APPLICATION PROJECT GRANT

1. INVESTIGATORS : Dr. Rashidul Haque
ICDDR,B
: Prof. W. A. Petri, Jr.
University of Virginia

2. COLLABORATOR : Prof. Barry M. Farr
University of Virginia

3. CONSULTANT : Prof. R. Bradly Sack
Johns Hopkins University

4. TITLE OF THE PROJECT : Field studies of human immunity to amebiasis in Bangladesh.

5. STARTING DATE : As soon as funds available

6. DATE OF COMPLETION : Four years after starting

7. TOTAL BUDGET : US $ 3,55,442 (for Bangladesh part)

8. HEAD OF PROGRAMME : _____
Director,
Laboratory Sciences Division

AA

Form Approved Through 9/30/97
OMB No. 0925-0001

Department of Health and Human Services
Public Health Service

Grant Application

Follow instructions carefully.
Do not exceed character length restrictions indicated on sample.

LEAVE BLANK—FOR PHS USE ONLY.		
Type	Activity	Number 98-009
Review Group	Formerly	
Council/Board *(Month, Year)*		Date Received 29-9-98

1. TITLE OF PROJECT *(Do not exceed 56 characters, including spaces and punctuation.)*
Field studies of human immunity to amebiasis in Bangladesh

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT ☐ NO ☒ YES *(If "Yes," state number and title)*
Number: Title: TMP Pilot--NIAID and PA-96-048 Expanded Research on Emerging Diseases

3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR

3a. NAME *(Last, first, middle)*	3b. DEGREE(S)	3c. SOCIAL SECURITY NO.
Petri, William Arthur Jr.	M.D., Ph.D.	

3d. POSITION TITLE
Professor

3e. MAILING ADDRESS *(Street, city, state, zip code)*
Division of Infectious Diseases
Room 2115 MR4 Building
University of Virginia HSC
Charlottesville VA 22908

3f. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT
Medicine, Microbiology and Pathology

3g. MAJOR SUBDIVISION
School of Medicine

3h. TELEPHONE AND FAX *(Area code, number and extension)*
TEL: 804 924-5621
FAX: 804 924-0075

E-MAIL ADDRESS: wap3g@virginia.edu

4. HUMAN SUBJECTS	4a. If "Yes," Exemption no. or IRB approval date	4b. Assurance of compliance no.
☐ No ☒ Yes	10/01/97 ☐ Full IRB or ☒ Expedited Review	M-1343

5. VERTEBRATE ANIMALS	5a. If "Yes," IACUC approval date	5b. Animal welfare assurance no.
☒ No ☐ Yes		

6. DATES OF PROPOSED PERIOD OF SUPPORT *(month, day, year—MM/DD/YY)*		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)
07/01/98	06/30/02	159,541	193,891	637,222	780,930

9. APPLICANT ORGANIZATION
Name University of Virginia
Address P.O. Box 9003
Charlottesville VA 22903

10. TYPE OF ORGANIZATION
Public: → ☐ Federal ☒ State ☐ Local
Private: → ☐ Private Nonprofit
Forprofit: → ☐ General ☐ Small Business

11. ORGANIZATIONAL COMPONENT CODE 01

12. ENTITY IDENTIFICATION NUMBER 1546-001-796A1 Congressional District 5th

13. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE
Name D. Wayne Jennings
Title Director, Sponsored Programs
Address P.O. Box 9003 University of Virginia
Charlottesville VA 22906

Telephone 804 924-4270
FAX 804 982-3096
E-Mail Address dwj@virginia.edu

14. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name Norma S. Miller
Title Manager Grants & Contracts Admin
Address University of Virginia
Office of Sponsored Programs
P.O. Box 9003
Charlottesville, Va 22906

Phone 804 924-4270
FAX 804 982-3096
E-Mail Address dwj@virginia.edu

15. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: 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 / PD NAMED IN 3a. *(In ink. "Per" signature not acceptable.)* William A Petri Jr. DATE 9/23/97

16. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF OFFICIAL NAMED IN 14. *(In ink. "Per" signature not acceptable.)* Norma S Miller DATE 10/29/97

DESCRIPTION. State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This description is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. **DO NOT EXCEED THE SPACE PROVIDED.**

Amebiasis is a common worldwide parasitic infection, responsible annually for 50 million cases of amebic colitis and liver abscess and 100,000 deaths. *Our hypothesis is that immunity to E. histolytica infection is acquired from previous infection* In Specific Aim 1 a three year prospective study will measure *E. histolytica* infection and invasion in cohorts of children ages 2-5 in the Mirpur district of Dhaka. If our hypothesis is correct, we would predict the following outcomes: (a) children who have had prior *E. histolytica* infection will be less likely to have future episodes of invasive amebiasis; (b) it is possible that they will also be less likely to be re-infected with *E. histolytica;* and (c) if re-infected may clear infection more rapidly. In Specific Aim 2 we will test the related *hypothesis that clearance of asymptomatic infection by E. histolytica is associated with production of adherence-inhibitory antibodies against the carbohydrate-binding domain of the Gal/GalNAc adherence lectin.* Children with asymptomatic, noninvasive, *E. histolytica* infection (identified in Aim 1) will be prospectively followed from the onset of infection to its clearance. The children will be monitored for serum and salivary antibody responses against the native Gal/GalNAc lectin, and its carbohydrate-binding domain (CBD). If our hypothesis is correct, we would predict that clearance of *E. histolytica* infection will be associated with the development of an adherence-inhibitory antibody response against the carbohydrate- binding domain of the lectin. Completion of these studies should provide a foundation for understanding the mechanisms of protective immunity to amebiasis, and provide a baseline for evaluating the efficacy of vaccines as they enter clinical trials.

PERFORMANCE SITE(S) *(organization, city, state)*

University of Virginia
Department of Medicine
Charlottesville VA 22908

International Centre for Diarrhoeal Disease Research, Bangladesh
Parasitology Laboratory
Dhaka, Bangladesh

KEY PERSONNEL. See instructions on Page 11. *Use continuation pages as needed* to provide the required information in the format shown below.

Name	Organization	Role on Project
William A. Petri, Jr., M.D., Ph.D.	University of Virginia	Principal Investigator
M. Rashidul Haque, M.B., Ph.D.	International Centre for Diarrhoeal Disease Research, Bangladesh	Co-Principal Investigator
Barry M. Farr, M.Sc., M.D.	University of Virginia	Collaborator
R. Bradley Sack, M.D., Sc.D.	Johns Hopkins University	Consultant

Type the name of the principal investigator/program director at the top of each printed page and each continuation page. (For type specifications, see instructions on page 6.)

RESEARCH GRANT

TABLE OF CONTENTS

*Type density and type size of the entire application must conform to limits provided in instructions on page 6.

Appendix *(Five collated sets. No page numbering necessary for Appendix.)*

[X] Check if Appendix is included

Number of publications and manuscripts accepted or submitted for publication *(not to exceed 10)* 5

Other items *(list):*

Protection of Human Subjects documentation.

DETAILED BUDGET FOR INITIAL BUDGET PERIOD
DIRECT COSTS ONLY

FROM 07/01/98 THROUGH 06/30/99

PERSONNEL (Applicant organization only) NAME	ROLE ON PROJECT	TYPE APPT. (months)	% EFFORT ON PROJ.	INST. BASE SALARY	DOLLAR AMOUNT REQUESTED (omit cents) SALARY REQUESTED	FRINGE BENEFITS	TOTALS
William A. Petri, Jr.	Principal Investigator	12	20		25,000	5,500	30,500
Barry M. Farr	Collaborator	12	4		5,000	1,100	6,100
Joshua Rogers	Laboratory Specialist	12	40		9,572	2,680	12,252
SUBTOTALS →					39,572	9,280	48,852

CONSULTANT COSTS Dr. R. Bradley Sack, Johns Hopkins University	5,000
EQUIPMENT (Itemize)	
SUPPLIES (Itemize by category) Cell culture media/sera ($3,000) Chemicals/solvents ($1,000) Glassware/plasticware ($1,500) Chromatography/electrophoresis ($2,000) Molecular biology reagents ($2,000)	9,500
TRAVEL International travel (USA - Bangladesh) ($3,000)	3,000
PATIENT CARE COSTS — INPATIENT	
PATIENT CARE COSTS — OUTPATIENT	
ALTERATIONS AND RENOVATIONS (Itemize by category)	
OTHER EXPENSES (Itemize by category) Publication costs, illustrations, overseas shipping	1,000
SUBTOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD	$ 67,352
CONSORTIUM/CONTRACTUAL COSTS — DIRECT COSTS	76,189
CONSORTIUM/CONTRACTUAL COSTS — INDIRECT COSTS	16,000
TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD (Item 7a, Face Page) →	$ 159,541

BUDGET FOR ENTIRE PROPOSED PERIOD OF SUPPORT
DIRECT COSTS ONLY

BUDGET CATEGORY TOTALS		INITIAL BUDGET PERIOD (from Form Page 4)	ADDITIONAL YEARS OF SUPPORT REQUESTED			
			2nd	3rd	4th	5th
PERSONNEL: *Salary and fringe benefits Applicant organization only*		48,852	50,318	51,828	53,383	
CONSULTANT COSTS		5,000	5,150	5,305	5,464	
EQUIPMENT						
SUPPLIES		9,500	9,785	10,079	10,381	
TRAVEL		3,000	3,090	3,183	3,278	
PATIENT CARE COSTS	INPATIENT					
	OUTPATIENT					
ALTERATIONS AND RENOVATIONS						
OTHER EXPENSES		1,000	1,030	1,061	1,093	
SUBTOTAL DIRECT COSTS		67,352	69,373	71,456	73,599	
CONSORTIUM/ CONTRACTUAL COSTS	DIRECT	76,189	68,690	70,751	72,875	
	INDIRECT	16,000	16,480	16,974	17,483	
TOTAL DIRECT COSTS		159,541	154,543	159,181	163,957	

TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PERIOD OF SUPPORT *(Item 8a, Face Page)* → $ 637,222

JUSTIFICATION. Follow the budget justification instructions exactly. Use continuation pages as needed.

Please see Budget Justification on page 9.

PROJECT : IMMUNITY TO ENTAMOEBA HISTOLYTICA INFECTION

The project will be submitted by the University of Virginia and ICDDR,B to the NIH for funding.

