Attachment 1.

Date 29-6-19

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Principal Investigator Dr.Mahmudul Haque Trainee Investigator (if any)

Application No. Rc/03/90 & Dr. Firdousi Qadri Supporting Agency (if Non-ICDDR,B)

Title of Study Studies on virulence factors of Helicobacter pylori strains isolated from peptic ulcer patients. Project status:

( ) New Study
( ) Continuation with change
( ) No change (do not fill out rest of fo

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 herew Committee:
   ___ Umbrella proposal - Initially sub overview (all other requirements be submitted with individual stud
   ✓ Protocol (Required)
   ✓ Abstract Summary (Required)
   ___ Statement given or read to subjec nature of study, risks, types of ions to be asked, and right to re to participate or withdraw (Requi
   ✓ Informed consent form for subject
   ___ Informed consent form for parent guardian
   ___ Procedure for maintaining confide ity
   ___ Questionnaire or interview schedu

* If the final instrument is not compl prior to review, the following infor should be included in the abstract s

1. A description of the areas to be covered in the questionnaire or interview which could be conside either sensitive or which would constitute an invasion of privac
2. Examples of the type of specific questions to be asked in the sen areas.
3. An indication as to when the qu naire will be presented to the (for review.

(PTO)

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

Dr. Hasan

SECTION I - RESEARCH PROTOCOL

1. TITLE : STUDIES ON VIRULENCE FACTORS OF HELICOBACTER PYLORI STRAINS ISOLATED FROM PEPTIC ULCER PATIENTS

2. PRINCIPAL INVESTIGATORS :
Dr. Mahmudul Haque
Medical Officer
Department of Microbiology
Institute of Postgraduate Medicine & Research(IPGM&R)

Dr. Firdousi Qadri
Associate Scientist
Lab. Science Division
ICDDR,B.

CO-INVESTIGATORS :
Dr. Kazi Masihur Rahman
Professor of Microbiology
IPGM&R

Dr. Mahmud Hasan
Professor of Gastroenterology
IPGM&R.

Dr. Ruhul Amin Miah
Associate Professor of Microbiology
IPGM&R

3. STARTING DATE : Shortly after receipt of the fund

4. COMPLETION DATE : One year from the starting date of the protocol.

5. TOTAL DIRECT COST : US$ 5,490

6. COLLABORATING AGENCIES : IPGM&R and P.C.C. of ICDDR,B

7. SIGNATUE OF PRINCIPAL INVESTIGATORS 1. 2. Firdousi Qadri

8. APPROVAL OF THE HEAD OF THE INSTITUTE/ DIVISION :

1. Director IPGM&R — 2/7/90

2. Division Head Laboratory Science Division, ICDDB,B

8. ABSTRACT:

Helicobacter pylori is one of the recent discoveries that has shown a significant association with gastritis and peptic ulcer diseases. Efforts are on to establish its causal relationship with the above disorders and explain the pathogenesis of the infection. The protocol has been designed to study the virulence factors of H. pylori. Strains isolated from peptic ulcer patients will be studied for the presence of presumptive colonization factors or adhesins by examining the pattern of haemagglutination of different species of erythrocytes by the bacterium and the inhibition of haemagglutination by various chemicals. For the non-specific factor influencing colonization, the cell surface hydrophobicity will be estimated. Enzyme urease, proposed to be a prominent virulence factor of H. pylori, will be purified, its profile in different strains will be compared and its immunogenic potentiality will be assessed by western blot technique using serum from H. pylori infected patients. This will have a diagnostic and prophylactic implications.

SECTION - RESEARCH PLAN

A. INTRODUCTION

1. Objective

To study the virulence factors of H. pylori which will assist to explain the pathogenesis of H. pylori infection and clarify its role in the aetiopathogenesis of gastritis and peptic ulcer diseases . The study will purify urease(proposed to be a prominent virulence factor of H. pylori), compare its profile in various strains and find out its immunogenic potentiality in infected patients.

2. Background

Peptic ulcer disease is an age old medical problem having widescale prevalence all over the world. An endoscopic study[1] carried out in Dhaka described a very high prevalence of peptic ulcer(11.9% and 3.5% of duodenal and gastric ulcer respectively) in this region. Despite the long history of the disease its definite aetiology is yet to be identified.

