Centre for Health & Population Research **RRC APPLICATION FORM**

RESEARCH PROTOCOL NUMBER: 2005-010

FOR OFFICE USE ONLY		
RRC Approval:	☐Yes / ☐No	Date:
ERC Approval:	☐Yes / ☐No	Date:
AEEC Approval:	☐Yes / ☐No	Date:

Project Title: Genetic diversity of *Helicobacter pylori* in colonization and infection: role in disease outcome (symptomatic and asymptomatic) and transmission.

Short protocol title (in 50 characters):
Genetic diversity of *H. pylori*

Theme: ***(Check all that apply)***

- ☐ Nutrition
- √☐ Emerging and Re-emerging Infectious Diseases
- ☐ Population Dynamics
- ☐ Reproductive Health
- ☐ Vaccine evaluation
- ☐ HIV/AIDS
- ☐ Environmental Health
- ☐ Health Services
- ☐ Child Health
- ☐ Clinical Case Management
- ☐ Social and Behavioural Sciences

Key words: __*H. pylori*, Infection, Genetic diversity, Transmission

Relevance of the protocol: Research on epidemiology and antimicrobial resistance pattern of *H. pylori* isolates in Bangladesh is conducted at LSD, CSD of ICDDR, B. In the current protocol we would like to understand the mechanism of transmission, genetic diversity in colonization and infection, role in disease outcome and as well as monitoring of antimicrobial resistance pattern of *H. pylori*. This will help us to understand the role of microbial diversity in human stomach in reducing the *H. pylori* related disease and better treatment.

Centre's priority: ICDDR, B: Centre for Health and Population Research has been working in the field of H. pylori related diseases for the last 10 years. *H. pylori* related diseases like ulcer and stomach cancer is one of the major health problem in developing countries including Bangladesh. The current study fell under the mission of the Centre, titles "To develop and promote realistic solutions to the major health, population and nutrition problems facing the poor people of Bangladesh and other settings."

Programmes

- ☐ Child Health Programme
- ☐ Nutrition Programme
- √☐ Programme on Infectious Diseases & Vaccine Science
- ☐ Poverty and Health Programme
- ☐ Health and Family Planning Systems Programme
- ☐ Population Programme
- ☐ Reproductive Health Programme
- ☐ HIV/AIDS Programme

Principal Investigator: Dr. Motiur Rahman **Division: LSD** **Phone:** 8811751-60, Extn. 2432, 2403
(should be a Centre's staff) **Address:** **Associate Scientist, LSD**
Email: motiur@icddrb.org

Co-Principal Investigator(s):
Internal:

Co-Principal Investigator(s): External: (Please provide full official address and Gender)
Lars Engstrand, Professor, Head, section of GI-infections, Dept. of Bacteriology ,
Swedish Institute for Infectious Disease Control, SE 171 82 Solna, Sweden phone + 46 8 457 2415.
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Co-Investigator(s):

Internal	**Shafiqul A sarker**	**Division:**	**CSD**	**Phone: 2333**	**(Male)**
	Pradip Kumar Bardhan	**Division:**	**CSD**	**Phone: 2313**	**(Male)**
	Shamsun Nahar	**Division:**	**LSD**	**Phone: 2424**	**(Male)**
	Rasel Khan	**Division:**	**LSD**	**Phone: 2424**	**(Male)**

Co-Investigator(s):
External: Mian Mashhud Ahmad Endoscopist, Dhaka Medical College Hospital, Dhaka, Bangladesh (Male)
Morten Kiwi**,** Swedish Institute for Infectious Disease Control, SE 171 82 Solna, Sweden (Male)

Student Investigator/Intern:
Internal (Centre's staff) **Jinath Sultana Division:** **LSD, Phone: 2403**

External
(Please provide full address of educational institution and Gender)

Collaborating Institute(s): Swedish Institute for Infectious Disease Control, SE 171 82 Solna, Sweden

Population: Inclusion of special groups (*Check all that apply)*:

Gender
√☐ Male
√☐ Females

Age
√☐ 0 – 5 years
√☐ 5 – 9 years
√☐ 10 – 19 years
√☐ 20 – 64 years
☐ 65 +

☐ Pregnant Women
☐ Fetuses
☐ Prisoners
☐ Destitutes
☐ Service providers
☐ Cognitively Impaired
☐ CSW
√☐ Others (specify , all general populations)
☐ Animal

Project / study Site (*Check all the apply)*:

√☐ Dhaka Hospital
☐ Matlab Hospital
☐ Matlab DSS area
☐ Matlab non-DSS area
☐ Mirzapur
√☐ Dhaka Community
☐ Chakaria
☐ Abhoynagar
☐ Mirsarai
☐ Patyia
☐ Other areas in Bangladesh _____
☐ Outside Bangladesh
name of country: _____
☐ Multi centre trial
(Name other countries involved)
_____

Type of Study *(Check all that apply)*:

☐ Case Control study
☐ Community based trial / intervention
☐ Program Project (Umbrella)
☐ Secondary Data Analysis
☐ Clinical Trial (Hospital/Clinic)
☐ Family follow-up study
☒ Cross sectional survey
☐ Longitudinal Study (cohort or follow-up)
☐ Record Review
☐ Prophylactic trial
☒ Surveillance / monitoring
☒ Others (Analysis of specimen for H. pylori)

Targeted Population *(Check all that apply)*:

√☐ No ethnic selection (Bangladeshi)
☐ Bangalee
☐ Tribal groups
☐ Expatriates
☐ Immigrants
☐ Refugee

Consent Process *(Check all that apply)*:

√☐ Written
☐ Oral
☐ None
√☐ Bengali language
☐ English language

Proposed Sample size: Total sample size: 42 index case

Subgroup 1- Asymptomatic 21
Subgroup 2- Symptomatic 21

Determination of Risk: Does the Research Involve *(Check all that apply)*:

☐ Human exposure to radioactive agents?
☐ Fetal tissue or abortus?
☐ Investigational new device?
(specify_____)
☐ Existing data available from Co-investigator
☐ Human exposure to infectious agents?
☐ Investigational new drug
☐ Existing data available via public archives/source
√☐ Pathological or diagnostic clinical specimen only
☐ Observation of public behaviour
☐ New treatment regime

Yes/No

√☐ ☐ Is the information recorded in such a manner that subjects can be identified from information provided directly or through identifiers linked to the subjects?

☐ √☐ Does the research deal with sensitive aspects of the subject's behaviour; sexual behaviour, alcohol use or illegal conduct such as drug use?

