and virulence properties.

b) Specific aims

1) To identify the plasmid-mediated changes in the OMP and/or LPS profile through (i) transfer of the plasmids to *E. coli* K-12 (ii) transfer to a plasmidless *S. dysenteriae* type 1 strain, and (iii) curing of the plasmids from virulent *S. dysenteriae* type 1 strain.

2) To study the effects of the above transformations on the virulence properties.

3) To identify essential regions on the plasmids that contribute to the synthesis of OMP and/or LPS and virulence by deletion of defined regions of the plasmids.

4) To confirm the phenotypic expression coded by the defined segments of the plasmid by cloning the defined segments in suitable vectors and by transforming laboratory strains of *E. coli* with such recombinant vectors.

5) To study the DNA-DNA homology of the plasmids with other plasmids in *Shigella* and pathogenic *E. coli* strains.

c) Significance

Many different determinants, both chromosomal and plasmid mediated, contribute to the virulence of shigellae. We have shown that *S. dysenteriae* type 1 contains three core plasmids of 140, 6 and 2 megadalton (Mdal) size. Studying the role of these plasmids in *S. dysenteriae* type 1 and identifing the plasmid-borne virulence genes, and factors influencing their expression, will help us to understand more clearly the pathogenic mechanisms of *S. dysenteriae* type 1 strains. This will be an important step towards the prevention and treatment of the disease.

9. ETHICAL IMPLICATIONS

This is a laboratory based study and laboratory strains of *S. dysenteriae* type 1 and *E. coli* will be used in the study. No studies on human beings are involved.

10. BACKGROUND

Shigella dysenteriae type 1 causes the most severe form of bacillary dysentery (1) and has been the cause of epidemics in developing countries. Among the genus *Shigella*, this is the most virulent species. The mechanism of its virulence is not yet clearly understood.

10.1. *Outer membrane protein (OMP)*

OMPs plays an important role in virulence and pathogenicity of *Shigella flexneri*, *S. sonnei* and Enteroinvasive *Escherichia coli* (2). OMPs also carry serotype and group-specific antigens (3). Interest has focussed on the role of plasmids in the virulence properties of shigellae (Table 1). A series of studies conducted over the past eight years have demonstrated that the ability to invade epithelial cells by all shigellae (4,5) and enteroinvasive *E. coli* (EIEC) (6) is mediated by a large plasmid. This plasmid (120-140 Mdal) also contains genes responsible for Congo red binding (7); contact hemolysin (8) and unique outer membrane proteins (OMP) (2). Seven of these OMPs appeared to be associated with the virulent phenotype in both *Shigella flexneri* and EIEC. Recently we have reported (9) that in addition to the 140 Mdal plasmid, two other plasmids with molecular sizes of 6 and 2 Mdal, are present in a significant number of *S. dysenteriae* type 1 strains obtained from widely scattered geographic locations. We have observed that loss of either, 140, 6 or 2 Mdal plasmid does not affect the synthesis of major polypeptides in *S. dysenteriae* type 1 strains (10). However simultaneous loss of all the three plasmids lead to a significant difference in the OMP profiles (10). These results indicate that genes carried by all the three unique plasmids and perhaps also the chromosome might be required for the synthesis of major OMPs

in *S. dysenteriae* type 1. The possible interactions between these different locations of genes might be important, because pathogenicity in these bacteria has been reported to be multifactorial and requires a number of chromosomal as well as plasmid-mediated factors for its expression (4,11,12).

