38

Attachment 1. Date _____

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Principal Investigator L. Gothefors

Application No. 81-001 (P)

Title of Study Identification of Colonization Factors (CFA) in E. coli

Trainee Investigator (if any) _____

Supporting Agency (if Non-ICDDR,B) _____

Project status:
(X) 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 } N.A.
   (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 NA
   (h) Compensation &/or treatment where there are risks or privacy is involved in any particular procedure Yes No NA
5. Will signed consent form be required:
   (a) From subjects Yes (No)
   (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)
   NA 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)
   NA Informed consent form for subjects
   NA Informed consent form for parent or guardian
   NA Procedure for maintaining confidentiality
   NA 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.

Principal Investigator

Trainee

81-001(P)
recd 16/1/81

SECTION I - PILOT STUDY

1. Title : IDENTIFICATION OF COLONIZATION FACTORS (CFA) IN E. COLI

2. Investigators : L. Gothefors, I. Huq, A.M. Svennerholm, C.Ahren, B. Stoll

3. Starting Date : 1st January, 1981

4. Completion Date : 31st March, 1981

5. Total Direct Cost : US $ 2,600

6. Scientific Program Head :

This protocol has been approved by the combined Pathogenesis & Host Defence Working Group.

Signature of Scientific Program Head M M Rahaman

Date 7 - 1 - 1981

7. Abstract Summary:

A limited protocol is proposed to examine faecal E.coli strains for the presence of different colonization factor antigens as well as for the production of enterotoxin. The strains will be fresh isolates from patients in the ongoing surveillance study (protocol 80-005), patients in our hospital and patients (without diarrhoea) from the surgical wards at the Medical College Hospital. The CFAs will be identified by their capacity to agglutinate various types of erythrocytes. Finally, the prevalence of toxin production and CFA will be evaluated versus the clinical features of the patients.

SECTION II - PLAN OF PILOT STUDY

A. INTRODUCTION

The study of the enterotoxic enteropathies is clearly shifting from early emphasis on the toxins themselves to the sequence of events leading to efficient toxin delivery. These include safe passage of vibrios through the stomach, adherence to and later colonization of the mucosal surface.

It is necessary to assess the importance of each of these events. It is then possible to determine more precisely the contribution of immune mechanisms directed against each of these factors to the overall resistance to reinfection which follows clinical illness.

1. Objectives

To study various E. coli strains for the presence of colonization factors and their relation to toxin production.

2. Background Information

ETEC is isolated from 25% of diarrhoea cases at Matlab Hospital (1) and thus has a major impact on the morbidity and mortality of the inhabitants of rural Bangladesh. Most strains isolated produce ST alone or ST and LT and this toxin production is used as an epidemiologic tool, sometimes in parallel to routine serotyping.

ETEC, however, appear to require accessory virulence properties - in addition to the enterotoxin - to be pathogenic for humans or animals. The best recognized accessory virulence properties are adherence or

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colonization factors that enable ETEC to attach to the mucosa of the small intestine.

Some animal ETEC strains possess plasmid mediated, pili-like surface organelles of attachment, such as K88 and K99 antigens, that are associated with mannose-resistant hemagglutination (MRHA). Evans et al (2) have described analogous pili in human ETEC strains that are identifiable by their patterns of MRHA with various types of erythrocytes; these pili have been labelled colonization factor antigens I and II (CFA/I, CFA/II).

Controversy exists as to the prevalence of these antigens in human ETEC strains and whether they are required by all (or most) ETEC strains in order to manifest virulence, intestinal colonization and immune response in humans. Evans et. al [3] reported that 25(86%) of 29 ETEC strains from traveller's diarrhoea possessed CFA/I. In contrast Gross et. al [4] examined 89 ETEC pathogens and found that < 10% reacted with antibody to CFA/I. Levine et.al in a recent study [5] of ETEC strains used in volunteer challenge studies reported that out of eight strains causing diarrhoea only two produced CFA. Because the MRHA-type pili are plasmid-mediated [6], one possible explanation for the divergent results is that in the survey recording a low prevalence, the strains could have lost their CFA plasmids prior to the time of testing [5]. (Testing for toxin production is also recommended to be done as early as possible after isolation.)