Could the information recorded about the individual if it became known outside of the research:

☐ √☐ a. place the subject at risk of criminal or civil liability?

☐ √☐ b. damage the subject's financial standing, reputation or employability; social rejection, lead to stigma, divorce etc.

Do you consider this research *(Check one)*:

√☐ greater than minimal risk ☐ no more than minimal risk

☐ only part of the diagnostic test Not Applicable

Minimal Risk is "a risk where the probability and magnitude of harm or discomfort anticipated in the proposed research are not greater in and of themselves than those ordinarily encountered in daily life or during the performance of routine physical, psychological examinations or tests. For example, the risk of drawing a small amount of blood from a healthy individual for research purposes is no greater than the risk of doing so as a part of routine physical examination".

Yes/No

☐ ☐ Is the proposal funded? Yes

If yes, sponsor Name: SIDA

Yes/No Not applicable

☐ ☐ Is the proposal being submitted for funding ?

If yes, name of funding agency: (1)

(2)

Do any of the participating investigators and/or their immediate families have an equity relationship (e.g. stockholder) with the sponsor of the project or manufacturer and/or owner of the test product or device to be studied or serve as a consultant to any of the above?

IF YES, submit a written statement of disclosure to the Executive Director.

Dates of Proposed Period of Support

(Day, Month, Year - DD/MM/YY)

Beginning date: June 2005

End date: Dec 2007

Cost Required for the Budget Period ($)

	Direct Cost	Indirect Cost	Total Cost
Year-1	52753	11116	63869
Year-2	79259	19307	98566
Year-3	66400	15964	82364
TOTAL: $	**198412**	**46384**	**244799**

Approval of the Project by the Division Director of the Applicant

The above-mentioned project has been discussed and reviewed at the Division level as well by the external reviewers. The protocol has been revised according to the reviewer's comments and is approved.

Dr. G B Nair

Name of the Division Director | Signature | Date of Approval

Certification by the Principal Investigator

I certify that the statements herein are true, complete and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

Signature of PI Dr. Motiur Rahman

Date:

Name of Contact Person (if applicable)

Table of Contents

X Check here if appendix is included

PROJECT SUMMARY: Describe in concise terms, the hypothesis, objectives, and the relevant background of the project. Describe concisely the experimental design and research methods for achieving the objectives. This description will serve as a succinct and precise and accurate description of the proposed research is required. This summary must be understandable and interpretable when removed from the main application. **(TYPE TEXT WITHIN THE SPACE PROVIDED).**

Principal Investigator: Dr. Motiur Rahman

Project Name: Genetic diversity of *Helicobacter pylori* in colonization and infection: role in disease outcome (symptomatic and asymptomatic) and transmission.

Total Budget	Beginning Date	Ending Date
244799US $	June 2005	Dec 2007

Hypothesis: The genetic diversity of *H. pylori* isolates (individual isolates collected from a single patient) within a single individual is associated with diseases outcome and contributes to transmission of infection.

Objectives:

1. To document the prevalence of genetic diversity of *H. pylori* isolates (genetic diversity of isolates within individual patient) in *H. pylori* infected patients with and without symptoms (gastroduodenal symptoms).
2. To study the role of genetic diversity of *H. pylori* isolates (genetic diversity of isolates within individual patient) in the transmission of infection among family contracts.
3. To study the role of genetic diversity of *H. pylori* isolates (genetic diversity of isolates within individual patient) in pathogenesis, diseases manifestation, and antimicrobial susceptibility.
4. To compare the prevalence of genetic diversity of isolates (genetic diversity of isolates within individual patient) from the present study with similar data from other countries.

Background & methods:

Helicobacter pylori is a genetically diverse gram-negative bacterial species that chronically infects more than half of all people worldwide and can provoke development of peptic ulcer disease and contribute to the risk of gastric cancer. It is still unknown why only a minority (10-20%) of infected individuals progresses to clinical disease. *H. pylori* infection is mainly acquired in childhood and may predispose to peptic ulcer or gastric cancer later in life. In most children, the acquisition of *H. pylori* infection does not lead to clinically apparent disease, even when the organism colonizing the gastric mucosa and causes chronic active gastritis. Knowledge about *H. pylori* infection is evolving, particularly in the pediatric age group for which there are still large gaps in knowledge. Additional multicenter, randomized, placebo-controlled treatment trials in children infected by *H. pylori* are critically needed to characterize the effect of *H. pylori* eradication treatment during childhood on symptoms and gastroduodenal mucosal disease. The risk factors for acquiring infection include poor socio-economic conditions, family overcrowding, and possibly an ethnic or genetic predisposition. The route of transmission of *H. pylori* in humans is not known but is postulated to be fecal-oral, gastric-oral (in vomitus), or oral-oral. The role diversity of isolates within a single individual and within different individual is poorly understood. To see the role of genetic diversity among *H. pylori* isolates in pathogenesis, survival and transmission of infection, we propose to conduct a study involving both asymptomatic and symptomatic patient population with gastroduodenal ulcer. A descriptive, cross-sectional survey will be conducted among asymptomatic and symptomatic patient population with *H. pylori* infection. Asymptomatic subjects will be recruited from Nandipara, a periurban community near Dhaka City where ICDDR, B. whereas, symptomatic subjects will be recruited from Dhaka Medical College Hospital (DMCH). All household heads of children attending with unrelated symptoms and fulfilling enrollment criteria will be primarily invited for stool antigen test for *H. pylori*. Those who are positive for stool antigen will be requested for endoscopic examination or nasogastric intubation for collection of gastric juice at the Clinical Research and Service Centre (CRSC) of ICDDR, B. On the other hand, patients attending the Department of Gastroenterology, DMCH for upper gastrointestinal tract endoscopy will be eligible for the study. Among the patients who are positive for *H. pylori* will be considered as index case and their family members will be screened for *H. pylori*. Family members of the index case who are positive for *H. pylori* stool antigen test will be invited to enroll in the study and will undergo upper GI endoscopy for biopsy or nasogastric intubation for gastric juice. From each individual, 20 single colonies will be isolated and the *H. pylori* diversity will be assessed as described below. 1. All single colony isolates will be typed by: (a) PCR-based molecular typing methods, primarily RAPD; (b) PCR-based determination of presence or absence of known virulence factors (e.g. *cag* PAI, *vacA* alleles, *iceA1, iceA2, oipA, babA*) and neutral markers (e.g. IS *605*, *606* and *608*). 2. A subset of the isolates will be subjected to more

in-depth analyses by: (a) DNA sequencing of a number of selected genes (e.g. *vacA*, *recA*, *glmM*, *picA*, *picB*, *glr, ppA, sysS*, *cag* PAI genes). (b) Multilucous enzyme typing (MLST) and (c) DNA microarray analysis.