10.2. *Lipopolysaccharide (LPS)*

Studies carried out recently showed that plasmids may also be involved in the expression of lipopolysaccharide (LPS) in several *Shigella* spp. (Table 1) (13,14). The importance of plasmids in virulence of shigellae has also been shown by the observation that a 120 Mdal plasmid was present in virulent Form I of *S. sonnei* but absent in avirulent Form II (15). Further studies showed that in *S. sonnei* most and probably all genes necessary for the expression of the O-antigen are located on a 120-Mdal plasmid which, in addition, carries genes essential for invasiveness. The O-antigen has been suspected as an essential virulence factor in *Shigella* spp and is encoded by the 6 Mdal plasmid (13), as well as chromosomal DNA in *S. dysenteriae* type 1 strains (16). Recently we have found that each one of the 140, 6 and 2 Mdal plasmids in *S. dysenteriae* type 1 strains have some sort of involvement in the synthesis of the O-antigen (10). Qadri *et al.* have shown that *S. dysenteriae* type 1 can agglutinate erythrocytes (17) and that hemagglutination observed with *S. dysenteriae* type 1 is associated with LPS (unpublished observation). This again suggests the importance of LPS in the virulence of *S. dysenteriae* type 1 strains and it indicates that the "core" plasmids might have some role to play in the pathogenic mechanisms of *S. dysenteriae* type 1 strains.

Sugar analysis of LPS has suggested that the LPS of the mutants lacking any one of the three plasmids, is deficient in either Rhamnose, Galactose or *N*-acetylglucosamine. Strain PSD-10 lacking all three plasmids (140, 6 and 2 Mdal) is autoagglutinable in normal saline and is completely deficient in

all three sugars (unpublished observation). This indicates that these strains might be deficient in the gene(s) coding for an enzyme(s) responsible for addition of O-antigen repeat sugar units to the LPS-core. Thus they produce lesser amounts of the O-antigen or not. It is also possible that plasmid-borne genes may encode factors that regulate chromosomal determinants of the LPS. However, detailed biochemical and genetic studies are required to understand the mechanism.

The role of Shiga toxin in the pathogenesis of dysentery due to *S. dysenteriae* type 1 is not completely clear, but appears to be important. Little information is available on the genetics of Shiga toxin production but the genes for this toxin appear to be located on the chromosome (17). However, it will be important to know whether toxin production by strains of *S. dysenteriae* type 1 is influenced by the different 'core' plasmids.

In relation to cellular invasions by *Shigella* spp. there are two steps where the molecular basis is still unclear. These are the process of bacteria-induced cell phagocytosis and the lysis of the pagocytic vaccules. Although it has been reported that these steps are mediated by extrachromosomal determinants in some *Shigella* spp. (6) but the molecular basis of such observations yet remains to be determined.

We have isolated strains of *S. dysenteriae* type 1 which carry either one or two of these three plasmids in different combinations (Table 1). Although, the role of the large 140 Mdal plasmid in pathogenesis of *Shigella* spp. has been studied to some extent, the role of the smaller unique plasmids (the 6 and the 2 Mdal) in the pathogenicity of *S. dysenteriae* type 1 is not clear. The 2-Mdal plasmid is also present in some *S. flexneri* (18) and in *S. dysenteriae* type 2 strains (19), which indicate that many different

determinants, both chromosomal and plasmids, may contribute to the pathogenesis of *Shigella* spp.

The apparent complexity of the factors determining virulence of shigellae has been the main obstacle in the development of an effective vaccine. A better understanding of the functions of the small plasmids in *Shigella* spp. is the major objective of this study.

Table 1

Virulence plasmids in *Shigella* spp: Summary

Size of Plasmid (Mdal)	Species	Virulence determinants
140	*S. flexneri* *S. dysenteriae* type 1 *S. boydii*	invasiveness, congo red binding, contact hemolysin
140	*S. flexneri*	OMP
120	*S. sonnei*	invasiveness, congo red binding, LPS, OMP
6	*S. dysenteriae* type 1	LPS
2	*S. dysenteriae* type 1 *S. dysenteriae* type 2 *S. flexneri*	Not known

* The 120-140 Mdal plasmids are referred to as large plasmid and the 6 and 2 Mdal plasmids are referred to as small plasmids in the text.

MATERIALS AND METHODS

A) Bacterial Strains

Nine strains of *Shigella dysenteriae* type 1 will be studied (Table 1). Strain Z-24623 was isolated from a patient at the ICDDR,B, Dhaka treatment centre. The other 8 strains were derived from virulent strains after repeated subculturing (approximately 15 times) on MacConkey's agar (DIFCO Laboratories, Detroit, MI). The roughness of the avirulent mutants was ascertained by autoagglutination in physiological saline and were tested with rough specific phages. All these strains are stored at -70°C in trypticase soy (TS) broth (GISBCO Diagnostics, Madison, WI) with 15% glycerol.