PI : Prof. W. A. Petri, Jr., University of Virginia
Co-PI: Dr. Rashidul Haque, ICDDR,B

Budget for initial budget period (year 1)

I. Local Salary	Number	% effort	Amount in US $
Investigator	1	40%	5,725
Project Physician	1	100%	7,584
Research Officer	1	100%	4,164
Laboratory Technicians	2	100%	6,592
Health Assistants	3	100%	8,064
Health Workers	2	100%	4,080
Lab Attendant	1	100%	1,480
		Sub total	37,689
II. Supplies and and materials			
Chemicals, reagents etc			5,000
Plastic, consumables			2,000
Office supplies			1,000
Antigen detection kits			5,000
Medicine and care for study subjects			4,000
		Sub total	17,000
III. Contractual			
Rents, utilities etc			3,000
Use of common facilities			16,000
Contractual others			1,500
		Sub-total	20,500
IV. Travel			
Local travel (Project area-ICDDR,B)			2,000
International Travel (Dhaka-USA)			3,000
		Sub-total	5,000
V. Interdepartental service (Lab tests and others)			2,500
VI. Capital Expenditure (Equipment)			
ELISA reader (one)			6,000
Freezer (one)			1,000
Computer and UPS			2,500
		Subtotal	9,500

Total cost for initial budget period (Year 1) : $ 92,189

19/10/97
Shamima Moin
Controller, Budget & Costing

11

12

BUDGET JUSTIFICATION:

(A) UNITED STATES

1. Personnel: Dr. Petri is the Principal Investigator and will be responsible for the entire project. He will oversee the conduct of the field study, review data collection, prepare manuscripts and progress reports, and meet in person with Dr. Haque twice a year to assess progress and plans (one meeting in Dhaka and one in Charlottesville each year). Dr. Farr is a collaborator who will provide expert assistance in the design and statistical analyses of the field studies. Mr. Joshua Rogers will prepare purified lectin and recombinant carbohydrate binding domain for the serologic studies, assess epitope-specific antibody responses, and perform adherence assays with human sera.

2. Consultant: The consultant Dr. Brad Sack brings to the project tremendous experience and expertise in the design and conduct of prospective human clinical trials in Bangladesh. He has been invaluable in the design of these studies and will consult on the practical design and follow-through of the cohort study in Mirpur.

3. Travel: Funds are requested for one visit per year of Dr. Petri to Bangladesh to oversee the field work and consult in person with Dr. Haque.

4. Supplies: Cell culture media/sera and glassware/plasticware are for culture of *E. histolytica* trophozoites (the source of the purified lectin for the anti-lectin antibody measurements). Chromatography and electrophoresis and molecular biology reagents are for the purification of the native lectin and recombinantly-expressed carbohydrate domain and preparation of microtiter plates for assessing the antibody response of children.

5. Other Expenses: Project-related publication costs, medical illustrations and overseas shipping costs.

(B) BANGLADESH

1. Personnel: The co-Principal Investigator will be responsible for the conduct of all of the field work in Dhaka. The Project Physician will work out of the Project Field Office six days per week. This physician will be available to examine and treat any of the children in the study who become ill. Several field workers are required for the project because of the frequent visits required to monitor the children in the study. Three Health assistants and two Health workers will be working in the community for registration of the family, motivation of the family, collection of stool, blood and saliva samples and for routine morbidity surveillance and surveillance for *E. histolytica* infection. Of the two laboratory technicians one will be working in the field for collection of blood sample from the study population and one will be working for stool microscopic examination and antigen detection in the lab. The lab attendant will assist the technicians in performing the stool and antigen detection analyses. The research officer will perform all serological tests required in the study with the help of the co-Principal Investigator.

2. Supplies: Chemicals and reagents are required for formal-ether concentration of stool samples and culture of all stool samples that will be collected in the study. PCR reagents will also be required, and antigen detection tests for *E. histolytica* will need to be procured.

3. Other Expenses: The project will pay the rent of a field office where the project workers will have their working station. The project will not pay any remuneration to the study subjects but free primary health care services will be provided to all the family members and for this medicine and other necessary will be procured. The cost for this purpose is shown under "patient care cost".

4. Equipment: An ELISA reader and a freezer will be required for the project. The ELISA reader that we have in our lab is very old and in need of replacement. A large number of stool , serum and saliva samples need to be stored at -20°C and space available at present is not sufficient.

5. Travel: Funds are requested for travel from the ICDDR,B to the study site (15 minutes by auto) for study personnel, and to transport children requiring medical attention from the study site to ICDDR,B. One trip per year for Dr. Haque to the laboratory of Dr. Petri is requested so that results can be assessed and study planning and analysis conducted in person.

BIOGRAPHICAL SKETCH

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME	POSITION TITLE
William A. Petri, Jr., M.D., Ph.D.	Professor

EDUCATION *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
University of Wisconsin-Madison			Chemistry
University of Virginia	Ph.D.	1980	Microbiology
University of Virginia	M.D.	1982	

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

PROFESSIONAL EXPERIENCE

1978 - 1982 Graduate student and postdoctoral fellow, laboratory of Robert R. Wagner M.D., Department of Microbiology, University of Virginia.
1982 - 1985 Intern and Resident in Internal Medicine at University and Veterans Hospitals of Cleveland, Case Western Reserve University.
1985 - 1988 Fellow in Infectious Diseases, Department of Medicine, University of Virginia
1988 - 1992 Assistant Professor of Medicine and Microbiology, University of Virginia
1992 - 1996 Associate Professor of Medicine, Pathology, and Microbiology, University of Virginia
1996 Professor of Medicine, Pathology, and Microbiology, University of Virginia

HONORS/COMMITTEES

Burroughs Wellcome Fund Scholar Award in Molecular Parasitology (1996-2001)
Member, NIH Tropical Medicine and Parasitology Study Section (1993-1997)
Chair, Scientific Program Committee, American Society for Tropical Medicine & Hygiene (1992-1998)
Board of Directors, American Type Culture Collection (1993-1996)
American Society for Clinical Investigation (1995)
Editorial Boards: Molecular Medicine Today; Invasion & Metastasis; Parasitology International
Phi Beta Kappa (Wisconsin); Alpha Omega Alpha (Virginia)
Burroughs Wellcome Fund New Investigator in Molecular Parasitology (1992-1995)
Lucille P. Markey Scholar in Biomedical Research (1985-1993)
Ad hoc Reviewer, NIH Bacteriology & Mycology-2 Study Section (1992-1996)

SELECTED PUBLICATIONS

Petri WA Jr, Wagner RR. Reconstitution into liposomes of the glycoprotein of vesicular stomatitis virus by detergent dialysis. J Biol Chem 254:4313-4316, 1979.

Petri WA Jr, Estep TN, Pal R, Thompson TE, Biltonen RL, Wagner RR. Thermotropic behavior of dipalmitoylphosphatidylcholine vesicles reconstituted with the glycoprotein of vesicular stomatitis virus. Biochemistry 19:3088-3091, 1980.

Petri WA Jr. Vesicular stomatitis virus glycoprotein-lipid interactions. Ph.D. Dissertation, University of Virginia, 1980.

Ravdin JI, Petri WA Jr, Murphy C, Smith RD. Production of mouse monoclonal antibodies which inhibit in vitro adherence by *Entamoeba histolytica* trophozoites. Infect Immun 53:1-5, 1986.

Petri WA Jr, Smith RD, Schlesinger PH, Murphy CF, Ravdin JI. Isolation of the galactose binding adherence lectin of *Entamoeba histolytica.* J Clin Invest 80:1238-1244, 1987.

Chadee K, Petri WA Jr, Innes DJ, Ravdin JI. Rat and human colonic mucins bind to and inhibit the adherence lectin of Entamoeba histolytica. J Clin Invest 80:1245-1254, 1987.

Petri WA, Jr, Joyce MP, Broman J, Smith RD, Murphy CF, Ravdin JI. Recognition of the Gal/Gal NAc adherence lectin of *Entamoeba histolytica* by human immune sera. Infect Immun 55:2327-2331, 1987.

Petri WA Jr., Chapman MD, Snodgrass T, Mann BJ, Broman J. and Ravdin JI. Subunit structure of the galactose and N-Acetyl-D-galactosamine-inhibitable adherence lectin of *E. histolytica.* J Biol Chem 264:3007-3012, 1989.

Petri WA Jr., Jackson TFHG, Gathiram V, Kress K, Saffer LD, Snodgrass TL, Chapman MD, Keren Z, and Mirelman D. Pathogenic and nonpathogenic strains of *Entamoeba histolytica* can be differentiated by monoclonal antibodies to the galactose-specific adherence lectin. Infect Immun 58:1802-6, 1990.

Petri WA Jr., Snodgrass TL, Jackson TFHG, Gathiram V, Simjee AE, Chadee K, Chapman MD. Monoclonal antibodies directed against the galactose-binding lectin of *Entamoeba histolytica* enhance adherence. J Immunol 144:4803-9, 1990.

Petri WA Jr. and Ravdin JI. Protection of gerbils from amebic liver abscess by immunization with the galactose-specific adherence lectin of *Entamoeba histolytica.* Infect Immun. 59:97- 101, 1991.

Mann BJ, Vedvick T, Torian B, Petri WA Jr. Cloning of the 170 kDa subunit of the galactose-specific adherence lectin of *Entamoeba histolytica.* Proc Natl Acad Sci USA, 88:3248-3252, 1991.

Saffer LD, Petri WA Jr. Role of the galactose-specific lectin of *Entamoeba histolytica* in contact-dependent killing of mammalian cells. Infect Immun. 59:4681-4683, 1991.

Braga LL, Ninomiya H, McCoy JJ, Eacker S, Wiedmer T, Pham C, Wood S, Sims PJ, Petri WA Jr. Inhibition of the Complement Membrane Attack Complex by the Galactose-Specific Adhesin of *Entamoeba histolytica.* J. Clin. Invest. 90:1131-1137, 1992.

Haque R, Kress K, Wood S, Jackson TFHG, Lyerly D, Wilkins T, and Petri WA Jr. Diagnosis of pathogenic *Entamoeba histolytica* infection using a stool ELISA based on monoclonal antibodies to the galactose-specific adhesin. J Infect Dis. 167:247-9, 1993.

Petri WA Jr, Mann BJ. Molecular Mechanisms of Invasion by *Entamoeba histolytica.* Sem. Cell Biol.4:305-13, 1993.