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In an attempt to resolve this confusion we will take stools from patients in the surveillance study, from hospital patients and from non-diarrhoeal patients. Fresh E. coli isolates will be examined for the presence of CFA/I, CFA/II and type 1 somatic pili and possible other hemagglutinins. The same isolates will also be tested for LT and ST production.

B. SPECIFIC AIMS

a. To study the distribution of colonization factors in E. coli causing diarrhoea and in non-diarrhoeagenic E. coli from the Dacca area.

b. To specify "new" colonization factors by using an extended haemagglutination system.

c. To set up haemagglutination-methods in our laboratory which later on can be used as epidemiological tools in parallel to testing for LT and/or ST.

C. METHODS OF PROCEDURE

1. SUBJECTS

Samples from three categories of patients will be studied (50 individuals in each group):

a. patients already enrolled in the surveillance study. Stool samples from them are already tested for LT/ST after storage for some time, but this will now be done on fresh isolates.

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b. hospital patients with moderate to severe watery diarrhoea of 36 hrs. duration:

- dark-field and cholera culture negative
- clinical picture not suggesting dysentery

c. patients from the surgical ward of Dacca Medical College with non-diarrhoeal problems.

2. LABORATORY INVESTIGATIONS

Three E.coli colonies will - without further storing or repeated sub-culturing - be tested for:

1. LT production - GM$_1$ELISA
2. ST production - infant mouse essay
3. CFA - haemagglutination

For details of the haemaglutination please see attached Addendum.

3. CLINICAL FEATURES

As this is a pilot study only, the severity of the clinical symptoms will be roughly estimated from the already existing data sheets (surveillance study), and from hospital records.

D. SIGNIFICANCE

This study will enable us to know the frequency of various colonization factors in E. coli strains causing diarrhoea in Bangladesh. If these antigens are proven to be responsible for the colonization,

they may have an important role as a component in a combined vaccine to prevent human diarrhoea due to ETEC.

They can also be used in clinical and epidemiological studies in parallel to essay of enterotoxinproduction.

E. FACILITIES REQUIRED

1. Office space	:	No additional space
2. Laboratory space	:	For the haemagglutination in the microbiological branch
3. Hospital resources	:	No extra
4. Animal resources	:	Nil
5. Logistic support	:	Personnel and transport for sampling at Dacca Medical College
6. Major items of equipment	:	Nil

Bacteria

Media

For diagnosis of pili on "new" strain fresh isolates are cultivated on CFA-agar. (18 hrs. 37°C).

1%	Casamino acids	Solve in H_2O, autoclave and pour in Petri dishes.
0.15%	Yeast extract	
0.005%	Mg SO_4	
0.0005%	Mn Cl_2	
2% agar		

Type 1 pili (mannosesensitive) are most readily identified on bacteria grown in broth (still - not shaken, large surface) over night at 37°C.

Strains with other pili could be cultivated on ordinary blood agar plates (or corresponding media).

Storage

CFA I and CFA II strains should be stored in aliquots in -70°C. Other strains may be stored as deep agar stabsor freeze dried.

Haemagglutination (HA)

HA is performed in blood with 1% mannose added and with guineapig erythrocytes also without added mannose. 15 ul of bloodsuspension (+4°C) is put on a glass slide. Several colonies of the bacteria to study are harvested and carefully mixed in saline on the slide with the erythrocyte suspension. Read at room temperature. Then leave the slide on ice (+4°C) for a couple of minutes before reading the test again. If positive "true" agglutinates (different from what is seen with the bacteria in saline or in the buffer with blood) should have been performed. The tubes with broth cultures are centrifuged and decanted and the pellets are resuspended with saline to a concentration of 10^{10} organisms/ml. Do always make positive controls (see reference systems) every second week.