Smooth strains were serologically identified by slide agglutination tests with *S. dysenteriae* type 1 antisera. The characteristics of these strains are as follows :

TABLE 2

Strains No.	Plasmid(s) size (Mdal)						*Drug resistance pattern	Salt aggregation test values
	140	90	20	6	4	2		
Z-24623	+	-	-	+	+	+	CST Sxt	1.5
PSD-1	-	-	-	-	-	+	Sensitive	0.25
PSD-10	-	-	+	-	+	-	CS	aa
PSD-20	-	-	-	+	-	-	Sensitive	1.0
PSD-30 (128 Mdal)	+	-	-	-	-	-	CS	aa**
PSD-40	-	+	-	-	-	-	ACS	aa
PSD-50	+	-	-	+	-	-	CSTSxt	1.0
PSD-60	-	-	-	+	-	+	CST	1.0

*C = chloramphenicol; S = streptomycin; T = tetracycline; Sxt = trimethoprim sulphamethexazole; A = ampicillin

**aa = auto aggregation

B) Research Plan

1. The small plasmids will be cured and or transferred to a strain of *S. dysenteriae* type 1 lacking this plasmid and/or into *E. coli* K-12 strain either by conjugation or transformation to study the following phenotypic expressions: Methods are given in Appendix A.

   a) Change in patterns of OMPs

   b) Change in pattern of LPS

   c) Change in the following virulence properties:

      i) Invasiveness as determined by Sereny's test and tissue culture invasion

      ii) Haemagglutinating ability

      iii) Adherance properties

      iv) Level of toxin production

      iv) Contact hemolysis

2. The smaller plasmids will be isolated and analyzed by restriction digestions and a restriction map will be prepared to define segments of the plasmids (Appendix B).

3. Defined regions of the plasmids will be deleted and the modified plasmids will be introduced into *S. dysenteriae* type 1 and other strains (mentioned in step 1) to study the effect of deletions. This will identify essential regions associated with the expression of properties mentioned in step 1.

4. Defined essential regions of plasmids (step 3) will be cloned in suitable vectors to study the phenotypic expression of such defined fragments of the plasmids. The procedures involved are given in Appendix B.

5. Defined segments will be used as probes to study DNA-DNA homology with other plasmids from *Shigella* spp. and pathogenic *E. coli* strains (Appendix B).

Abstract

Shigella dysenteriae type 1 strains contain three "core" plasmids of molecular size 140, 6 and 2 megadalton (Mdal). Preliminary results of our previous studies indicate that the core plasmids have some role in the virulence of *S. dysenteriae* type 1 strains. This study aims to identify the roles of these plasmids in infections due to *S. dysenteriae* type 1 and strains to identify the genetic determinants responsible. In the first part of this study the phenotypic expression coded by these plasmids will be studied by curing the plasmids and/or by transferring them into both *E. coli* K-12 and a plasmid-less strain of *S. dysenteriae* type 1 to produce isogenic strains. This will also help us understand whether all the three unique plasmids are essential in the expression of virulence properties. The essential regions of the plasmids associated with synthesis of OMP and/or LPS will also be identified through deletion and/or colonies of defined segments of the plasmids. This study will clarify the function of plasmids in virulence of *Shigella* spp. elucidate the pathogenesis of disease and possibly contribute towards developing an effective vaccine in future.

FLOW CHART I

Activities of the Laboratory, 1-3 years

First year

1) To transfer plasmids to plasmidless strains of both *Shigella* and *E. coli* K-12 by conjugation and/or transformation technique

2) Curing of plasmids by standard methods

3) Changes in OMP profiles due to the effect of transferring the plasmids (step 1 and 2)

4) To study the effect of transformations on the expression of properties, such as invasiveness, adherence, haemagglutination, level of toxin production, etc.