Purdy, JE, Mann, BJ, Shugart, EC, and Petri, WA Jr. Analysis of the gene family encoding the *Entamoeba histolytica* galactose-specific adhesin 170 kDa subunit. Molec Biochem Parasitol 62:53-60, 1993.

McCoy, JJ, Mann, BJ, Vedvick T, Pak Y, Heimark DB, and Petri WA Jr. Structural analysis of the light subunit of the *Entamoeba histolytica* adherence lectin. J Biol Chem 268:24223-31, 1993.

McCoy, JJ, Mann, BJ, Vedvick T, and Petri WA Jr. Sequence analysis of genes encoding the *Entamoeba histolytica* galactose-specific adhesin light subunit. Molec Biochem Parasitol 61:325-8, 1993.

Haque, R, Lyerly, D, Wood, S, and Petri, WA Jr. Detection of *Entamoeba histolytica* and *Entamoeba dispar* directly in stool. Amer J Trop Med Hyg 50:595-6, 1994.

Purdy, JE, Mann, BJ, Pho, LT, and Petri, WA Jr. Transient transfection of the enteric parasite *Entamoeba histolytica* and expression of firefly luciferase. Proc Natl Acad Sci USA, 91:7099-7103, 1994.

McCoy JJ, Weaver AM, and Petri WA Jr. Use of Monoclonal Anti-light Subunit Antibodies to Study the Structure and Function of the *Entamoeba histolytica* Gal/GalNAc Adherence Lectin. Glycoconjugate Journal 11:432-436, 1994.

Ragland BD, Ashley LS, Vaux DL, and Petri WA Jr. *Entamoeba histolytica:* Target cells killed by trophozoites undergo apoptosis which is not blocked by bcl-2. Exp Parasitol 79:460-467, 1994.

McCoy JJ, Mann BJ, Petri WA Jr. Adherence and Cytotoxicity of *Entamoeba histolytica,* or How Lectins Let Parasites Stick Around. Infection Immunity 62: 3045-3050, 1994.

Petri WA Jr, Mann BJ. Microbial Adherence. In Principles and Practice of Infectious Diseases, 4th Edition. (Mandell, GL, RG Douglas Jr., and JR Bennett, eds.), Churchill Livingstone Inc., New York, pp11-19, 1995.

Mueller DE and Petri WA Jr. Clonal growth in Petri dishes of *Entamoeba histolytica.* Trans R Soc Trop Med Hyg, 89:123, 1995.

Adler P, Wood SJ, Lee YC, Lee RT, Petri WA Jr, and Schnaar RL. High affinity binding of the *E. histolytica* lectin to polyvalent N-acetylgalactosaminides. J Biol Chem, 270:5164-71, 1995.

Petri WA Jr, Schnaar RL. Purification and Characterization of the Galactose- and N-acetylgalactosamine-(Gal/GalNAc) Specific Adherence Lectin of *Entamoeba histolytica.* Methods Enzymology 253:98-104, 1995.

Vines RR, Purdy JE, Ragland BD, Samuelson J, Mann BJ, and Petri WA Jr. Stable transfection of *Entamoeba histolytica.* Molec Biochem Parasitol 71:265-7, 1995.

Haque R, Neville LM, Hahn P, and Petri WA Jr. Rapid diagnosis of *Entamoeba* infection using the*Entamoeba* and *Entamoeba histolytica* stool antigen detection kits. J Clin Microbiology 1995; 33:2558-61.

Petri WA Jr. Amebiasis and the *Entamoeba histolytica* Gal/GalNAc lectin: from lab bench to bedside. J Invest Med 44:24-35, 1996.

Purdy JE, Pho LT, Mann BJ, Petri WA Jr. Upstream regulatory elements controlling expression of the *Entamoeba histolytica* lectin. Molec Biochem Parasitol 1996; 78:91-103.

Haque R, Faruque ASG, Hahn P, Lyerly DM, Petri WA Jr. *Entamoeba histolytica* and *Entamoeba dispar* infection in Bangladesh. J Infect Dis 1997; 175:734-6.

Ramakrishnan G, Vines RR, Mann BJ, Petri WA Jr. A tetracycline-inducible gene expression system in *Entamoeba hi* Molec Biochem Parasitol 1997; 84:93-100.

Singh U, Rogers JB, Mann BJ, Petri WA Jr. Transcription initiation is controlled by three core promoter elements in the protozoan parasite *Entamoeba histolytica.* Submitted for publication, 1997.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
Photocopy this page or follow this format for each person.

NAME	POSITION TITLE
Mohamed Rashidul Haque, M.B., Ph.D.	Associate Scientist

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Medical Academy, Sofia, Bulgaria	M.B.	1985	
Medical Academy, Sofia, Bulgaria	Diploma	1985	
Bulgarian Academy of Sciences, Sofia	Ph.D.	1988	Health Education

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. **DO NOT EXCEED TWO PAGES.**

PROFESSIONAL EXPERIENCE

1983 - 1984	Internship training in Medicine, Medical Academy, Sofia, Bulgaria
1986 - 1987	Clinical Ordinator Training, Institute of Infectious and Parasitic Disease, Medical Academy, Sofia, Bulgaria
1989 - 1995	Assistant Scientist/Head, Parasitology Laboratory, International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B), Dhaka, Bangladesh
1995 - Present	Associate Scientist/Head, Parasitology Laboratory, ICDDR, B

SELECTED PUBLICATIONS

Haque R, Komandarev S. Immunological analysis of antigens of *T. canis* and *A. lumbricoides* and attempt to obtain species specific antiserum agsinst these helminths. Helminthology, 26:67-71, 1988.

Haque R, Komandarev S, Dragneva N, Michov L. Purification of *T. canis* antigens by means of affinity chromatography and their application in serodiagnosis of Visceral larva Migrans. Helminthology, 27:55-63, 1989.

Haque R, Hall A, Tzipori S. Zymodemes of *Entamoeba histolytica* in Dhaka, Bangladesh. Annals of Tropical Medicine and Parasitology, 84:629-32, 1990.

Gonzalez-Ruiz A, Haque R, Reheman T, Aguirre A, Jarmillo C, Castanon G, Hall A, Guhl F, Ruiz-Palacios A, Warhurst DC, Miles MA. A monoclonal antibody for the distinction of invasive and non-invasive clinical isolates of *Entamoeba histolytica*. J Clin Microb, 30:2807-13, 1992.

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Gonzalez-Ruiz A, Haque R, Aguirre A, Castanon G, Hall A, Guhl F, Ruiz-Palacios G, Miles MA, Warhurst DC. Value of microscopy in the diagnosis of dysentery associated with invasive *Entamoeba histolytica*. J Clin Pathol, 47:236-39, 1994.

Palmer DR, Hall A, Haque R, Anwar KS. Antibody isotype responses to antigens of *Ascaris lumbricoides* in a case control study of persistently heavily infected Bangladeshi children. Parasitology, 111:385-93, 1995.

Haque R, Faruque ASG, Petri WAJr. Prevalence of *Entamoeba dispar* and *Entamoeba histolytica* infection in children in Bangladesh. J Infect Dis 1997; 175:734-6.

Petri, William Arthur Jr.

BIOGRAPHICAL SKETCH

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME	POSITION TITLE
R. Bradley Sack, MD, Sc.D.	Professor, Department of International Health

EDUCATION *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Lewis and Clark College, Portland, OR	BS	1956	Pre-Med
University of Oregon Medical School, OR	MD,MS	1960	Medicine, Bacteriology
Johns Hopkins University, Baltimore, MD	ScD	1968	Pathobiology

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

960-61 Intern in Medicine, University Hospital, University Hospital, University of Washington, Seattle, WA
961-62 Resident in Medicine, University of Washington, Seattle, WA
962-64 Fellow, Dept. of Medicine, Johns Hopkins University, School of Medicine, Center for Medical Research and Training, Calcutta, India
962-64 Instructor, Dept. of Medicine, Johns Hopkins University, School of Hygiene and Public Health, Baltimore, MD
964-65 Assistant Resident in Medicine, University of Washington, Seattle, WA
965-70 Research Associate, Dept. of Pathobiology, Johns Hopkins University, School of Hygiene and Public Health, Baltimore, MD
967-68 Instructor, Dept. of Medicine, Johns Hopkins University, School of Medicine, Baltimore, MD
968-70 Resident Coordinator, Johns Hopkins University, Center for Medical Research and Training, Calcutta, India
968-70 Assistant Professor of Medicine, Johns Hopkins University, School of Medicine, Baltimore, MD
970-72 Associate Professor of Medicine, Head, Division of Infectious Diseases, University of Oregon Medical School, Portland, OR
972-79 Associate Professor of Medicine, Johns Hopkins University, School of Medicine, Baltimore, MD. Associate Director, Johns Hopkins University International Center for Medical Research, Baltimore, MD
977-80 Director, Johns Hopkins University International Center for Medical Research, Baltimore, MD
977-90 Chief, Division of Geographic Medicine, Johns Hopkins University School of Medicine, and School of Hygiene and Public Health, Baltimore, MD
79- Professor of Medicine, Johns Hopkins University School of Medicine, Dept. of Medicine, Baltimore, MD
esent
85- Professor of International Health, Johns Hopkins, University, School of Hygiene and Public Health, Dept. of Present International Health, Baltimore, MD
91-94 Associate Director, Divisions of Community Health and Laboratory Sciences, International Center for Diarrheal Present Diseases Research, Bangladesh; Dhaka, Bangladesh

nors and membership: AOA, Sigma Xi, AFCR, ASCI, IDSA, ASTM&H, ASM, U.S.-Japan Cholera Panel

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ikel CS,Grieko FD,Reuben J,Myers LL, Sack RB. Human Colonic Epithelial Cells,HT29/C1, Treated with Crude Bacteroides fragilis Enterotoxin Dramatically Alter their Morphology. Infect Immun 60:321-327,1992.

k **RB**,Myers LL,Almeido-Hill J,Shoop DS,Bradbury WC, Reid R,Santosham M. Enterotoxigenic Bacteroides fragilis: Epidemiologic Studies of its role as a Human Diarrhoeal Pathogen. J Diarrh Dis Res 10:4-9, 1992.

dique AK,Zaman K,Baqui AH,Akram K,Mutsuddy P,Eusof A,Haider K,Islam S,**Sack RB**. Cholera Epidemics in Bangladesh:1985-1991. J Diarrh Dis Res 10:79-86,1992.

n C,Unicomb L,Gentsch J,Banul N,**Sack RB**, and Glass R. Rotavirus Diarrhea in Bangladeshi Children: Correlation of Disease Severity with Serotypes. J Clin Microb 30:3234-3238,1992.

ique SM,Abdul Alim AR,Rahman MM,Siddique AK,**Sack RB**,Albert MJ. Clonal relationships among Classical Vibrio cholerae 01 strains isolated between 1961 and 1992 in Bangladesh. J Clin Microb 31:2513-16,1993.