KEY PERSONNEL (List names of all investigators including PI and their respective specialties)

Name	Professional Discipline/ Specialty	Role in the Project
1. Dr. Motiur Rahman	Microbiologist & Molecular biologist	Principal investigator
2. Dr. Shafiqul Sarker	Gastroenterologist	Co investigator
3. Dr. Pradip Bhardhan	Gastroenterologist	Co investigator
4. Mrs. Shamsunnahar	Microbiologist	Co investigator
5. Dr. Lars Engstrand	Molecular Biologist (SMI, Sweden)	Co P.I
6. Dr. Mian Mashhud Ahmad	Endoscopist DMCH	Co investigator
7. Rasel Khan	Microbiologist	Co investigator
8. Morten Kiwi	Epidemiologist	Co investigator

DESCRIPTION OF THE RESEARCH PROJECT

Hypothesis to be tested:

Concisely list in order, in the space provided, the hypothesis to be tested and the Specific Aims of the proposed study. Provide the scientific basis of the hypothesis, critically examining the observations leading to the formulation of the hypothesis.

Hypothesis: The genetic diversity of *H. pylori* isolates (individual isolates collected from a single patient) within a single individual are associated with diseases outcome and contributes to transmission of infection.

Specific Aims:

Describe the specific aims of the proposed study. State the specific parameters, biological functions/ rates/ processes that will be assessed by specific methods **(TYPE WITHIN LIMITS).**

Objectives:

1. To document the prevalence of genetic diversity of *H. pylori* isolates (genetic diversity of isolates within individual patient) in *H. pylori* infected patients with and without symptoms (gastroduodenal symptoms).

2. To study the role of genetic diversity of *H. pylori* isolates (genetic diversity of isolates within individual patient) in the transmission of infection among family contracts.

3. To study the role of genetic diversity of *H. pylori* isolates (genetic diversity of isolates within individual patient) in pathogenesis, diseases manifestation, and antimicrobial susceptibility.

4. To compare the prevalence of genetic diversity of isolates (genetic diversity of isolates within individual patient) from the present study with similar data from other countries.

Background of the Project including Preliminary Observations

Describe the relevant background of the proposed study. Discuss the previous related works on the subject by citing specific references. Describe logically how the present hypothesis is supported by the relevant background observations including any preliminary results that may be available. Critically analyze available knowledge in the field of the proposed study and discuss the questions and gaps in the knowledge that need to be fulfilled to achieve the proposed goals. Provide scientific validity of the hypothesis on the basis of background information. If there is no sufficient information on the subject, indicate the need to develop new knowledge. Also include the **significance and rationale** of the proposed work by specifically discussing how these accomplishments will bring benefit to human health in relation to biomedical, social, and environmental perspectives. **(DO NOT EXCEED 5 PAGES, USE CONTINUATION SHEETS).**

Helicobacter pylori is a genetically diverse gram-negative bacterial species that chronically infects more than half of all people worldwide and can provoke development of peptic ulcer disease and contribute to the risk of gastric cancer [1]. Since 1995 it has been proved that the bacterium *H. pylori* is the main cause of chronic gastritis and the majority of other gastric diseases. Recent data suggests that the bacterium is found at 95% duodenum ulceration and at 70-80% cases of gastric ulcer [3]. *H. pylori* infection one of the most widespread infections of human race. Such a mass infection of the population by the *H. pylori* and accordingly high abundance of gastric diseases, caused by it undoubtedly testifies to insufficient efficiency of present methods of understanding the underlying mechanism of pathogenicity of *H. pylori* infection [7].

H. pylori infection is mainly acquired in childhood and may predispose to peptic ulcer or gastric cancer later in life [2]. In most children, the acquisition of *H. pylori* infection does not lead to clinically apparent disease, even when the organism colonizing the gastric mucosa and causes chronic active gastritis [3]. Knowledge about *H. pylori* infection is evolving, particularly in the pediatric age group for which there are still large gaps in knowledge. Additional multicenter, randomized, placebo-controlled treatment trials in children infected by *H. pylori* are critically needed to characterize the effect of *H. pylori* eradication treatment during childhood on symptoms and gastroduodenal mucosal disease. There is compelling evidence that this organism is associated with a significant proportion of duodenal ulcers and, to a lesser extent, with gastric ulcers in children and adults [4]. There are epidemiologic data linking chronic *H. pylori* infection, probably beginning in childhood, with the development of gastric adenocarcinoma and gastric lymphoma [5]. Findings in recently reported animal models support the role of *H. pylori* in the pathogenesis of gastric cancers [6]. The incidence of *H. pylori* infection in industrialized countries is estimated to be approximately 0.5% of the susceptible population per year. In contrast, there is a significantly higher estimated incidence of *H. pylori* infection in developing countries of approximately 3% to 10% per year [7]. The limited data on the incidence of *H. pylori* infection in children consist largely of retrospective seroprevalence studies. Humans appear to be the primary natural reservoir of *H. pylori* although other reservoirs such as water, domestic cats, and houseflies have been proposed [8], [9], [10].

The risk factors for acquiring infection include poor socio-economic conditions, family overcrowding, and possibly an ethnic or genetic predisposition. In North America, the prevalence rates of *H. pylori* among Asian-Americans, African-Americans and Hispanics are similar to those of residents of developing countries [11]. The route of transmission of *H. pylori* in humans is not known but is postulated to be fecal-oral, gastric-oral (in vomitus), or oral-oral [12]. Many researchers believe that *H. pylori* are transmitted by the oro-fecal route through the ingestion of food or water. In addition, it is also possible that *H. pylori* are transported from the stomach to the mouth in association with gastro-esophageal reflux (in which the contents of the stomach are refluxed in small amounts into the

esophagus) or belching, both common symptoms of gastritis; the bacterium could then be transmitted via the oro-oral route. Although the oral-oral route of transmission between spouses is unlikely to be an important mode for *H. pylori* infection [13]. Transmission among family members is considered to constitute the main route of *H. pylori* infection [14]. Previous investigations using serological or histological diagnosis and/or the 13C urea breath test have demonstrated an intrafamilial clustering of *H. pylori* infections in Canada [15], the United States [16], Italy [17], [18], and England [19], [20]. It has also been suggested that H. pylori is transmitted from infected parents, especially infected mothers, to children in Germany [21], [22], [23], the United States [24], and Japan [25], [26]. H. pylori transmission among siblings has been suggested in Colombia [27] and Japan [26]. Previous studies in England using DNA fingerprinting demonstrated a familial infection of a mother, father, and child due to a single *H. pylori* strain [19]. In contrast, in developing countries, environmental factors may be more important than intrafamilial transmission [23], [28].