Since the first description of the presence of spiral bacterium in human stomach by Krimtz in 1906 (cited from Freedberg & Barron[2]) there have been sporadic reports[3,4,5,6] on the relationship of spiral and other bacterium with pathologic states like peptic ulcer, gastritis etc. but none could establish any definite relationship. The description of a spiral organism by Marshall[7] & Warren[8] in 1983 revived the issue of microbial aetiology of petic ulcer. The bacterium was initially named Campylobacter pyloridis.[9] The name was later readjusted as Campylobacter pylori.[10] With the availability of detailed information on the morphology, ultrastructure, ribosomal RNA sequencing pattern, the bacterium showed major differences from other species of Campylobacters and thus placed in a new genus and named Helicobacter pylori.[11]

H. pylori is a Gram-negative, microaerophilic, spiral(seen in vivo only), ox-bow shaped, curved or occasionally rod shaped bacterium having a smooth cell wall with a layer of glycocalyx external sheath and multiple sheathed flagella arising from one or occasionally both poles; produces enzyme urease and shows resistance to nalidixic

acid. Whereas Campylobacters possess rough surfaced wall with a single unsheathed flagellum arising from one or both poles and do not produce urease.

Since its first description a series of reports have described a strong association between this organism and pathologcal states like gastritis, gastric ulcer, duodenitis and duodenal ulcer [12-18]. Despite such an association, the exact mechanism of possible bacterial pathogenicity are so far poorly understood.

Microbial pathogenesis is usually complex and multifactorial. Pathogens have several biochemical mechanism which may act individually or in concert to produce infectio and disease. It is useful to understand the pathogenesis of infection so that new therapeutic and preventive schemes can be developed. Microorganisms enter the host by a limited number of routes. Interactions with the h host surfaces are usually involved at or soon after entry. The first major interaction batwwen a pathogenic microorganism and its host entails attachment to eucaryotic cell surface. Some organisms multiply at and remain on the surface of the host while others use attachment as the first essential step before proceeding to deeper tissues or other locations.

The attachment stage is the best characteristic of host -parasite interactions as it is one of the easiest aspect to address experimentally.[19,20,21] In its simplest form, microbial adherence requires the participation of two factors: a receptor and an adhesin. The receptos defined so far are usually specific carbohydrate residues on the eucaryotic cell sufface. The bacterial adhesin is typically a protein structure on the bacterial eell surface possesing specific carbohydrate binding properties. Adhesins which attach to erythrocytes are termed haemagglutinins and have been morphologically identified as thin hair-like structures commonly known as either fimbriae or pili.[22]

Haemagglutination (HA) tests have been found to be useful for detecting the presence of fimbriae. But surface adhesins showing properties may also be non-fimbrial in nature. They are referred to as the non-fimbrial adhesins(NFA) to differentiate them from fimbrial

adhesins.[23,24,25]

Besides binding of bacteria via specific adhesins to epithelial cell receptors non-specific contribution of cell surface hydrophobicity is also considered important. Hydrophobicity involves intraction between non-polar groups present on bacteria and host cell. Besides the nom-polar hydrophobic component polar hydrophilic components are also present on the bacterial cell surface and it is the interaction between the hydrophobic and hydrophilic components that determine the overall contribution og hydrophobic interaction to adhesin.[28] A number of intestinal pathogens express a hydrophobic cell surface which permits colonization of mucus layer in the small bowel.[29] Hydrophobicity of the H. pylori strains isolated from peptic ulcer patients needs to be estimated which will assist to have an idea on the colonization factors of H. pylori.

Evans et al tested 10 strains of H.pylori for haemagglutnating activity.[26] Those strains showed HA with the crythrocytes of all the tested species. However, variable species specific HA were observed by other authors.[27,28] Studies may be carried out with erythrocytes of larger number of species and describe the pattern of haemagglutination. In order to have an idea on the nature of haemagglutinin inhibition tests (HAI) have been carried out with limited number of chemicals. HAI with strains from widely divergent geographical location may be carried out with larger number of chemicals and the pattern of HA and the nature of haemagglutinin noted.

Adherence properties of H. pylori can more specifically be shown by its adherence to different epithelial cell lines, more particularly to cell line of intestinal origin. Only one report[29] showing adherence of H. pylori to epithelial cell line Hep-2 and intestinal cell line Int-407 has been published. Strains isolated from a divergent area may be used to study the adherence properties.