FF Principal Investigator/Program Director (Last, first, middle): Petri, William Arthur, Jr.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
Photocopy this page or follow this format for each person.

NAME	POSITION TITLE
Barry M. Farr, M.D., M.Sc.	Professor of Medicine and Epidemiology

EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)

INSTITUTION AND LOCATION	DEGREE (If applicable)	YEAR(s)	FIELD OF STUDY
Univ. of Mississippi, Oxford, Mississippi	B.A.	1970-74	Chemistry
Univ. of Mississippi, Oxford, Mississippi		1974-76	Medicine
Washington Univ., St. Louis, Missouri	M.D.	1976-78	Medicine
London School of Hygiene & Trop. Medicine, London, U.K.	M.Sc.	1983-84	Epidemiology

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. DO NOT EXCEED TWO PAGES.

Education

1976-1978 M.D., Washington University School of Medicine, St. Louis, Missouri
1978-1981 Residency, Internal Medicine, University of Virginia School of Medicine; Charlottesville, VA
1981-1983 Fellowship, Infectious Diseases, University of Virginia School of Medicine, Charlottesville, VA
1984 M.Sc. in Epidemiology, London School of Hygiene and Tropical Medicine
1984-1985 Research Associate with David L. Miller, Professor of Community Medicine, St. Mary's Hospital Medical School, London and Dr. Christopher L.R. Bartlett, Communicable Disease Surveillance Centre, London

Positions Held

1983-1989 Assistant Professor, Department of Internal Medicine, University of Virginia School of Medicine, Charlottesville, VA
1986- Hospital Epidemiologist, University of Virginia Hospital, Charlottesville, VA
1986- Director, Master of Science Program in Epidemiology, University of Virginia School of Medicine, Charlottesville, VA
1989-1995 Associate Professor, Department of Internal Medicine, University of Virginia School of Medicine, Charlottesville, VA
1989- Chairholder, William S. Jordan, Jr. Professorship of Internal Medicine in Epidemiology
1995- Professor, Department of Internal Medicine, University of Virginia School of Medicine, Charlottesville, VA

Honors and Committees

Milbank Clinical Epidemiology Scholar Award, University of Virginia School of Medicine, 1983-1988
Member of Scientific Committee on Respiratory Disease, International Union against Tuberculosis and Lung Disease, Paris, 1987 - 1990
Member, Food and Drug Administration Committee for Prevention of Complications Associated with Central Venous Catheters, Twinbrook, MD, 1989 - 1991
Consultant to Hospital Infection Control Practice Advisory Committee regarding Guideline for Prevention of Intravascular Device Associated Infections, Centers for Disease Control, 1994
Member and Scientific Program Chairman, Annual Meeting Planning Committee, Society for Healthcare Epidemiology of America, 1994 - 1997
Chair, Section on Nosocomial Infections, American Society for Microbiology,1995-1996
Young Investigator Award, Society for Healthcare Epidemiology of America, 1995
Academic Councilor, Society for Healthcare Epidemiology of America, 1996 -

Lanata CF,Black RE,Maurtua D,Gil A,Gabilindo A,Yi A,Miranda E,Gilman RH,Leon-Barua R, and **Sack RB**. Etiologic Agents in Acute vs. Persistent Diarrhea in Children under three years of age in peri-urban Lima, Peru. Acta Paediatr Scand (Suppl) 381:32-8,1992.

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Baqui AH,Black RE,**Sack RB**,Chowdhury HR,Yunus M,Siddique AK. Malnutrition, Cell-Mediated Immune Deficiency, and Diarrhea: a Community -Based Longitudinal Study in Rural Bangladeshi Children. Am J Epidemiol 137:355-365,1993.

Sack RB,Gyr K. Helicobacter pylori infection in the Developing World. Lancet 341:1274,1993.

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Figueroa-Quitanilla D,Salazar-Lindo E,**Sack RB**,Leon-Barua R,Sarabia-Arce S,Campos-Samchez M,Eyzaguirre-Maccan E. A Controlled Trial of Bismuth Subsalycylate in Infants with Acute Watery Diarrheal Disease. N Engl J Med 328:1653-1658,1993

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Sack RB,Castrellon J,Della Sera E,Goepp J,Burns B,Croll J,Tseng P,Reid R,Carizzo H,Santosham M. Hydrolyzed Lactalbumin-based Oral Rehydration Solution for Acute Diarrhea in Infants. Acta Paed 83:819-823,1994.

Qadri F,Chowdhury A,Hossain J,Chowdhury J,Azim T,Shimada T,Islam KMN,**Sack RB**, and Albert MJ. Development and Evaluation of Rapid Monoclonal Antibody-Based Coagglutination Test for Direct Detection of Vibrio cholerae 0139 Synonym Bengal in Stool Samples. J Clin Microb 32:1589-1590,1994.

Islam MS,Miah MA,Hasan MK,**Sack RB**,Albert MJ. Detection of non-culturable Vibrio cholerae 01 associated with a Cyanobacterium from an Aquatic Environment in Bangladesh. Trans Roy Soc Trop Med & Hyg 88:298-299,1994.

Sack RB. The Diarrhea of Travelers. Chpt 319,1705-1706, in: Cecils Textbook of Medicine,19th Ed.,Wyngaarden,Smith, and Bennett,Ed.WB Saunders Co., Philadelphia,1992.

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Sack RB. Prospects for the Control of Cholera with Oral Vaccines. J Diarr Dis Res 19:1-3,1992.

Sack RB,Santosham M,Reid R,Black R,Croll J,Yolken R,Aurelian A,Wolff M,Chan E,Garrett S,Froehlich J. Diarrhoeal Diseases in the White Mountain Apaches: Clinical Studies. J Diarrh Dis Res 13:12-17,1995.

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Ahmed ZU,Hoque MM,Rahman ASMH,**Sack RB**. Thermal Stability of an Oral Killed-Cholera-Whole-Cell Vaccine Containing Recombinant B-Subunit of Cholera Toxin. Microb Immun 38:837-842,1994.

Zaman K, Baqui AH, Yunus Md, **Sack RB**, Bateman OM, Chowdhury HR, Black RE. Association between nutritional status, cell-mediated immune status and acute lower respiratory infections in Bangladeshi children. European J Clin Nutr 50:309-14,1996.

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Siddique AK, Akram K, Zaman K, Mutsuddy P, Eusof A, **Sack RB**. Vibrio cholerae O139: How great is the threat of a pandemic? Trop Med Intl Hlth 1(3):393-98, 1996.

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Selected Publications

Scheld WM, Sydnor A Jr, Farr BM, Gratz JC, Gwaltney JM Jr. Comparison of cyclacillin and amoxicillin for therapy of acute maxillary sinusitis. *Antimicrob Agents Chemother* 30:350-353, September 1986.

Farr BM, Gwaltney JM Jr. The problems of taste in placebo matching: zinc gluconate for the common cold. *J Chronic Dis* 40(9):875-879, 1987.

Farr EM, Conner EM, Betts RF, Oleske J, Minnefor A, and Gwaltney JM Jr. Two randomized controlled trials of zinc gluconate lozenge therapy of experimentally induced rhinovirus colds. *Antimicrob Agents Chemother* 31(8):1183-1187, 1987.

Donowitz LG, Hunt EM, Pugh VG, Farr BM, Hendley JO. A comparison of historical and serologic immunity to varicella zoster virus in 373 hospital employees. *Am J Infect Control* 15:212-214, 1987.

Farr BM, Hendley JO, Kaiser DL, Gwaltney JM. Two randomized controlled trials of virucidal nasal tissues in the prevention of upper respiratory infections. *Am J Epidemiology* 128(5):1162-1172, 1988.

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Lima NL, Pereira CRB, Souza IC, Facanha MC, Lima AAM, Guerrant RL, Farr BM. Selective surveillance for nosocomial infections in a Brazilian hospital. *Infect Control Hosp Epidemiol* 14:197-202, 1993.

Jernigan JA, Lowry BS, Hayden FG, Gröschel DHM, Farr BM. Adenovirus type 8 epidemic keratoconjunctivitis in an eye clinic: Risk factors and control. *J Infect Dis* 167:1307-1313, 1993.

Adal KA, Anglim AM, Palumbo CL, Titus MG, Coyner BJ, Farr BM. The use of high-efficiency particulate air-filter respirators to protect hospital workers from tuberculosis: a cost-effectiveness analysis. *New Engl J Med.* 331:169-173, 1994.

Howell PB, Walters PE, Donowitz GR, Farr BM. Risk factors for central venous catheter infection in cancer patients. *Cancer.* 75:1367-1375, 1995.

Farr BM, Johnston BL, Cobb DK, Fisch MJ, Germanson TP, Adal KA, Anglim AM. Preventing pneumococcal bacteremia in patients at risk: results of a matched case-control study. *Arch Intern Med.* 155;2336-2340, 1995.

Adal KA, Flowers RH, Anglim AM, Hayden FG, Titus MG, Coyner BJ, Farr BM. Rates of nosocomial influenza and compliance with preventive strategies at a university hospital. *Infect Control Hosp Epidemiol.* In Press.

Roberts NE, Collmer JE, Wispelwey B, Farr BM. Urbs in rure redux: Changing risk factors for rural HIV infection. Submitted for publication.

Jernigan JA, Siegman-Igra Y, Giuliano K, Guerrant RL, Farr BM. Preventing *Clostridium difficile* and other nosocomial infections with disposable thermometers: a randomized crossover study. Submitted for publication.

Anglim AM, Klym MB, Byers KE, Scheld WM, Farr BM. Effect of a vancomycin restriction policy on ordering practices during an outbreak of vancomycin-resistant Enterococcus faecium. Submitted for publication.

Other Support

Petri, William A., Jr.