Clinical isolates of *H. pylori* from different individuals show enormous variation in their genomic fingerprint as identified by restriction fragment length polymorphism or arbitrary primed PCR. This genetic diversity represents a combination of point mutation, inserted sequence and horizontal gene transfer [29]. This represents the plasticity of *H. pylori* genome. This plasticity offers selective advantage to the bacterium for co-evolving with its host over the course of long colonization and infection period. The evolution of new genotypic and phenotypic traits reflects the net outcome of two opposing forces e.g. point mutation, recombination and horizontal transfer versus functional barrier to gene transfer, and restriction modification system.

During the past years, a great deal of progress has been made in defining genetic variation among *H. pylori* isolates, however it is difficult to predict the outcome of *H. pylori* infection in a colonized untreated individual or to identify human population at risk for significant pathology. All available data indicate that the outcome is determined by factors emanating from the host, the microorganism, and the environment [30], [31], [32].

Although isolates from different individuals are rarely identical, information on the genetic diversity of strains within a single individual is scanty. It is conceivable that the variation can give rise to cohabiting organism that differ in phenotype and might play significant role in outcome of infection. The mechanism of underlined genetic diversity has under intense research and it has been shown that mutation in *H. pylori* is unusually high (~10x7) [29]. *H. pylori* have shown to be panmictic species, i.e. recombination is frequent and in addition, selective sweeps where only a subset of population survives is rare. As a result, *H. pylori* lack the sequential bottleneck that purifies the population of the bacteria. It has been proposed that the net virulence of the population can be determined by relative ratio of more and less virulent sub clones, and a shift to a more virulent composition can lead to host bacterial relationship towards pathogenesis. However, clonal groupings of *H. pylori* have also been detected from different ethnic groups in New Zealand, China, The Netherlands, and other countries [33], [34], [35], [36], [37], [38]. Moreover, comparison of the complete genomic sequences of two unrelated *H. pylori* isolates [39], [40] reveals that the overall genomic organizations, gene orders, and predicted proteomes are quite similar, with the majority of nucleotide changes representing synonymous substitutions. Based on these observations, Wang et al. suggested that mutation must be of major importance in generating the genetic variation of *H. pylori* [41].

H. pylori isolates are divided into two main types, Type I expressing the 38.5 kb Cag PAI containing 27 genes essential for translocation of bacterial components to cell [42]. It has been conclusively shown that inactivation of such translocation system (type IV secretory system) markedly reduces

the virulence potential of the organism. It has been shown that the *cag* PAI is involved in the induction of interleukin-8 (IL-8) secretion by human cells colonized by *H. pylori*. This property may be related to the proinflammatory power of a strain and thus to its virulence. Strains inducing IL-8 secretion have shown to be associated with the presence of the *cagA* gene and with severe diseases. Knockout of most of the genes of this region (except *cagA* and *cagN*) resulted in a decrease or a suppression of the IL-8 induction ability of a strain. The mechanism of IL-8 induction is not yet understood. However, some genes of the *cag* PAI are homologous to genes of a type IV secretion pathway, suggesting that this region encodes a secretion system involved in the export of virulence determinants (Audibert *et al.*, 2000). Because the *cag* PAI has a variable genetic structure and because this structure may influence the IL-8 induction ability of the strain, it is important to whether CagPAI+ strains from Bangladeshi patients with overt disease tend to have CagPAI genes that are more "active", in terms of ability to induce synthesis of the pro inflammatory cytokine IL-8, than do CagPAI+ strains from controls with benign infections. Besides this in a panel of genetically diverse bacteria some isolates are more active in terms of ability to induce synthesis of the pro inflammatory cytokine IL-8.

The role diversity of isolates within a single individual and within different individual is poorly understood. We plan to study the role of genetic diversity among *H. pylori* isolates in pathogenesis, survival and transmission of infection.

Operational definition for the current study:

Genetic diversity: Genetic diversity refers to the variation at the level of individual genes (polymorphism), and provides a mechanism for populations to adapt to their ever-changing environment. The more variation, the better the chance that at least some of the individuals will have an allelic variant that is suited for the new environment, and will produce offspring with the variant that will in turn reproduce and continue the population into subsequent generations. Clinical isolates of *H. pylori* from different individuals show enormous variation in their genomic fingerprint as identified by restriction fragment length polymorphism (RFLP) or arbitrary primed PCR (AP-PCR). This genetic diversity represents a combination of point mutation, inserted sequence and horizontal gene transfer [29]. Point mutation may be house keeping genes or virulence genes. Insertion sequence may be associated with certain antimicrobial resistance or may be associated with virulence properties of the organism. This represents the plasticity of *H. pylori* genome. This plasticity offers selective advantage to the bacterium for co-evolving with its host over the course of long colonization and infection period. The evolution of new genotypic and phenotypic traits reflects the net outcome of two opposing forces e.g. point mutation, recombination and horizontal transfer versus functional barrier to gene transfer, and restriction modification system. The genetic diversity is continuous phenomenon and in the present study is defined as genetic difference of *H. pylori* isolates from individual subject. The gentic diversity in the present study will be measured as a combined diversity of virulence gene(s) (Vac allelic variation and cag PAI variation) and house keeping gene(s) (as determined by MLST typing using eight gene). Measuring the genetic diversity of virulence gene is based on the presence of certain virulence gene and is a dichotomous variable. Genetic diversity of house keeping gene is a continuous variable this measurement of genetic diversity in a given bacterial population is being done by MSLT analysis system which uses a complex algorithm and is documented MLST website. This depends on synonymous and asynomouous mutation of selected genes. The MLST website calculates the diversity using internationally accepted algorithm and converts the continuous variable diversity to a dichotomous variable. Therefore in the current study genetic diversity is dichotomous.

Asymptomatic subject: Asymptomatic *H. pylori* positive subject may be defined as individuals who are positive for *H. pylori* (as determined by stool antigen test) but do not have signs or symptoms for H. pylori infection (signs and symptoms of gastric or duodenal ulcer, non ulcer dyspepsia).