Second year

5) Changes in LPS profiles due to the effect of transformation and curing of the plasmid

6) Restriction mapping of the plasmids to define segments of the plasmid(s)

7) Construction of deletions

8) Studying the effects of deletions on expression of virulence properties

Third year

9) Cloning of defined segments of plasmid(s)

10) Studying the expression of cloned fragments

11) Study of DNA-DNA homology of such fragments with other plasmids and chromosomes

12) Analysis of data

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LIST OF PUBLICATIONS OF DR. KHALEDA HAIDER (last five years)

1. Haider K, Albert J, Tzipori S. Suitability of MacConkey's medium for direct testing of enterotoxigenic *E. coli*. J Med Microbiol [under review process].

2. Haider K, Huq MI, Talukder KA, Ahmad QS. Electropherotyping of plasmid deoxyribonucleic acid (DNA) of different serotypes of *Shigella flexneri* strains isolated in Bangladesh. Epidem Infect 1989; 102(3):421-8.

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TASK OF INDIVIDUAL INVESTIGATORS

Dr. Khaleda Haider

1. Perform transfomation, conjugation and curing of plasmids

2. Analysis of OMP and/or LPS profiles on SDS-PAGE of both donor and recipient strains

3. Test virulence properties, such as Sereny's test, HeLa cell invasion, contat haemolysis, adhererence, haemagglutination, etc. of both donor and recipient strains.

Dr. S. M. Faruque

1. Isolation and restriction mapping of plasmids

2. Construction deleted of plasmids

3. Cloning of defined plasmid fragments

Dr. S. Tzipori

1. Coordinate the work
2. Provide intellectual guidance
3. Assist with analysis of the data
4. Assist with documentation

KH:pm:mh/J6:PLASMID1.PRO

BUDGET SUMMARY

[in US$]

First year			
Persosnnel	...	$ 0.00	
Capital equipment	...	800.00	
Operating cost	...	10.00.00	
			$ 10,800.00
Second year			
Personnel	...	0.00	
Operating cost	...	12,000.00	
			12,000.00
Third year			
Personnel	...	33,000.00	
Operating cost	...	14,150.00	
			47,150.00
TOTAL COST			$ 69,950.00

DETAILED BUDGET

a) Personnel cost

1st and 2nd year

Salaries of Dr. Khaleda Haider
Dr. S. M. Faruque
Ms. Shamsun Nahar
will be supported by other protocols for the first and the second year

3rd year

Dr. Khaleda Haider NO-B (100%)...	10,000.00
Dr. S. M. Faruque NO-C (100%) ...	18,000.00
Ms. Shamsun Nahar GS-V (100%) ...	5,000.00
	33,000.00

b) Operating cost

1)	Supplies	
	- Stationaries ...	1,500.00
	- Xerox and duplication	300.00
	- Medical illustration	300.00
	- Library ...	50.00
		2,150.00
2)	Cost for laboratory tests	
	- Glasswares	
	- Reagents and chemicals	
	- Media	
	- Animal	
	- Restriction enzymes	30,500.00
	- Radioisotopes	
	- Filter papers	
	- X-ray films	
3)	Travel ...	3,000.00
4)	Publications ...	500.00

c). Capital equipment

Electrophoresis kit ...	600.00
Micropipettes ...	200.00
	800.00

APPENDIX A

Sereny test

Sereny's test will be performed by inoculating 20 µl of 3 x 10^8 CFU ml^{-1} of *S. dysenteriae* 1 into the eye of adult guinea pigs (20). Strains producing keratoconjunctivities within 72 h will be considered virulent.

Invasion of HeLa cells

18 h culture of all strains adjusted to a concentration of 2 x 10^8 CFU/ml will be used to infect nonconfluent HeLa cell monolayers by using the method described by Hale *et al.* (21).

Analysis of plasmid deoxyribonucleic acid (DNA)

Plasmid DNA will be extracted from the strains by a modification of the method of Birnboim and Doly (22). Separation will be carried out by agarose gel electrophoresis (23). Reference marker plasmids of pDK-9 (140-, 105-, 2.7-, and 2.0-Mdal), R1 (62-Mdal), RP4 (36-Mdal) and Sa (23-Mdal) will be used to determine the molecular mass of the plasmid DNA of *S. dysenteriae* type 1 strains.