ACTIVE

R01 AI26649-08 (PI: Petri) 8/1/96 - 7/31/01 30% effort
NIH/NIAID $191,000 direct costs annually
(including FIRCA supplement R03 TW 00848-01)
Title: "Structure and Function of *E. histolytica* Lectin"
The major goals of this project are structural/functional studies of Gal/GalNAc lectin of *E. histolytica*. The FIRCA supplement with Dr. Rashidul Haque is to examine genetic diversity within *E. histolytica*.
Overlap: There is no scientific or budgetary overlap.

R01 AI-37941 (PI: Petri) 12/1/95 - 11/30/98 20% effort
NIH/NIAID $136,634 direct costs annually
(including FIRCA supplement R03 TW 00747-01)
Title: "Gene Expression in *E. histolytica*"
The major goals of this project are to identify stability sequences on the *E. histolytica* rDNA episome and analyze the amebic lectin *hgl5* promoter. The FIRCA supplement with Dr. Bhattacharya is to examine the rDNA episome origin of replication.
Overlap: There is no scientific or budgetary overlap.

2T32AI07046-21 (PI: Petri) 7/1/97 - 6/30/02 10% effort
NIH/NIAID $246,518 direct costs annually
Title: "Infectious Diseases Training Program"
This grant provides 5 postdoctoral and 3 predoctoral training positions in infectious diseases.
Overlap: There is no scientific or budgetary overlap.

Burroughs Wellcome Fund (PI: Petri) 7/1/96 - 6/30/01 No % effort specified
Title: "Scholar Award in Molecular Parasitology" $80,000 direct costs annually
Overlap: None; this is a career development award.

PENDING

Phase II STTR AI36587 (PI: Lyerly) 11/1/97 - 10/31/99 proposed 10% effort for Dr. Petri
NIH/NIAID $87,152 direct costs annually proposed
Title: "Serodiagnosis and Immunoprophylaxis of Amebiasis"
Overlap: None. This grant proposal is designed to develop new serodiagnostic and vaccine reagents for *E. histolytica*.

Haque, M. Rashidul

ACTIVE

Thrasher Research Fund (PI: Haque) 8/1/96 - 6/30/98 40% effort
Title: "Field trial of beta carotene and anti-helminthic therapy" $55,814 direct costs annually
Overlap: None. This grant will conclude prior to the start of the pending NIH proposal.

R03 TW 00848-01 (PI: Petri) 4/1/97 - 3/31/00 No % effort specified
NIH/NIAID $20,000 direct costs annually
Fogarty International Collaborative Award "Intraspecies variation in *E. histolytica*"

The major goals of this project are to define the genetic diversity within *E. histolytica* and its relationship to virulence. Dr. Rashidul Haque is the foreign collaborator for this grant.
Overlap: None

PENDING

USAID: (PI: Haque) 3 year proposal
Title: "Intraspecies variation in *E. histolytica* and protective immunity with *E. histolytica* infection"
Overlap: The protective immunity section of the USAID proposal, which specifically deals with the long-term consequences of colonization with *E. histolytica*, overlaps in its emphasis on immunity with the current NIH proposal. If both proposals were to be funded the budget of the NIH proposal would be commensurately reduced during the period of overlap.

Sack , R. Bradley

ACTIVE

EPA R824995-01-1 (PI Patz, Jonathan) 10/1/96 - 9/30/99 10% effort
Title: "Integrated assessment of public health effects of climate change for the U.S."
Overlap: None

PENDING

None

Farr, Barry

ACTIVE

2-U01-AI26512 (PI Guerrant, Richard) 6/94 - 5/99 10% effort
NIH/NIAID $165,123 direct costs annually
Title: "Recognition and expression of tropical infectious diseases"
Overlap: None

PENDING

None

RESOURCES

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory:

A new 940 sq. ft. laboratory committed solely to the research program of the Principal Investigator adjacent to other infectious diseases and microbiology laboratories was occupied in July, 1988. A 100 sq. ft. cold room is adjacent to the laboratory.

Clinical:

Animal:

Animal care facilities are located in the same building as the laboratory and are sufficient for the planned experiments.

Computer:

In addition to the numerous personal computers (Apple Macintosh) available for word processing and data analysis, the computer facilities of the University of Virginia are available, on-line via ethernet.

Office:

An office of approximately 120 sq. ft. is located in continuity with the lab space for the Principal Investigator.

Other:

These include glassware washing, autoclave, storage, radiation work areas and tissue culture facilities. Core facilities available at the University include a protein/ nucleic acid sequencing center, hybridoma center, FACs center, electron microscopy center and computer graphics center.

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

Dr. Petri's laboratory is equipped with a CHEF gel apparatus, fluorimeter, Beckman GPR centrifuge, microfuge, Speed Vac, UV/Vis spectrophotometer, CO_2 incubator, freezers (-20, -70°C), gel electrophoresis equipment, peristaltic pump and fraction collector, water baths, balances and fume hood. Equipment existing in the Division and Department of Microbiology that is available to Dr. Petri includes several Beckman ultracentrifuges and Sorvall superspeed centrifuges, liquid scintillation and gamma counters, lyophilizer and a preparative high performance liquid chromatography system with recorder, integrator, UV-Vis detector and precision pumps for gradient elutions.

RESOURCES

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory: The facilities of the parasitology laboratory of Laboratory Sciences Division (LSD), ICDDR, B will be used to conduct the proposed research. There are well-equipped and well-staffed laboratories for research in bacteriology, molecular biology, environmental microbiology, nutritional biochemistry, virology and parasitology under the Laboratory Sciences Division.

Clinical: Facilities of the Clinical Sciences Division (CSD) of ICDDR, B will be used to conduct the proposed research. The CSD operates a hospital in Dhaka for care of patients with diarrhoea and for conducting research and training. In addition to diarrhoea treatment, the division also operates a surveillance program which investigates every 25th patient attending the hospital. This system makes it possible to study different important issues

Animal: related to diarrhoeal diseases. The proposed research program will use the facilities of surveillance program of CSD.

A large animal house is available at the ICDDR, B. The Animal Resources Branch provides direct support to scientists in animal experiments and production of laboratory animals. No animal experiments are foreseen in the proposed projects.

Computer:

The Center operates an IBM mainframe computer. It is connected to 25 terminals. This system provides the capacity to analyze large data sets and is complemented by over 300 personal computers. New e-mail facilities have been established in the Center. Computer facilities will be available to the proposed project as necessary.

Office:

Adequate office facilities are available at the Center as well as for the proposed project in the parasitology laboratory.

Other:

The support services branch is well organized under the laboratory sciences division which includes managerial support, logistic support, bio-engineering cell and archive unit. These facilities will be used for the proposed project when necessary.

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

The parasitology laboratory has a cellulose-acetate electrophoresis system and power supply (Helena lab), four Olympus microscopes including an inverted microscope, freezer, liquid nitrogen tank, ELISA reader and many other pieces of equipment normally required for a parasitology laboratory. Beside this the parasitology laboratory uses equipment from other laboratories of the LSD when required.

A. SPECIFIC AIMS

It is not known if humans acquire immunity against colonization or invasion by *E. histolytica*. The World Health Organization in 1997 identified the study of human immunity as a priority research need, "essential for evaluating the feasibility of developing an *E. histolytica* vaccine". Drs. Petri (USA) and Haque (Bangladesh) have collaborated for the last six years on the study of human infection by *E. histolytica*. This collaboration has resulted in the development of sensitive and specific methods to identify *E. histolytica* infection. We have used these tests in over two thousand children in Bangladesh to demonstrate that *E. histolytica* infection is prevalent.

We now propose to conduct a prospective study to determine if children who have been infected with *E. histolytica* are less likely to be re-infected. Completion of these studies should establish the existence and nature of acquired immunity, and provide a foundation for the future understanding of mechanisms of protective immunity.

Hypothesis 1: *Immunity to invasive infection by E. histolytica is acquired from a previous (symptomatic or asymptomatic) infection.*

Specific Aim 1: A three year prospective study will measure *E. histolytica* infection and invasion in two cohorts of children ages 2-5 in the Mirpur district of Dhaka. One cohort will be composed of children with serologic evidence of prior *E. histolytica* infection, and the other cohort will be composed of children without evidence of prior infection. The children will be monitored for *E. histolytica* infection and invasion. The Mirpur district of Dhaka, Bangladesh, where the proposed studies will be conducted, has a high prevalence of *E. histolytica* infection. It is a stable community only minutes from the ICDDR,B laboratories, and is the site where Dr. Haque is successfully conducting other prospective trials.

If hypothesis (1) is correct, we would predict the following outcomes: (a) children who have had prior *E. histolytica* infection will be less likely to have future episodes of invasive amebiasis; (b) it is possible that they will also be less likely to be re-infected with *E. histolytica;* and (c) if re-infected may clear infection more rapidly.

Hypothesis 2: *Clearance of asymptomatic infection by E. histolytica is associated with production of adherence-inhibitory antibodies against the carbohydrate-binding domain of the Gal/GalNAc adherence lectin.*

Specific Aim 2: Children with asymptomatic, noninvasive, *E. histolytica* infection (identified in Aim 1) will be prospectively followed from the onset of infection to its clearance. The children will be monitored for serum and salivary antibody responses against (a) the native Gal/GalNAc lectin, (b) the lectin carbohydrate-binding domain (CBD), (c) lectin adherence-inhibitory, and (d) lectin adherence-enhancing epitopes. These studies are based our preliminary data that adherence-inhibitory anti-CBD antibodies confer passive protection to amebiasis, and are associated with clearance of *E. histolytica* colonization in humans.

If the hypothesis (2) is correct, we would predict that clearance of *E. histolytica* infection will be associated with the development of an adherence-inhibitory antibody response against the carbohydrate- binding domain of the lectin.