Symptomatic subjects: Symptomatic *H. pylori* positive subject may be defined as individuals who are positive for *H. pylori* as determined by stool antigen test and have signs or symptoms for *H. pylori* infection (signs and symptoms of gastric or duodenal ulcer, non ulcer dyspepsia such as epigastric distress on empty stomach or 45 -60 minute after meals, nocturnal epigastric pain relived by antacid or vomiting, dyspepsia, gastro-esophageal regurgitation and heart burn, anorexia, nausea, abdominal distension and bloating and flatulance).

Research Design and Methods

Describe in detail the methods and procedures that will be used to accomplish the objectives and specific aims of the project. Discuss the alternative methods that are available and justify the use of the method proposed in the study. Justify the scientific validity of the methodological approach (biomedical, social, or environmental) as an investigation tool to achieve the specific aims. Discuss the limitations and difficulties of the proposed procedures and sufficiently justify the use of them. Discuss the ethical issues related to biomedical and social research for employing special procedures, such as invasive procedures in sick children, use of isotopes or any other hazardous materials, or social questionnaires relating to individual privacy. Point out safety procedures to be observed for protection of individuals during any situations or materials that may be injurious to human health. The methodology section should be sufficiently descriptive to allow the reviewers to make valid and unambiguous assessment of the project. **(DO NOT EXCEED TEN PAGES, USE CONTINUATION SHEETS).**

Study design, site and subjects: The study will be a descriptive, cross-sectional survey and will be conducted both asymptomatic and symptomatic patient population with *H. pylori* infection. Asymptomatic population will be recruited from Nandipara, Nandipara, a periurban community 7 miles northeast of Dhaka city and populated by people of low socioeconomic status. A population of >3,000 with ~500 children <5 years of age live in a 2.5 square miles area. Among the dwellers, 75% of the males are classified as day laborers, 20% as rickshaw pullers, and 5% as carpenters, servicemen, or small businessmen. Fifteen percent of the women are day laborers and 85% are housewives. Most of the families live in poorly constructed houses with mud or thatched walls and tin or thatched roofs. The average family size is five members. The slum has a municipal waters supply for drinking and cooking, but for bathing, washing, and cleaning utensils, water from ponds and ditches is used. About 90% of the population uses hanging latrines, which are poorly constructed on bamboo poles near ponds or ditches, resulting in high fecal contamination of water. Most children defecate in open spaces. A clinic for minor illness has been run in Nadipara since 1986 by the International center for Diarrhoeal Disease Research, Bangladesh (ICDDR,B)[7]. The prevalence of *H. pylori* infection in children less than 5 years old is 60% in this community [43]. The field clinic provides primary health care facility to the local community. The clinic is operated 2 days in a week and approximately 100-125 patients are attending the clinic each day. Health care facility is provided to males, female and children. The population served by the clinic represents the general population of Nandipara community. The symptomatic patient population will be recruited from Dhaka Medical College Hospital (DMCH) who will undergo endoscopic procedures for diagnosis and treatment of peptic ulcer disease.

Enrollment criteria for asymptomatic subjects:

Inclusion criteria

1. Male and female of any age
2. Living in Nandipara
3. Stool antigen test for *H. pylori* is positive

Exclusion criteria:

1. Known contraindication for endoscopy.
2. Use of proton pump inhibitor in recent weeks.
3. Previous abdominal surgery

Enrollment criteria for symptomatic subjects:

Inclusion criteria:

1. Male and female of any age
2. History of epigastric pain/discomfort.
3. Gastro-duodenal ulcer, non-ulcer dyspepsia
4. Living in or around Dhaka city.

Exclusion criteria:

1. Known contraindication for endoscopy.
2. Use of proton pump inhibitor in recent weeks.
3. Previous abdominal surgery.

Enrollment of asymptomatic infection: Asymptomatic *H. pylori* subjects will be enrolled from patients attending at ICDDR, B Primary Health Care clinic at Nandipara. All household heads of patients attending with unrelated symptoms and fulfilling enrollment criteria will be primarily invited for stool antigen test for *H. pylori*. Stool antigen positive household heads will then be invited for final enrollment of the study. All information regarding the study will be provided and will be asked for informed written consent. After consenting of the household heads and all members of the family will be screened for stool antigen test. Those who are positive for *H. pylori* by stool antigen will be requested for endoscopic examination or nasogastric juice collection. Endoscopic examination or nasogastric intubation and gastric juice collection will be done at ICDDR, B under supervision of Dr. Shafiqul A. Sarker and Dr. Pradip Bhardhan.

Enrollment of symptomatic subjects: Symptomatic subjects will be will be recruited from DMCH endoscopic clinic. Patients attending for upper GI endoscopy who fulfilled the enrolled criteria will be requested to participate in the study. All information regarding the study will be provided and will be asked for informed written consent. After consenting of the patient biopsy specimen will be collected during routine endoscopic examination. If the subject is positive for *H. pylori* (index case) all other members of the family will be screened for *H. pylori* by stool antigen test. Those who are positive for *H. pylori* will be requested for endoscopic examination or nasogastric juice collection. Endoscopic examination or nasogastric intubation and gastric juice collection will be done as described above.

Treatment: All patients positive for *H. pylori* infection (by culture or by stool antigen test) will be treated for *H. pylori* infection using standard triple drug regimen (Omeprazole (20 mg twice daily) plus clarithromycin (500 mg twice daily) and amoxicillin (1 g twice daily) for 10 days.

Confirmation of *H. pylori* infection by stool antigen test: The initial diagnosis for *H. pylori* will be done by stool antigen test. Stool specimen will be collected from all enrolled individual and will be transported to ICDDR, B. 100 µl of semisolid stool or a 5-6 mm block of hard stool will be used for diagnosis of *H. pylori* as recommended by manufacturer (Premier Platinum HpSA, Meridian BioScience Inc, USA).

Endoscopy and gastric juice collection: Endoscopy of the subjects will be done by well-washed and disinfected fiber optic endoscopes (GIF XQ 30, Olympus, Japan). Two biopsy samples will be taken from each patient, one from the gastric antrum and one from the corpus (1 cm away from ulcer). Immediately after collection biopsy specimen will be kept in transport media and will be transported to ICDDR, B, *H. pylori* laboratory in cold chain. In children we will not perform endoscopy and gastric juice will be collected by nasogastric tube as described earlier [44], [45]. The gastric juice will be adjusted to pH 7.0 with Tris HCL and will be transported ICDDR, B, *H. pylori* laboratory in cold chain.