The enzyme urease has been proposed to play a major role as a virulence factor of H. pylori. It has been proposed to create an insulating alkaline blanket around the organism and thus allow its passage through the acid environment of stomach. The ammonia produced from the break down of urea by urease have been proposed

to cause back-diffusion of H^+ ions with resultant hyperchlorohydria and tissue damage. The study of urease profile of the strains will help to find out inter-strain variations in the proposed virulence factors of H. pylori. The enzyme may be purified and the purified or the crude form of urease may be used as an antigen in transblotting procedure to find our their immunogenicity in infected patients.

3. Rationale:

Peptic ulcer is a common medical problem of Bangladesh. On a global perspective this area happens to be one of the highest prevalent zone of peptic ulcer. In the absence of knowledge about the definite aetiology of peptic ulcer disease existing therapies are inadequate to give life long cure; treatment needs to be continued for prolonged period with frequent relapses. Establishment of H. pylori as one of the causal agent may help in planning more effective intervention measures. Identification of virulence factors of H. pylori will be able to explain the pethogenesis of lesions associated with its infection and assist in devising diagnostic and therapeutic measures.

B. SPECIFIC AIMS:

1. To measure the cell surface hydrophobicity of H .pylori isolates by using Salting -out aggregation test!

2. To detrmine the adhesive property of H. pylori by studying its haemagglutinating potentiality of erythrocytes from different species.of animals.

3. To characterize the agglutinims of H. pylori(if present) by inhibition of haemagglutination with different chemicals.

4. To study the adhesiveness of H. pylori in an in-vitro cell culture model.

5. To purify urease and find out its profile in the local isolates .

6. To study the immunogenicity of urease in H. pylori infected patients.

7. To establish a collaborative linkage between Department of Micorbiology and Gastroenterology of IPGM&R and Lab. Science Division of ICDDR,B.

C. METHODS AND PROCEDURES

A. Bacterial strains to be used in the study:

H. pylori strains will be isolated from patients referred for endoscopy at the Gastroenterology Department of IPGM&R. The strains will be identified using the following criteria :i. Colonial character, ii. Gram-stain, iii. oxidase, catalase and urease tests. Fresh isolates will be used for determination of SAT value and haemagglutination pattern. The strains will be preserved in liquid nitrogen for further study. Before each experiment they will be sub-cultured and tested again for reconfiramtion of their properties. Enterotoxigenic E. coli strain no. 045565 will be used as a positive control for haemagglutination assays while non-pathogenic E. coli strain no 36000 will be used as a negative control.

b. Sample collection, processing and culture:

The samples will be collected from patients undergoing routine endoscopy for peptic ulcer-like symptoms. Informed consent will be obtained from the patients and biopsy samples will be collected from endoscopically positive peptic ulcer patients. The samples will be collected from the following sites : i. gastric ulcer : three pieces from intact mucosa around the ulcer, ii. duodenal ulcer: three pieces from antrum within three centimeters of pylorus. Samples will be collected in 20% glucose broth, transported to the laboratory and preserved at $4^{o}C$ till tested.

The samples will be minced into pieces on a glass slide with the help of a sterile blade and inoculated on chocolate agar plates composed of Brain Heart Infusion Agar with 10% sheep blood and antibiotics (vancomycin- 6mg/L, amphotericin B 10 mg/L, nalidixic acid- 2 mg/L and trimethoprim- 5 mg/L. Plates will be incubated at 37°C in a microaerophilic atmosphere obtained either by using Campy Pak (BBL Microbiology Systems, Cockeysville, MD.) or by evacuating the gas jar upto 5300 mm Hg and refilling with a gas mixture (H_2 10%, CO_2- 10% and N_2- 80%).