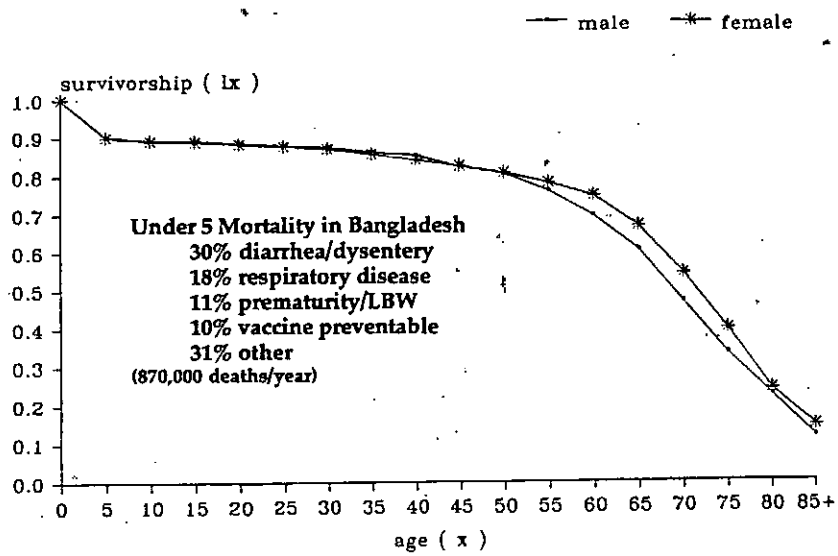
Responsiveness to PA-96-048 "Expanded Research on Emerging Diseases": *E. histolytica* has emerged in last three years as an important cause of dysentery in Bangladesh (see Table 1 and Fig. 8). Building on new and specific diagnostic tests, and the recent appreciation that *E. histolytica* and *E. dispar* are separate species, this proposal responds to PA-96-048 by using field studies to test the host factors influencing emergence of *E. histolytica* infection.

B. BACKGROUND AND SIGNIFICANCE

Importance of the disease amebiasis. Amebiasis is a common worldwide parasitic infection. It is estimated that annually 40 to 50 million cases of amebic colitis and liver abscess and 100,000 deaths result from *E. histolytica* infection (WHO, 1997). The preponderance of *E. histolytica* infection, morbidity and mortality is experienced in Central and South America, Africa and India. For example, in Mexico the 1987-8 national serosurvey of 67,668 human sera samples demonstrated anti-*E. histolytica* antibodies in 8.4% of the population (Caballero-Salcedo et al 1994).

In Bangladesh, where we propose to do these studies, diarrheal diseases are the leading cause of death in children under age 5 (**Figure 1**).

Figure 1: Probability of Survival from Birth to Age (x) by Sex, 1994



Data obtained from the Demographic Surveillance System (DSS)-Matlab, Health and Demographic Surveillance Programme, Community Health Division, International Centre for Diarrhoeal Disease Research, Dhaka, Bangladesh.

We have found *E. histolytica* infection in 7-8% of grade school aged children with diarrhea (Haque et al 1997a). Overall *E. histolytica* was identified in 4.2% of all patients, making it the fifth leading enteropathogen identified (**Table 1**).

Table 1. Enteropathogens identified in stool specimens of patients with diarrhea seen at the International Centre for Diarrhoeal Disease Research Hospital in Dhaka, Bangladesh. Data is for the 1994 - 1995 systematic surveillance of 2915 patients.

Organism	Patients Infected (%)
Vibrio cholerae O1 & O139	18.6
Other vibrios	12.0
Rotavirus	11.9
Shigella sp.	9.3
E. histolytica*	**4.2**
Salmonella sp.	1.7

**E. histolytica* prevalence was determined on a subset of 1049 patients ages 1-14 using an antigen detection test which differentiates *E.* histolytica from *E. dispar*

Redescription of *E. histolytica* as pathogenic *E. histolytica* and nonpathogenic *E. dispar*. *Entamoeba histolytica* has recently been redescribed as:

E. histolytica (formerly called the pathogenic zymodemes of *E. histolytica*), and
E. dispar (formerly called the nonpathogenic zymodemes of *E. histolytica*)

The two species are morphologically identical. They can be differentiated by isoenzyme analysis, antigen detection using monoclonal antibodies to the Gal/GalNAc lectin, sequences of single copy genes, and small subunit ribosomal RNA sequences (Diamond & Clark 1993). *E. dispar* has never been documented to cause colitis or liver abscess. *E. histolytica* is the cause of amebic colitis and liver abscess. This nomenclature was recently formalized in a joint statement by WHO, PAHO, and UNESCO (WHO, 1997).

Throughout this proposal *Entamoeba histolytica* is defined as the "pathogenic zymodemes" and *Entamoeba dispar* as the "nonpathogenic zymodemes" of what was formerly called *E. histolytica*. The term *E. histolytica/dispar* complex is used in cases where the two species have not been differentiated by specific tests.

Anti-amebic antibodies are an indicator of current or prior *E. histolytica* infection A serum antibody response against *E. histolytica* can be demonstrated in greater than 80% of patients with amebic colitis, 90-99% of patients with amebic liver abscess, and in 100% of asymptomatic carriers of *E. histolytica*. Serum antibodies persist for at least three years after infection (Kagan 1970; Ximenez et al 1991, Jackson et al 1985). The presence of anti-amebic antibodies is therefor an excellent indicator of past or current infection with *E. histolytica*. Patients with *E. dispar* infection do not have a detectable anti-amebic antibody response (Jackson et al 1985; Ravdin et al 1990). A transient (weeks to several months) secretory immune response has also been observed in patients with *E. histolytica* infection. Anti-amebic and anti-galactose adherence lectin IgA antibodies are present in the stool, saliva and colostrum of patients with clinical amebiasis (Aceti et al 1991; Kelsall et al 1994; Agarwal et al 1992). The presence of serum anti-amebic antibodies is not correlated with the absence of infection with *E. histolytica* (Jackson et al 1985). This is to be expected since almost all *E. histolytica*-infected people are seropositive.

Evidence in humans for immunity to *E. histolytica*. It is not known if acquired immunity to *E. histolytica* infection exists. Many prior studies are impossible to interpret, as *E. histolytica*-specific tests were not used (Choudhuri et al 1991; Krupp 1970). Studies that lump *E. histolytica* and *E. dispar* infection together suffer both from the fact that *E. dispar* infection is more common, and that the ratio of asymptomatic *E. histolytica* to *E. dispar* infection varies widely (from close to zero in North America to 1:3 in Bangladesh).

Longitudinal studies of individuals asymptomatically infected with *E. histolytica* (all of whom have serum anti-amebic antibodies) have shown that most spontaneously clear the infection in 3-9 months. However 10-14% of these *E. histolytica*-infected individuals have been shown to progress to invasive amebiasis, demonstrating that immunity to luminal infection, if it exists, is incomplete (Gathiram & Jackson 1985, 1987; Irusen et al 1992).

Case-series of patients with amebic colitis in Natal, South Africa (Gathiram & Jackson 1985) and in Dhaka, Bangladesh (Wanke et al 1985; Haque et al - Preliminary Studies) have demonstrated a decline in incidence above age 14, with a second peak of infection in adults > 40 years old. This could be interpreted as evidence of immunity

acquired in childhood which wanes in the elderly. The only other evidence for immunity to invasive amebiasis is the retrospective patient chart review by De Leon (1970) which found a low incidence of patients being re-admitted to the hospital with liver abscess. Because DeLeon's study was retrospective and lacked a control group, it is impossible to know if the apparently low rate of hospital readmissions with amebic liver abscess was due to acquired immunity, a low prevalence of *E. histolytica* reinfection, or loss of patients to follow-up.

We are left with a situation where there is no human "real world" data that acquired immunity exists. Development of a vaccine against amebiasis cannot optimally occur in a vacuum of knowledge of naturally acquired immunity.

In vitro or animal model evidence of potentially protective humoral and cell-mediated immune responses against *E. histolytica*. Immunization of animals with several *E. histolyica* antigens provides protection from an intra-hepatic challenge with *E. histolytica* (reviewed by Stanley 1997). These antigens include recombinant DNA prokaryotic-expressed serine-rich and cysteine-rich proteins and the Gal/GalNAc adherence lectin.

Support for a role for antibodies in immunization-mediated protection has come from studies using a severe combined immunodeficient (SCID) mouse model. SCID mice lack functional B and T cells, and unlike immunocompetent mice, are partially susceptible to amebic liver abscess. Passive transfer to SCID mice of antibodies against whole *E. histolytica* proteins, the serine-rich protein, or the cysteine-rich domain of the galactose lectin, resulted in faster resolution of amebic liver abscess (Cieslak et al 1992; Zhang et al 1994a, Lotter et al 1997). [Antibodies against the cysteine-poor domain of the galactose lectin increased liver abscess size when transferred to SCID mice, which was interesting in light of previous observations that immunization of gerbils with the intact lectin protected some animals and exacerbated disease in others (Petri & Ravdin 1990)].

The lack of severe amebiasis in patients with the acquired immunodeficiency syndrome suggest a less than stringent requirement of CD4 (+) T cells for protective immunity. However, lymphocytes from patients recovered from invasive amebic disease proliferate in response to amebic antigens, have amebicidal activity, and produce interleukin-2 and gamma interferon (IFN-γ) (Salata et al 1985, 1986). Macrophages and neutrophils, activated with IFN-γ and TNF-α, are endowed with the capability of killing *E. histolytica* trophozoites, while in the absence of IFN-γ, these effector cells were killed by the amebae (Salata et al 1985, 1986; Denis & Chadee 1989; Lin & Chadee 1992). In murine macrophages TNF-α was shown to play a central role in activating macrophages for nitric oxide-dependent cytotoxicity against *E. histolytica* (Denis & Chadee 1989; Lin & Chadee 1992; Lin et al 1994).

While the precise roles and importance of humoral and cellular responses in immunity remain to determined, the above data suggest that protective immunity (if it exists) is likely to involve elements of both.

Examples of other gastrointestinal pathogens for which acquired immunity is better understood. It is instructive to understand how mechanisms of immunity have been discovered for other gastrointestinal pathogens, and how that information is influencing vaccine development.

Rotavirus is an example of an infection in the GI tract for which protection is acquired with natural infection. Rotavirus is a non-enveloped RNA virus that infects the mature villous intestinal epithelial cells in the small intestine. Nearly all children are

infected within the first 3 years of life. Maximum susceptibility to infection is from ages 6 months to 2 years. Natural rotavirus infection has been demonstrated to protect against both subsequent infection and diarrhea: children with two previous infections had an adjusted relative risk of subsequent infection and diarrhea of 0.40 and 0.17 respectively (Velazquez et al 1996). One to 4 months after infection rotavirus-specific IgG and IgA antibodies can be detected in both serum and duodenal fluid. One year after infection it is not possible to detect rotavirus antibodies at the mucosal site, although IgG antibodies persist in serum. Animal models have supported the protective role of antibodies: transfer of serum antibodies provides protection in cows (Besser et al 1988). Evidence exists in humans that protection against re-infection correlates with levels of neutralizing rotavirus antibodies. A serum neutralizing antibody of 1:128 or greater was associated with protection from gastroenteritis with the same serotype of rotavirus (Chiba et al 1986). Rotavirus serum IgA and salivary IgA antibodies were also found to correlate with resistance to severe rotavirus infection (Ward et al 1992; Hjelt et al 1987). One non-empiric approach to produce an effective rotavirus vaccine is to attempt to maximize antibody production against neutralization epitopes on the outer capsid proteins VP4 and VP7 (Kapikian & Chanock 1996).