Toxin assay

Differences in level of toxin production will be tested using two fold dilution of toxin by standard techniques (24,25).

Outer membrane proteins

Shigella strains will be grown in 100 ml of TS broth containing 0.3% yeast extract at 37°C for 18 h. After centrifugation, the bacterial pellets will be suspended in 80 ml of deionized water and incubated at room temperature for 1 h with low shaking (60 rpm on a rotary shaker) by the method described previously (26). After removal of bacterial cells by centrifugation at 16,000 X *g* for 20 min, the supernatant will again be centrifuged at 100,000 X *g* for 2 h. The clear supernatant will be dialyzed against buffer and concentrated against polyethylene glycol (PEG 15,000-20,000) and will be stored at -20°C.

Polyacrylamide gel electrophoresis (PAGE)

Isolated OMPs will be analyzed by SDS-PAGE (27) employing separating gels containing 13.5% acrylamide and stacking gels containing 5% acrylamide. Electrophoresis will be carried out at a constant current of 10 mA per gel overnight. Gels will be stained with Coomassie brilliant blue R-250 solution (0.2%) for the detection of proteins.

Extraction and analysis of LPS

LPS will be extracted from the strains by the modified phenol-water extraction procedure (28) and purified by ultracentrifugation at 105,000 X *g* for 4 h. Purified preparations of LPS will be analysed by electrophoresis in 12% SDS-polyacrylamide gels (27). LPS separated on the gel will be visualized by the silver staining technique of Hitchcock and Brown (29).

Hemagglutination (HA) will be carried out on slides (17) by using 30 µl of 2% (vol/vol) erythrocytes in 0.01 M sodium phosphate buffer (pH 6.8) containing 0.154 M sodium chloride (PBS) and an equal volume of bacterial suspension (5 x 10^{10} CFU/ml). HA will be tested with guinea pig,human (types A,B,O) or erythrocytes obtained from other species as has been described previously (17). However for most purposes assays will be routinely carried out with guinea pig and human (type O) erythrocytes.

Bacterial adhesion to cultured epithelial cells.

Henle intestinal 407 cells (Int.407)(Flow laboratories, Inc., Herts, England) will be maintained in Basal Eagle Medium (Flow) supplemented with 15% newborn calf serum (Flow) and 2 mM glutamine. Eighteen hours before the adhesin assay cells will be seeded (1 x 10^5) in culture vials (Kimble, Toledo, Ohio) containing cover slips (diameter 12 mM). Bacterial inoculum (1 x 10^9 CFU in 100 µl) will be applied to culture vials and centrifuged at 500 X *g* for 10 min at 25°C. The vials will be incubated for a further 50 min at 37°C in 5% CO_2 and then vials will be washed for six times in PBS to remove non-adherent bacteria. Cover slips will be fixed with methanol and stained with Giemsa and will be examined under a microscope (X1000).

Transfer and curing of plasmids

Plasmids will be transferred between strains by either conjugation or by isolation of the plasmid DNA followed by transformation of the recipient strain. Plasmids will be isolated by modification of the alkaline lysis method of Birnboim and Doly (24) and will be purified by using a commercially available column (NACS-52 PREPAC, BRL). Bacterial strains will be transformed by the calcium chloride procedure (30). The experiments of the transformants will be selected either by using probes made with 2 or 6 Mdal plasmid or by tagging this plasmids with suitable marker and transformants will be selected accordingly. Plasmids will be cured according to standard procedures (31,32) and will be identified by using the above DNA probes. Plasmid-cured colonies will not hybridized with

APPENDIX B

Restriction analysis of plasmids

Plasmids will be digested with appropriate restriction enzymes by standard procedure and the digested DNA will be electrophoresed in agarose gels. The size of fragments produced will be determined by comparing with molecular size markers. The data will be used to construct a restriction endonuclease cleanage map for each of the plasmids.