The strains will stored in liquid nitrogen in 15% glyderol Brain Heart Infusion Broth.

c. Measurement of cell surface hydrophobicity:

Cell surface hydrophobicity will be measured by the salt aggregation test using the method of Faris et al.[24] Washed bacterial cells will be serially diluted with ammonium sulfate of varying molarity (0.02 M to 3.6 M) on glass slides and and will be observed for 30 seconds. The highest dilution of ammonium sulfate in which visible aggregation occurs will be the SAT value for surface hydrophobicity.

d. Adhesion assays:

To determine adhesion of bacteria two different techni ques will be used and are described as follows:

Haemagglutination Assays

Freshly prepared erythrocytes obtained from guineapig, sheep, bovine, rabbit and human (Blood gr. O &A) will be used and the procedure followed by Salit et al.[32] will be applied . Haemagglutination assays will be carried out on slides using erythrocytes at a concentration of 2% and in microtitre plates(U-well) at 0.5% conc. The slide agglutination assays will only be used for quick detection of HA.

For slide agglutination, bacteria will be suspended in phosphate buffered saline(PBS) and mixed directly with erythrocyte suspension according to Evans et al.[23]

For determination of HA titre serial two-fold dilution of bacterial suspension (starting with 10^{10} cells/ml) will be mixed with erythrocytes (30 ul of 0.5% suspension). The results will be recorded after 60-90 minutes of incubation at $4^{o}C$. Inhibition by specific antisera will be tested after preincubation of bacteria with approximately diluted antiserum for 1 hr at $37^{o}C$. To determine inhibition by carbohydrates, different sugars e.g. D-galactose, D-fucose, D-manose, N-acetyl neuraminic acid, N-acetyl glucosamine, N-acetyl galactosamine, Feutin etc. using the procedure of Old DC.[35]

Adhesion assay in tissue culture cells

This assay will be carried out on following cell lines: a. Hep-2 ,b. Henle-407 and c. HeLa cell lines using the standard laboratory techniques. Inhibition by sugars and antisera to H. pylori and Serum from H. pylori infected patients will be carried out according to the method of Dolors et al.[36]

e) Demonstration of urease profile

1) Preparation of sample for SDS-PAGE:

The bacteria will be grown in chcolate agar plates enriched with 1% isovitalex, harvested and washed twice in PBS by centrifuging at 5000 Rpm($4^{o}C$). The pellet will be resuspemded in a small volume of solubilization buffer (0.625 M Tris-Hcl(PH 6.5), sodium dodecyl sulphate(SDS) 3% w/v, 2-mercaptoethanol 5% w/v. Cell debris will be removed from the sonicate by centrifugation at 13000g and cell free extract will be preserved in small volumes at $-20^{o}C$.

2) Urease Assay:

Urease activity of the sonic extract will be determined by modified Barthelot reaction [37] The amount of ammonia formed from a standard excess amount of urea will be determined by mixing : 10 ul of extract with 240 ul of UPEM substrate(30 mM urea, 20 mM sodium phosphate buffer, pH 7.5, 1mM disodium EDTA, 1o mM 2-mercaptoethanol and incubating at room temperature for 5 minutes. The reaction will be

stopped by addition of phenol nitroprusside solution 0.5ml, alkaline hypochlorite 0.5 ml and distilled water 0.5ml. After 5 minute incubation at room temperature the intensity of the developed blue colour will be measured at 570 nm and compared with that given by standard solutions of ammonium chloride. One unit of u urease will be defined as the amount of extract required to liberate on umol of ammonia in 5 min at room temperature in the above condition.

3) Sodium dodecylsulphate -polyacrylamide gel electrophoresis(SDS-PAGE):

Extracts containing urease were analysed by SDS-PAGE according to Laemmli with some modification[37].

f) Purification of urease:

Purification of urease will be carried out by the following method:

i) Isolation of crude urease from gel:

A 1.5 mm gel as described above will be loaded with whole cell extracts and run as per the method of Senior et al.[37] The bands in the gel corresponding to urease activity will be cut off from the gel and macerated in a stabilizing buffer (0.1M sodium phosphate buffer(pH 7.0) containing 0.01M 2-mercaptoethanol), mixed up overnight in a magnetic stirrer($4^{o}C$) and centrifuged at 8000 rpm to pellet the gel. The supernatant will be tested for urease activity and preserved in small quantities at $-20^{o}C$.

ii) Purification of urease by affinity chromatography:

DEAE-Sephadex colum will be used to purify urease using the method of Stemke et al.[38]

iii) Production of antisera against purified urease:

Rabbit anti-urease serum will be prepared by injecting 0.1 ml urease extracts in complete Freund's adjuvant(0.3ml) to hind toe pads, followed in 3 weeks by subcapsular injection of 0.2 ml serum im incomplete Freund's adjuvant.

iv) Western blotting;

The purified urease will be run in SDS-polyacrylamide and then transferred electropheretically to nitrocellulose paper by the method of Towbin et al.[39] The membrane will be treated with rabbit anti-urease antisera and the sera from H. pylori infected patients and the immunogenic potentiality will be noted.