Cholera is another example of an enteric infection for which a great deal is known about acquired immunity. Recovery from infection with classical biotype of *V. cholerae* provides protection against re-infection for up to three years (Levine et al 1981). Seroepidemiologic surveys have demonstrated that with increasing age the titers of vibriocidal antibodies increases and the incidence of *V. cholerae* infection decreases (Mosley et al 1968). [This is reminiscent of the near perfect correlation of serum antibodies against the *Haemophilus influenzae* type b capsular polysaccharide and protection from *H. influenzae* meningitis (Anderson et al 1977)]. Serum titers of vibriocidal antibody ≥ 20 were associated with protection against both colonization and disease, whereas IgG and IgA responses against cholera toxin were not (Mosley et al 1968; Glass et al 1985; Clemens et al 1991). The association of vibriocidal antibodies with protection was also present for individuals who had received the whole cell *V. cholerae* vaccine (Clemens et al 1991). The cholera example demonstrates that serum antibody responses can be a useful measure of immunity to an enteric infection, and that it may be important to measure both the titer and the specificity of the antibody response to make a correlation with protection.

To summarize the above section, surprisingly little is known about immunity to *E. histolytica*. Other experts share our view of the importance of obtaining this information:

"The experience of clinicians in areas of endemic amebiasis appears to support this concept [that prior infection results in protective immunity], but carefully controlled prospective studies are simply not available" (Stanley 1997).

"Fundamental studies on the immunology of human amoebiasis are essential for evaluating the feasibility of developing an E. histolytica vaccine" (WHO, 1997)

Specific gaps in our knowledge of the immune response to *E. histolytica* to be addressed by this proposal:

- Is there acquired immunity to colonization?
- Is there acquired immunity to invasion or severe disease?
- If immunity exists, does it correlate with systemic or secretory antibody responses against the trophozoite, the galactose lectin, or other defined antigens?

C. PRELIMINARY STUDIES

Introduction: Section **C-I** of Preliminary Studies reviews the production and/or standardization of tests to detect prior and current *E. histolytica* infection. Section **C-II** demonstrates the application of these tests to the study population in Bangladesh. Section **C-II** also shows the correlation of serum antibodies against the lectin carbohydrate binding domain with clearance of *E. histolytica* infection.

C-I: 1. Serum antibodies to the native galactose-specific adherence lectin are a marker of current or past infection with *E. histolytica.* The Gal/GalNAc adherence lectin is almost universally recognized by serum antibodies of individuals who have recovered from amebic colitis and liver abscess (Petri et al 1987, 1989; Ravdin et al 1990). Anti-lectin antibodies were detected in 99% (82/83) of South African patients with amebic liver abscess (**Fig. 2**), and 95% (4/4 in **Fig. 2** plus 16/17 in **Fig. 10**) of individuals colonized with *E. histolytica.* Sera from healthy American controls and patients infected with other parasites were negative. The prevalence of anti-lectin antibodies in South Africans colonized with *E. dispar* and with negative stool exam for *E. histolytica* were identical (25%), suggesting that the anti-lectin antibodies seen in these 2 groups were from past infection with *E. histolytica* and that colonization with *E. dispar* does not lead to a serum anti-lectin antibody response detectable with this assay. Others also observed an absence of a serum antibody response in *E. dispar* colonized individuals (Gathiram & Jackson 1985, 1987).

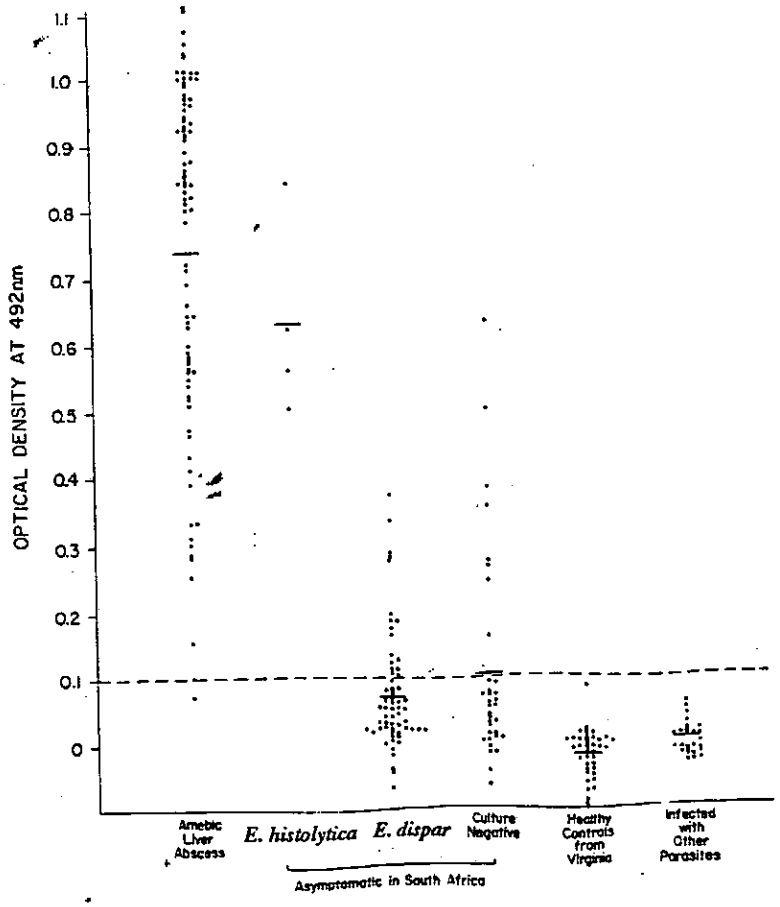


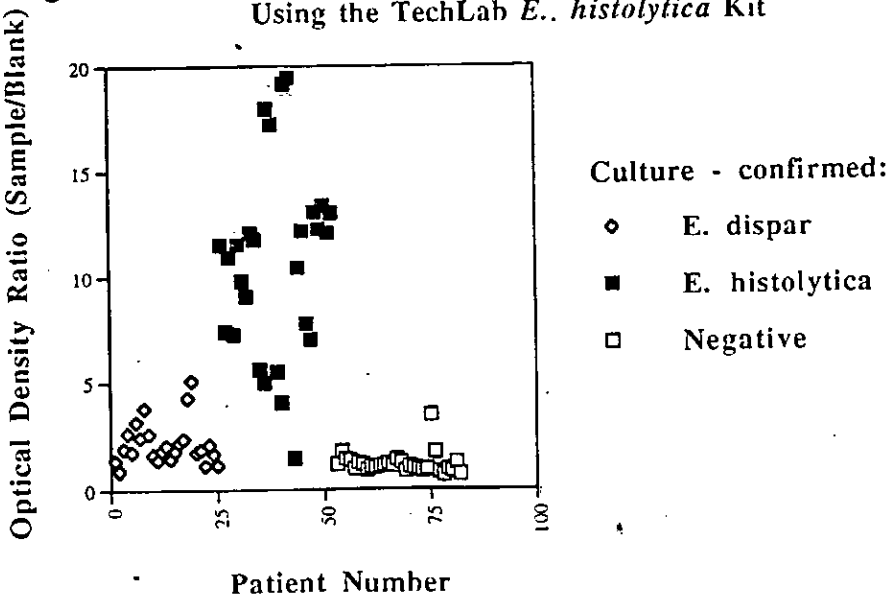
Figure 2. Detection of serum antibodies to lectin by ELISA. Each point represents average of duplicate tests of serum from one individual. Mean optical density values were 0.74 ± 0.26 for liver abscess, 0.63 ± 0.15 for asymptomatic *E. histolytica* infection, and 0.08 ± 0.09 for *E. dispar* infection.

Microtiter plates were coated with 0.1 μg/well of purified lectin, blocked in 1% bovine serum albumin, 0.05% Tween 20 in phosphate-buffered saline, and reacted with a 1:1,000 dilution of the serum sample. Unbound antibodies are washed from the wells, and human antibodies bound to the lectin detected with a peroxidase-conjugated anti-human IgG antibody (Ravdin et al 1990).

The anti-lectin ELISA has also been tested on patients from Cairo, Egypt with invasive amebiasis with comparable results (Abd-Alla et al 1992). The antigenic structure of the lectin is conserved in every isolate of *E. histolytica* we and others have tested, including hundreds of independent isolates from Bangladesh, South Africa, Egypt, Mexico, Brazil, the United States, Thailand and India (Petri et al 1990b; Haque et al 1993, 1995 [see Appendix A-E]; Abd-Alla et al 1993). Even at the level of gene family organization the *E. histolytica* lectin is remarkably conserved, a fact that not only reflects the probable clonal lineage of this asexual organism, but a fact which is of practical value if the protein is to be the basis of diagnostic tests (Ramkrishnan et al 1995). The anti-lectin ELISA therefore is a sensitive measure of prior or current infection with pathogenic *E. histolytica*.

C-I: 2. Specific identification of *E. histolytica* in stool using an antigen detection test based on anti-lectin monoclonal antibodies. A rapid and simple approach to the diagnosis of *E. histolytica* infection is an antigen-detection ELISA based on the antigenic differences in the Gal/GalNAc lectin in *E. histolytica* and *E. dispar* (Petri et al 1990a). The ELISA uses microtiter wells coated with rabbit polyclonal anti-lectin antibodies to "capture" the lectin from stool samples. Peroxidase-conjugated monoclonal antibodies (mAb) to lectin *E. histolytica*-specific epitopes 3 and 4 are used to detect *E. histolytica* in the stool specimen (**Figure 3**). Both *E. histolytica* and *E. dispar* are detected if the cross-reactive mAb to lectin epitopes 1 and 2 are used (**Figure 4**).