Positive HA is graded 0,+,++ and +++.

ADDENDUM

Identification of different pili structures (colonization factors) with hemagglutination

Reference Systems

Strain	Pili	Erythrocytes	Mannossensitive
H10407++	CFA I	Human A	-
411=5 +	CFA II	Bovine (ox)	-
286 C2	type 1 pili	guineapig	+

Erythrocytes

Besides the erythrocytes listed we are planning to use red blood cells from a few other species goat, chicken and rat for example.

Blood is collected once weekly in heparinized tubes. The erythrocytes are spinned down and washed 3-4 times in PBS. The cells are then diluted in saline to 3% final concentration with or without 1% mannose.

Erythrocytes should be fresh: not kept longer than 4-5 days (at +4°C)

REFERENCES

1. Merson, H.H. et al: Epidemiology of Cholera and Enterotoxigenic Escheridiea Coli Diarrhoea. In Cholera and Related Diarrhoeas, 43rd Nobel Symposium (1980).

2. Evan, D.G. and Evans, D.J. New surface-associated heat-labile colonization factor antigen CFA/II) produced by ETEC of serogroups 06 and 08. Infect. Immun. 21: 638 (1978).

3. Evans, D.G. et al : Detection and characterization of colonization factor of enterotoxigenic E. coli isolated from adults with diarrhoea. Infect. Immun. 19 : 727 (1978).

4. Gross R.J. et al : The occurrence of colonization factor (CF) in enterotoxigenic E. coli. Federation of European Microbiological Societies Letters 3 : 931 (1978).

5. Levine, M.M. et al : Haemagglutination and colonization Factors in enterotoxigenic and enteropathogenic Escherichia coli that cause diarrhoea. J. Infect Dis 141 : 733 (1980).

6. Evans, D.G. et al : Plasmid-controlled colonization factor associated with virulance in Eschericia coli enterotoxigenic for humans. Infect. Immun. 12 : 656 (1975).

Abstract Summary

A limited protocol is proposed to examine faecal E.coli strains for the presence of different colonization factors (CFA) as well as for the production of enterotoxin. The strains will be fresh isolates from patients in the ongoing surveillance study (protocol 80-005), patients in our hospital and patients (without diarrhoea) from the surgical wards at the Medical College Hospital. The CFA's will be identified by their capacity to agglutinate various types of erythrocytes. Finally, the prevalence of toxinproduction and CFA will be evaluated versus the clinical features of the patients.

The main objective of this study is to establish the frequency of different CFA in E.coli in Bangladesh.

This knowledge can be useful in epidemiological studies, but can also give information crucial for the production of a vaccine.

This project involves no interviews, physical, psychological, social, legal or any other risks, and a signed consent is therefore not required.

SECTION III - BUDGET

					Project Requirement	
1.	Personnel	Effort	Time	Amount	Taka	Dollars
	L. Gothefors	10%	3 months	$ 40,000	-	1,000.00
	Field worker	-	1 month	-	4,000.00	-
	Lab. technician	100%	3 months	-	6,000.00	
2.	Supplies and Materials					
	100 ST-test (300 mice)				600.00	
	150 LT-test (GM_1 Elisa)				2,250.00	
	Plastics glassware					300.00
	750ml blood for HA				750.00	
	One ox (to be bleeded weekly)				2,500.00	
3.	Equipment					
	0					
4.	Hospitalization cost					
	0					
5.	Outpatient cost					
	0					
6.	ICDDR,B Transport					
	To DMCH: 6 miles/ day x 20 days = 120 miles a Taka 2/mile				240.00	
7.	Travel					
	0					
8.	Transport of things					
	0					
9.	Rent, communications, utilities					
	0					
10.	Printing : Forms, stencil, xerox				1,000.00	100.00
11.	Contractual service					
	0					
12.	Construction					
	0					
				Grand Total :	17,340.00	1,400.00
				US $	2,600.00	