Culture of *H. pylori* from biopsy specimen and gastric juice: Immediately after collection biopsy sample will be homozinised and cultured on Brucella agar containing 5% yeast extract, 7% horse blood, vancomycin (10 μg/ml) and amphotericin B (15mg/L) in microaerophilic condition (0_2 5%, Co_2 15%, N_2 80%) at 37C for 5 days. *H. pylori* will be confirmed by Gram staining, urease test, catalase test, oxidase activity and by PCR using primers specific for 16S rRNA [46]. 20 randomly selected colonies and a pool of the culture will be propagated and stored at –86C for further analysis. The gastric juice will be centrifuges at 10,000xg for 15 minutes. The pallet will be used for culture on Brucella agar containing 5% yeast extract, 7% horse blood, vancomycin (10 μg/ml) and amphotericin B (15mg/L) in microaerophilic condition (0_2 5%, Co_2 15%, N_2 80%) at 37C for 3-5 days. The plates will be examined for suspected colonies of *H. pylori* very day. Suspected colonies will be patched on new plates and 20 randomly selected colonies and a pool of the culture will be propagated and stored at –86C for further analysis.

DNA extraction: Chromosomal DNA was prepared by the CTAB (hexadecyl-trimethyl ammonium bromide) extraction method [47] from confluent BHI agar plate cultures. Plasmid DNA from each isolates will also be studied by plasmid DNA extraction method.

Analysis of diversity of the isolates:

Typing of isolates: All bacterial isolates (20) from each individual patient will be analyzed for the presence of cag pathogenicity island (cag PAI) by PCR. The cag positive and negative isolates will be further analyzed for the presence and absence of all 27 genes encoded in the cag PAI by DNA microarray analysis. Cag PAI partial deletion isolated will be further studied for specific deletion and deletion motifs. DNA microarray analysis will be done in SMI Stockholm, Sweden.

Antimicrobial susceptibility phenotype analysis: All twenty bacterial isolates from each individual patient will be analyzed for antimicrobial susceptibility to metronidazole, tetracycline, clarithromycin and amoxicilline. The MICs for clarithromycin, amoxicillin, metronidazole and tetracycline of *H. pylori* isolates were determined by agar dilution method as approved by National Committee for Clinical Laboratory Standards. Two fold serial dilutions of antibiotics were used: clarithromycin (Abbott Laboratories, Abbott Park, Ill.) 0.125 – 8 μg/ml, amoxicillin (Sigma, St. Louis, MO) 0.05 – 8 μg/ml, metronidazole (Sigma, St. Louis, MO) 0.05 – 64.0 μg/ml, tetracycline (Sigma, St. Louis, MO) 0.05 – 64 μg/ml. The MIC breakpoint for resistance for clarithromycin will be MIC≥1 μg/ml, metronidazole will be MIC≥8 μg/ml, amoxicillin will be MIC≥2 μg/ml and tetracycline will be MIC≥2 μg/ml. Isolates with difference in antimicrobial susceptibility will be further analyzed (genetic mutation responsible for resistance) to understand the molecular mechanism of resistance.

Genotyping of *vac*A: The *vacA* genotype of all twenty bacterial isolates from each individual patient will be determined by PCR using s1a, s1b and s1c and s2 specific primers for signal sequences and m1 and m2 specific primers for the middle region of *vacA* gene [48]. All single colony isolates will be further typed by: (a) PCR-based molecular typing methods, primarily RAPD; (b) PCR-based determination of presence or absence of *iceA1, iceA2, oipA, babA,* IS *605*, *606* and *60*).

IL-8 induction: All twenty bacterial isolates from each individual patient will be analyzed for IL-8 induction on HEp-2 cells as described previously [49]. Briefly, HEp-2 cells will be co-cultured for 24 h with an *H. pylori* suspension containing 5×10^8 bacteria/ml. The medium will be removed, and the IL-8 produced in the supernatant will be evaluated by an enzyme-linked immunosorbent assay (ELISA)

using the specific ELISA kit provided by Diaclone (Besançon, France) according to the manufacturer's instructions.

DNA sequencing: Variation in the DNA sequence of a number of selected gene(s) (vacA m region, sysS, glmM, recA, glr, picA, and picB gene etc.) will be determined by DNA sequencing of PCR amplified gene product. The DNA sequence of all twenty bacterial isolates from each individual patient will be compared and analyzed.

MLST of *Helicobacter pylori* (http://pubmlst.org/helicobacter/): The *Helicobacter* MLST scheme uses internal fragments of the following eight housekeeping genes: atpA, efp, mutY, ppa, trpC, ureI, vacA, yphC. The primer pairs listed in the website <http://pubmlst.org/helicobacter/info/primers.shtml> will be used for the PCR amplification of internal fragments of these genes. MLST typing will primarily be used for identifying genetic diversity of isolates in a patient.

Sample size: The genetic diversity of *H. pylori* isolates in symptomatic and asymptomatic is not known. We assume that in 75% symptomatic patients and 30% of the asymptomatic patients will have genetically diverged bacteria. Assuming that 75% of the isolates from symptomatic patients and 30% of the isolates from asymptomatic are diverge the minimum number of index patients required in this study will be 42 (21 isolates in each group)(90% power and significance level of 0.05). Considering 60% prevalence of *H. pylori* infection we need to enroll 35 index cases for 21 subjects. Considering a minimum of 4 member infected with *H. pylori* we will examine a total of 140 subject in each group.

The required number of patients on each group n is calculated as follows:

$$n = \frac{p_1 \times (100 - p_1) + p_2 \times (100 - p_2)}{(p_2 - p_1)^2} \times f(\alpha, \beta)$$

p_1 = % with genetically diverse bacteria in symptomatic group
p_2 = % with genetically diverse bacteria in asymptomatic group
α = probability of type I error (0.05)
β = probability of type II error (0.9)
1-β= power to detect a difference of magnitude $p_1 - p_2$

Facilities Available

Describe the availability of physical facilities at the place where the study will be carried out. For clinical and laboratory-based studies, indicate the provision of hospital and other types of patient's care facilities and adequate laboratory support. Point out the laboratory facilities and major equipment that will be required for the study. For field studies, describe the field area including its size, population, and means of communications. **(TYPE WITHIN THE PROVIDED SPACE).**

The study will be carried out in LSD & CSD of ICDDR, B, DMCH, and SMI Stockholm, Sweden. LSD has all necessary equipments for caring out the research, incubation facilities and instruments for molecular biology is available at LSD. We are planning to do the DNA microarray analysis at SMI, who has the most modern H. pylori related essential lab facilities. Patients material (biopsy sample) will be collected from DMCH and the available facilities are optimum for the present study. Endoscopy of the field and healthy selected subjects will be done at Endoscopy Unit of CSD, ICDDR, B by Dr. Bardhan, who has a long experience and routinely performed endoscopy of the patients of ICDDR, B.