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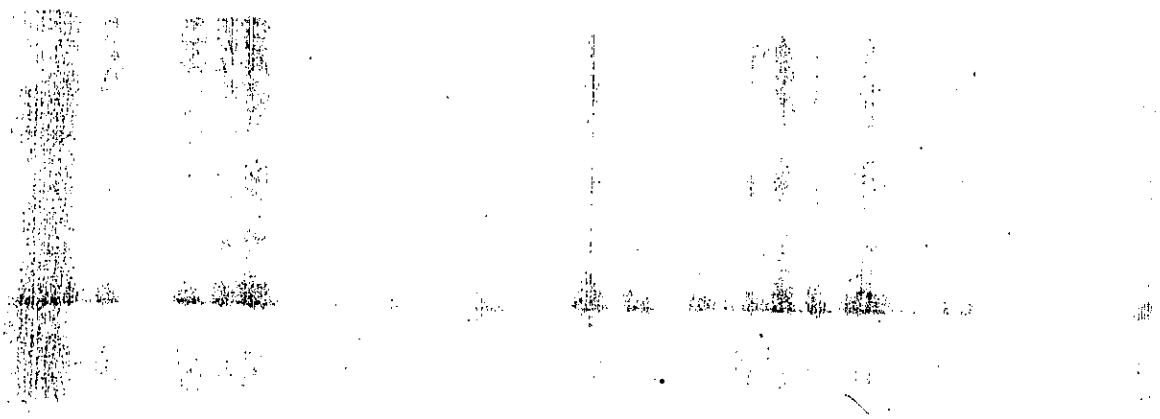

SECTION III - ESTIMATED BUDGET

1. Personnel Services:

Name	Position	Time Effort	Monthly Amount (US$)	Months	Total (US$)
PGM&R:					
Dr. Mahmudul Haque	P.I.	30%	60	12 X 1	720
Dr. Ruhul Amin Miah	Coinvestigator	10%	30	12 X 1	360
Dr. Mahmud Hasan	Coinvestigator	5%	-	-	-
Dr. Kazi Mashhur Rahman	Do	5%	-	-	-
To be named	Research physician	100%	135	12 X 1	1,620
To be named	Research Assistant	100%	60	12X1	720
DDDR,B					
Dr. Firdausi Qadri	P.I.	10%	-	-	-

2. SUPPLIES AND MATERIALS

a. Glasswares	200.00
b. Chemicals and media, diagnostics reagents	1,500.00
c. Animal resources	50.00
d. Equipments (Translab chamber)	500.00
e. Travel and tramsport	50.00
f. Transport of materials	-
g. Medical illustration	50.00
h. Xerox	50.00
Total US$	5,940.00

সম্মতি-পত্র

কোত্তর চিকিৎসা ও গবেষণা প্রতিষ্ঠানের মাইক্রোবায়োলজী ও
স্ট্রোএন্টারোলজী বিভাগ কর্তৃক পেপটিক আলসারের সঠিক
ণ নির্ণয়সংক্রান্ত বিষয়ে গবেষণা করছে। এই গবেষণার ফলে
পটিক আলসারের কারণ সম্পর্কে বর্তমান জ্ঞানের আরো
গতি হবে। তাতে এই রোগের জন্য আরো উপযোগী চিকিৎসা
তি চালু করা সম্ভব হতে পারে।

পনি এই গবেষণায় স্বেচ্ছায় অংশগ্রহণ করতে চাইলে আপনাকে
পাকস্থলী থেকে পরীক্ষার জন্য দু' টুকরো বায়পসী নমুনা এবং
৫ মি.লি. রক্ত প্রদান করতে হবে।

াপনি উক্ত গবেষণায় অংশগ্রহণে রাজী থাকলে নীচে আপনার
াক্ষর প্রদান করে সম্মতি দিন।

রোগীর নাম : ----------

স্বাক্ষর :