Preparation of probes

Defined restriction fragments of plasmids will be isolated from agarose gels by electroelution (30) and will be used as probes in determining homology between other plasmids, genomic DNA and total RNA preparation.

Radioactive labelling of DNA probes

The probe DNAs will be radioactively labelled by the method of Feinberg and Volgelstein (33,34) with α-^{32} P-dATP (10 µCi/µl, 3000 Ci/mM, AMERSHAM) and oligonucleotide primers ($P(dN)_6$, PHARMACIA) using the Large Fragment of *E. coli* DNA polymerase I. In case of small DNA probes (less than 200 base pairs) the method of choice will be 5-end labelling with $-^{32}$P-dATP using a 5 end-labelling kit (BRL). Radio-labelled probes will be denatured by boiling followed by quick chilling on ice, before using these for hybridization experiments.

Hybridization of DNA blots

Hybridization with labelled probes will be carried out as described by Maniatis *et al.* (30). The filters will be prehybridized in the presence of denatured salmon sperm DNA to block non-specific binding sites on the filters, and will then be hybridized with the denatured probe DNA for 12-16 hours at the appropriate temperature. After hybridization, the filters will be washed under conditions of increasing stringency.

Preparation of autoradiographs

The hybridized filters will be exposed to X-ray films in metal cassettes at -70^{o}C for the appropriate time. In case of weak signals, intensifying screens will be used. The exposed X-ray films will be developed and fixed by standard procedure.

Construction of modified plasmids

Specific DNA fragments will be deleted from plasmids by digesting with appropriate restriction endonuclease and or an appropriate exonnclease. The desired fragment of plasmid containing an origin of replication will be resealed by T4 DNA ligase to produce a smaller modified plasmid. Alternatively small restriction fragments will be cloned into other plasmid vector to produce recombinant plasmid as described by Maniatis *et al* (30).

Project title: Genetic analysis and phenotypic correlation of the plasmids universally present in strains of S. dysenteriae type 1

Principal Investigator(s): Drs. K. Haider, S.M. Faruque

Summary of Referee's Opinions: Please see the following table to evaluate the various aspects of the proposal by checking the appropriate boxes. Your detailed comments are sought on a separate, attached page.

	Rank Score		
	High	Medium	Low
Quality of Project			
Adequacy of Project Design			
Suitability of Methodology			
Feasibility within time period			
Appropriateness of Budget			
Potential value to field of knowledge			

CONCLUSIONS

I support the application:

a) without qualification ☐

b) with qualification:
- on technical grounds ☑
- on level of financial support ☐

I do not support the application ☐

Name of Referee: T. Glenn Morris Jr. MD

Position: Associate Professor of Medicine

Institution: University of Maryland School of Medicine

T. Glenn Morris

Signature

4/19/90

Date

This proposal is designed to evaluate the significance of two small plasmids that have been consistently found in strains of S. dysentariae type 1. The investigators propose to evaluate the function of these plasmids by transferring them into E. coli; to screen other enteric bacteria for the presence of homologous plasmids; to define critical regions on the plasmids by deleting defined plasmid regions; and to do mRNA and minicell studies to study gene expression.

The proposal is an interesting and ambitious one. Its major problem is its lack of focus. Specifically, there is no clearly defined hypothesis that is being tested. There is a suggestion that the two plasmids under study influence OMP or LPS expression. If this is true, then I would suggest that the investigators focus on one of these factors, and attempt to identify and clone the responsible genes from the plasmids. The methodology outlined is very reasonable, but I would prefer to see it directed toward characterization of a single factor - such as an OMP. If these studies are to lead toward vaccine development (a very reasonable goal) then I would also like to see some consideration of the role of plasmid factors in pathogenicity - i.e., are deletion mutants more or less virulent in animal models?

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Bangladesh.

April 3, 1990

CONFIDENTIAL

Dr. Saul Tzipori
Associate Director
Laboratory Sciences Division
ICDDR,B

Dear Dr. Tzipori :

Attached please find my comments on the protocol entitled "Genetic analysis and phenotypic correlation of the plasmids universally present in strains of S. dysenteriae type 1".