Data Analysis

Describe plans for data analysis. Indicate whether data will be analyzed by the investigators themselves or by other professionals. Specify what statistical software packages will be used and if the study is blinded, when the code will be opened. For clinical trials, indicate if interim data analysis will be required to monitor further progress of the study. **(TYPE WITHIN THE PROVIDED SPACE).**

Data analysis will be based on following objectives:

1. Prevalence of genetic diversity of *H. pylori* isolates (genetic diversity of isolates within individual patient) from patients with gastroduodenal symptoms and asymptomatic carriers.
    a. The prevalence of genetic diversity of the isolates collected from symptomatic and asymptomatic subjects as determined by RAPD, DNA sequencing and MLST typing and the diversity among two groups will be compared.

2. Role of genetic diversity of *H. pylori* isolates in the transmission of infection among family contracts.
    a. The prevalence of *H. pylori* infection among family members of the index case will be compared between symptomatic and asymptomatic groups and between index cases with and without genetically diverse bacteria.

3. Role of genetic diversity of *H. pylori* isolates (genetic diversity of isolates within individual patient) in pathogenesis, diseases manifestation, and antimicrobial susceptibility.
    a. The role of genetic diversity will be studied by comparing the prevalence of infection in two groups (symptomatic and asymptomatic) and by analyzing the antimicrobial susceptibility and IL 8 induction of isolates from individual patients. If diverge isolates differs in antimicrobial susceptibility, and IL 8 induction this will provide preliminary information regarding role of genetic diversity in pathogenesis and survival.

4. To compare the prevalence of genetic diversity of isolates (genetic diversity of isolates within individual patient) from the present study with similar data from other countries.
    a. Our collaborator in Sweden is conducting a similar study in Sweden and we plan to compare our data with data from Sweden.

Ethical Assurance for Protection of Human Rights

Describe in the space provided the justifications for conducting this research in human subjects. If the study needs observations on sick individuals, provide sufficient reasons for using them. Indicate how subject's rights are protected and if there is any benefit or risk to each subject of the study.

This study will be conducted in ICDDR, B filed site at Nandipara and Dhaka Medical College Hospital (DMCH). All patients attending the Department of Gastroenterology, DMCH for upper gastrointestinal tract endoscopy will be eligible for the study. Among the patients who are positive for *H. pylori* will be considered as index case and their family members will be screened for *H. pylori* by stool antigen test. Family members of the index case who are positive for *H. pylori* stool antigen test will be invited to enroll in the study and will undergo upper GI endoscopy (for adults) or gastric juice collection (for children below 12 years).

Healthy carrier will recruited from Nandipara ICDDR, B filed study site. Randomly selected household heads (both father and mother) will be screened for *H. pylori* infection by stool antigen test. Positive household heads will be invited to participate the study and those who agree will undergo upper GI endoscopy for biopsy or nasogastric intubation for gastric juice. After explaining the aim of the study, written consent will be obtained and subjects will be included in the study. The study will not interfere with the management and treatment of the patients and none of the procedures will be harmful. Endoscopy will be performed by trained experts in the field at ICDDR,B and all necessary precautions will be taken. All patients will be treated h. pylori infection based on stool antigen test and culture.

Use of Animals

Describe in the space provided the type and species of animal that will be used in the study. Justify with reasons the use of particular animal species in the experiment and the compliance of the animal ethical guidelines for conducting the proposed procedures.

No animal will be used in the study.

Literature Cited

Identify all cited references to published literature in the text by number in parentheses. List all cited references sequentially as they appear in the text. For unpublished references, provide complete information in the text and do not include them in the list of Literature Cited. There is no page limit for this section, however exercise judgment in assessing the "standard" length.

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Dissemination and Use of Findings

Describe explicitly the plans for disseminating the accomplished results. Describe what type of publication is anticipated: working papers, internal (institutional) publication, international publications, international conferences and agencies, workshops etc. Mention if the project is linked to the Government of Bangladesh through a training programme.

It is hoped that the information generated from this study will result in better understanding of the mechanism of transmission of H. pylori. This study will generate knowledge to understand the role of stomach flora in peptic ulcer and stomach cancer and it will generate valuable information regarding transmission, disease development, and antimicrobial resistance in both symptomatic and asymptomatic patient and will open a new or modified treatment guidelines for the management of H. pylori related

diseases in both adults and children. The results of this study will be disseminated in international journals and in international conferences.

Collaborative Arrangements

Describe briefly if this study involves any scientific, administrative, fiscal, or programmatic arrangements with other national or international organizations or individuals. Indicate the nature and extent of collaboration and include a letter of agreement between the applicant or his/her organization and the collaborating organization. **(DO NOT EXCEED ONE PAGE)**

This study is a collaborative one among LSD, CSD, ICDDR, B, DMCH and the Infections disease institute (SMI), Karolinska Institute, Stockholm, Sweden.

The endoscopy unit of Dept. of Gastroenterology, DMCH receives around 20 to 30 patients everyday for endoscopy. Our co-investigator Dr. Mian Mashhud performs endoscopy one day in a week and we have selected that particular day for enrollment. The patients at endoscopy Dept. are either referred form indoor or from outdoor.

CSD, ICDDR,B will do endoscopy of patients from Nandipara, and gastric juice collection. LSD of ICDDR,B will do culture of H. pylori, antimicrobial susceptibility test and primary genotyping assays. LSD will also perform Virulence factor expression, IL-8 induction tests, and MLST typing.

The investigators of SMI will interact closely in the study by transferring recently adapted techniques for culture of *H. pylori*, and molecular biology technology for genotyping of strains continuous linkers will be maintained by visits of scientists to each other's laboratory. SMI will be involved in more advance analysis of selected isolates by microarray analysis. This collaboration will help us to set up a advance molecular biology set up for molecular characterization of *H. pylori* strain isolated from Bangladeshi patients.

Biography of the Investigators:

Give biographical data in the following table for key personnel including the Principal Investigator. Use a photocopy of this page for each investigator.