Thanking you.

Sincerely yours,

Dr. Zia U. Ahmed
Senior Scientist

Enclo: as above.

ZUA:pm

Comments on protocol entitled "Genetic analysis and phenotypic correlation of the plasmids universally present in strains of *S. dysenteriae* type 1".

It is interesting to note that the authors have proposed to carry out a large number of experiments within a relatively short span of time (2 years) and at almost negligible cost ($ 35,000).

Much time and energy have been put into developing the protocol. However, considerable room exists to substantially improve upon the conception and design of the study.

Three plasmids - 140,6 and 2 MDa-seem to occur in most clinical isolates of *Shigella dysenteriae* 1. Loss of one of these plasmids does not change OMP profile, but in one strain where all the three plasmids were absent, there was a change in the OMP profile. This suggested to the authors that these plasmids may be related to the synthesis of some OMP or other virulence-related components. Based on this assumption, the authors developed a rather long list of experiments to test the functional biology of the plasmids which includes tests as to whether these plasmids

a. change the OMP and LPS profiles,

b. affect the Sereny-reaction, invasion of cultured cells and cytotoxicity,

(The former two properties are mediated by the 140 MDa plasmid and the latter effect is caused by the Shiga-toxin of which there are about 10 genes scattered in the chromosome. Thus, these experiments will not produce meaningful results).

c. cause changes in the level of Shiga-toxin production.

(Assay of Shiga-toxin quantitatively using a cell culture method is not likely to reveal much information. One should use an ELISA based on a monoclonal antibody).

The plasmids also be tested for a host of other effects which are poorly defined. For example, some competition with *E. coli* and *Klebsiella* during mixed growth *in vitro* is to be expected because it is a common phenomenon that one encounters during mixed growth of microbes. It is not easy to establish the basis of such competition and its *in vivo* significance is certainly most difficult to appreciate.

The important questions that have not been addressed by the authors are :

1. How these cryptic plasmids will be selected in a conjugation or transformation experiment or, how will these be cured? One need to develop a sensitive selection to detect plasmid recipients at a frequency of 10^{-6} or less. This is a critical requirement for the study and yet it has not been mentioned anywhere in the protocol.

2. The authors seem to assume (as evident in Research plan, p.8) that genes for OMP, LPS, Sereny reaction, toxin production, invasion of cultured cells, contact hemolysis, hydrophobicity, antibiotic resistance, growth rate, nutritional traits and tolerance to low pH are likely to be carried by these plasmids. A careful look at the literature would suggest otherwise.

3. Further studies proposed in the Research Plan include mRNA analysis of a sort the significance of which is not clear to me.

4. Minicells will be used , it has been said, to analyse plasmid-coded peptides but methods have not been outlined.

On the whole, it seems to me that the authors are out to test many interesting but disjointed questions. To localize and characterize only one virulence function could take a few years. It is interesting to note that proposal aims at studying a number of such functions in only two years. The statement made in line 8, p.4 under reference # 9 seems to be quite a sweeping remark on O-antigen synthesis. It is in press, but a careful evaluation of the evidence is necessary.

The authors should evaluate the assumption that since these two plasmids are almost always found in clinical isolates, these must relate to virulence. Tempting as this assumption may be, it is most difficult to approach experimentally. There is much seemingly useless DNA in the biosphere. If the biology of these plasmids is of genuine interest to the authors for some reasons, then attention should be focused on only one or two most propable questions (which have to be carefully discerned) and methodology should be developed to answer those questions as clearly as possible. The team of scientists seems to be very enterprising and well-read. They could be productive if their theoretical knowledge is propelled to addressing more defined and test-worthy questions.

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rincipal Investigator(s): Drs K. Haider, S. M. Faruque

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otential value to field of knowledge			✓

CONCLUSIONS

I support the application:

a) without qualification ☐

b) with qualification:
- on technical grounds ☑
- on level of financial support ☐

I do not support the application ☐

Name of Referee: Zia U. Ahmed

Position: Senior Scientist

Institution: ICDDR, B

Zia U. Ahmed
Signature

3 April 1990
Date