1. **Name:** Motiur Rahman, M.D, PhD

2. **Present position:** Associate Scientist and Head RTI/STI Laboratory, Laboratory Sciences Division (LSD)

3. **Educational background:**

Institution and location	Degree	Year	Field of study
Karolinska Institute, Stockholm, Sweden	PhD	1997	Microbiology, Molecular Biology
Medical College Hospital, Rangpur, Bangladesh	MBBS	1986	Medicine, Surgery Gynaecology

4. **List of ongoing research protocols** (Start and end dates; and percentage of time)

4.1 As principal Investigator

Protocol Number	Starting date	End date	% time
2002-008	7/02	6/05	10%
2004-034	12/04	12/06	25%
2003-035	7/04	09/05	20%
2004-043	1/05	1/06	20%

4.2 As Co-principal Investigator

Protocol Number	Starting date	End date	% time
HIV surveillance	01/04	12/05	5%

5. **Publications**

Types of publications	Numbers
a) Original scientific papers in peer reviewed journals	33
b) Peer reviewed articles and book chapters	-
c) Papers in conference proceedings	18
d) Letters, editorials, annotations, and abstracts in peer-reviewed journals	-
e) Working papers	-
f) Monographs or books	2

6. Five recent publications including publications relevant to the present research protocol

- Rasel Khan, Rahman M **(2005)** Response, T2182C Mutation is not associated with Clarithromycin Resistance in Helicobacter pylori. **Antimicrobial Agent & Chemotherapy.** 49(2):868-870.

- SA Sarker, S Nahar, **M Rahman** PK Bardhan, GB Nair, C Beglinger N Gyr. **(2004).** High prevalence of cagA and vacA seropositivity in asymptomatic Bangladeshi children with Helicobacter pylori infection. **Acta Paediatr** 93: 1-6.

- Shamsun Nahar, Asish K. Mukhopadhyay, Rasel Khan, Mian Mashhud Ahmad, Simanti Datta, Santanu Chattopadhyay, Swapan Chandra Dhar, Safique Sarker, Lars Engstrand, Douglas E. Berg, G. Balakrish Nair, **Motiur Rahman**. **(2004).** Antimicrobial susceptibility of *Helicobacter pylori* strains isolated from Bangladesh. **J Clin Microbial**. 42(10): 4856-4858.

- Rasel Khan, Shamsun Nahar, Jinath Sultana, Mian Mashhud Ahmad, **Motiur Rahman. (2004).** T2182C Mutation in 23S rRNA Associated with Clarithromycin Resistance in *Helicobacter pylori* Isolates from Bangladesh. **Antimicrobial Agent & Chemotherapy** 44(9):3567-69.

- Mokhlasur Rahman, Inger Kühn, **Motiur Rahman**, Barbro Olsson-Liljequist, Roland Möllby **(2004)**. Evaluation of a scanner assisted colorimetric MIC method for susceptibility testing of environmental Gram-negative fermentative bacteria. Applied and Environmental Microbiology 70(4): 2398-2404.

- Khairun Nessa, Shama-A-Waris, Zafar Sultan, Shirajum Monira, Maqsud Hossain, Shamsun Nahar, Habibur Rahman, Mahbub Alam, Pam Baatsen, **Motiur Rahman (2004)** Epidemiology and etiology of sexually transmitted infection among hotel based sex workers (HBSWs) in Bangladesh. **J Clin Microbial**. 42(2): 618-21

- **Motiur Rahman,** Asish K. Mukhopadhyay, Shamsun Nahar, Simanti Datta , Mian Mashhud Ahmed, Safique Sarker, Ibna M. Masud, Lars Engstrand, M. John Albert, G. Balakrish Nairand Douglas E. Berg. **(2003).** DNA level characterization of *Helicobacter pylori* strains from patients with overt disease and with benign infections in Bangladesh. J Clin Microbial 41(5): 2008-2014

Budget Justifications

Please provide one page statement justifying the budgeted amount for each major item. Justify use of man power, major equipment, and laboratory services.

The budget presented here represents a minimum estimation of the costs:

Personnel: We have budgeted 25% salary of PI Dr. Motiur Rahman and 10% salary of Dr. Shafiqul Sarker and Dr. Pradip Bardhan. Two research officer, one study physician and 2 FRA was budgeted.

Travel: Travel cost for two visits to Sweden was budgeted and travel and living expenses of one PhD student for training was budgeted. Travel cost of US $ 4000 was budgeted for local travel including the transport cost of the enrolled subjects.

Consultant: We have budgeted for one consultant from NICED India to visit us during the study to period to help in molecular aspect of the study.

Capital: US $ 20,000 was budgeted for capitals, which includes H. pylori incubator and one other instrument for the laboratory work.

Other Support

Describe sources, amount, duration, and grant number of all other research funding currently granted to PI or under consideration. (DO NOT EXCEED ONE PAGE FOR EACH INVESTIGATOR)

1. RTI/STI service delivery to FHI-BWHC clinics for Hotel Based Sex Workers in Dhaka. US $ 26,000. (2005)

2. Molecular analysis of virulence genes of *Helicobacter pylori* and identification of genotypes associated with overt disease (gastro duodenal ulcer, non ulcer dyspepsia and gastric cancer) in Bangladeshi population. 120,000 (2002-2005)

3. Field evaluation of simple rapid tests in the diagnosis of syphilis US $ 48765 (2004-2005)

4. Molecular and biochemical analysis of intestinal microflora in severely malnourished children with cholera treated with oral rehydration solution with and without amylase resistant starch US $ 22910 (2004-2005).

5. Diagnostic services to HIV/STI surveillance project among men having sex with men (MSMs) in Kathmandu, Nepal (US $ 40, 811)

6. A comparison of two methods (Enhanced Syndromic Management and Periodic Presumptive Treatment) of systematic prevention and control of STIs among hotel based female sex workers in Dhaka, Bangladesh (2004-2006) FHI Dhaka (US $ 24213) and UNICEF (US $ 70,000).

Check List

After completing the protocol, please check that the following selected items have been included.

1. **Face Sheet Included** [x]
2. **Approval of the Division Director on Face Sheet** [x]
3. **Certification and Signature of PI on Face Sheet, #9 and #10** [x] [ ]
4. **Table on Contents** [x]
5. **Project Summary** [x]
6. **Literature Cited** [x]
7. **Biography of Investigators** [x]
8. **Ethical Assurance** [x]
9. **Consent Forms** [x]
10. **Detailed Budget** [x]