Figure 3 Detection of *E. histolytica* Infection in Stool Using the TechLab *E.. histolytica* Kit



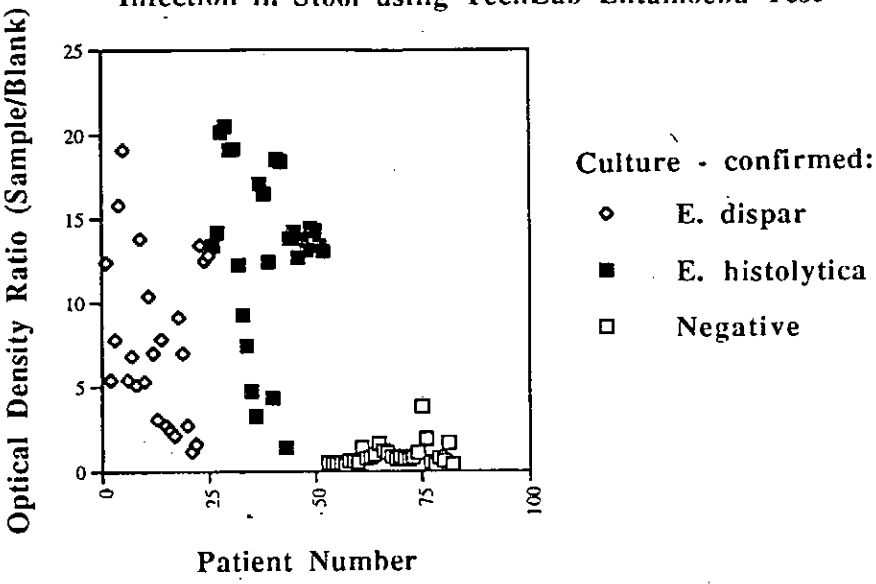
This assay has been applied to single stool specimens from over a thousand patients with diarrhea or dysentery in Dhaka, Bangladesh (Haque et al 1993, 1994, 1995, 1997a). Compared to culture plus zymodeme analysis, differentiation of *E. dispar* from *E. histolytica* was 95% sensitive and 93% specific. Ravdin and colleagues have reproduced these results in Egypt (Abd-Alla et al 1993; Abou-El-Magd et al 1996). We demonstrated that stool specimens from patients diagnosed with *E. histolytica* infection by ELISA were much more likely to have visible blood and trophozoites containing ingested red cells than specimens from patients diagnosed with *E. dispar* infection (58% vs 3.3% - **Table 2**). Thus, as expected, signs of dysentery were most common in patients diagnosed with infection with the disease-causing species *E. histolytica* (Haque et al 1995). The *E. histolytica* test (which detects only *E. histolytica*) and the *Entamoeba* test (which detects

Table 2. Some microscopic characteristics of stool specimens from patients infected with *E. histolytica* and *E. dispar* (diagnosed by *Entamoeba* and *E. histolytica* Tests).

	E. histolytica No. (%)		*E. dispar* No. (%)	
Visible blood in stool	14	(58)	1	(3.3)
Microscopic blood in stool	17	(70)	4	(13.3)
Ingested RBC	13	(68)	3	(15.7)

both *E. histolytica* and *E.dispar* in stool) have both received FDA 510k approval for in vitro diagnosis of amebiasis in humans. The ability of these tests to rapidly and specifically identify *E. histolytica* in stool will be an important part of the proposed studies.

Figure 4 Detection of *E. histolytica/E. dispar* Complex Infection in Stool using TechLab Entamoeba Test



C-I; 3. Development of PCR-based techniques for detection of *E. histolytica* in stool. We have developed a PCR technique for detection of *E. histolytica* in stool, and demonstrated that it has excellent correlation with culture and the TechLab *E. histolytica*-specific antigen detection test (**Figure 5**). The nested PCR test we used is based on amplification of the small subunit ribosomal RNA gene of *E. histolytica* and *E. dispar*, followed by restriction digest analysis of the PCR product. Single stool samples were obtained from 88 patients diagnosed by microscopy and/or culture with *E. histolytica/E. dispar* complex infection and 10 without. Isoenzyme analysis identified 53 of the infections as *E. histolytica*, and 28 as *E. dispar*. PCR and antigen detection on stool had comparable sensitivities, identifying 87% (46/53) and 85% (45/53) respectively *of E. histolytica* infections identified by isoenzyme analysis. The correlation of antigen detection with PCR for identification of *E. histolytica* in stool was 93% (45/48). PCR for *E. histolytica* was negative in all 10 samples that were negative for *E. histolytica* by isoenzyme and antigen detection. Mixed infections with *E. histolytica* and *E. dispar* were detected by PCR in 14% (12/88) of cases.

The nested PCR described in our work is comparable to culture and to the TechLab *E. histolytica* antigen detection test for the diagnosis of *E. histolytica* infection (Haque et al 1997b). While much more labor intensive than antigen detection, the PCR test will be a useful confirmatory test in the proposed studies, and has the added capability of detecting mixed infections with *E. histolytica* & *E. dispar* which will be missed by the antigen detection test.

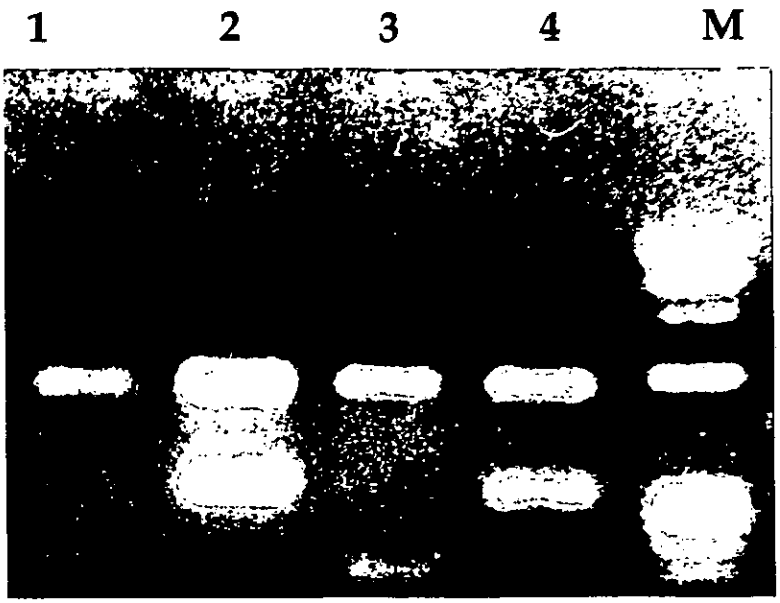


Figure 5. Restriction endonuclease digestion of the products of nested PCR. The restriction fragments of DNA amplified with the *E. dispar*-specific nested primers (lanes 1 and 3) and *E. histolytica*-specific nested primers (lanes 2 and 4) are shown. The starting materials for the PCR reactions were stool samples from patients with culture-confirmed infections with *E. dispar* (lane 1), *E. histolytica* (lane 2), and *E. histolytica* (lanes 3 and 4 - a mixed infection based on PCR). The marker (M) is øx 174 DNA digested with *Hae* III. (Haque et al, *J. Clin Microbiol 1997; in press).*

C-I: 4. Identification of the carbohydrate-binding domain of the lectin, and demonstration that antibodies against it provide passive protection from amebiasis. The location of the carbohydrate-binding domain in the native 260 kDa Gal/GalNAc lectin is of more than academic interest. The native lectin contains both adherence-inhibitory and adherence-enhancing epitopes, which confound its use in a vaccine. Immunization of gerbils with the intact lectin produces both adherence-enhancing and inhibitory antibody responses, and exacerbates disease in a subset of animals (Petri & Ravdin 1990). Humans with invasive amebiasis also produce antibodies against both adherence-inhibitory and adherence-enhancing epitopes of the lectin (Petri et al 1990b). We have recently identified the carbohydrate-binding domain and have tested whether it is a more effective vaccine than the native lectin (Dodson, Haque and Petri, unpublished).

To identify the carbohydrate binding domain of the lectin, a series of fragments of the lectin heavy subunit were expressed in *E. coli* and tested for reactivity against a series of anti-lectin monoclonal antibodies. The region of the lectin 170 kDa subunit designated Δ3A (lectin heavy subunit amino acids 895-998) was recognized only by the adherence-inhibitory epitope 4 mAb, and not by adherence-enhancing mAb (**Figure 6**). The Δ3A fragment of the lectin had similar Gal/GalNAc carbohydrate binding activity as the native

lectin (**Figure 7**). Immunization of gerbils with Δ3A provided substantial protection from amebic liver abscess, and passive transfer to naive mice of sera from Δ3A-immunized mice provided a similar degree of protection (**Table 3**). We conclude that the region of the lectin encompassed by Δ3A contains a carbohydrate binding site, and that antibodies against this region of the lectin confer protection from invasive amebiasis. In Specific Aims 1 and 2 we will measure antibody responses against the carbohydrate-binding domain, to test the hypothesis that anti-carbohydrate-binding domain antibodies protect from infection.

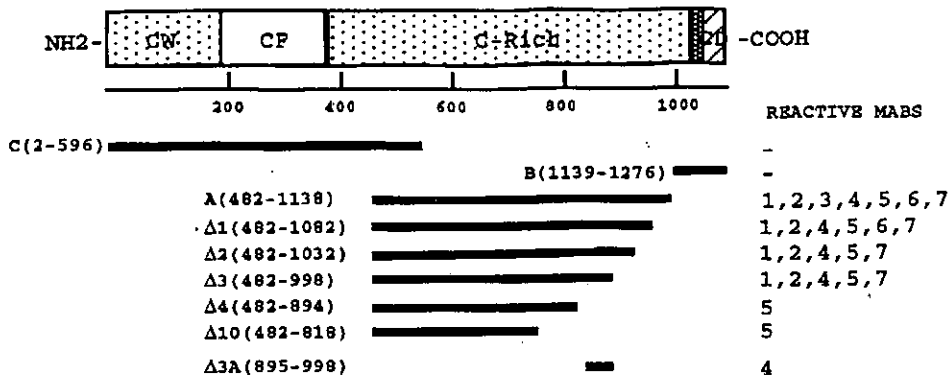


Figure 6. Binding sites of monoclonal antibodies that block Gal/GalNAc binding activity of the lectin. Fragments of the heavy subunit (A, B, C) were expressed as GST fusions in *E. coli* and tested for reactivity with anti-lectin mAb. Monoclonal antibodies against all 7 epitopes were located in fragment A, which contains the extracellular cysteine-rich region of the heavy subunit. A series of carboxy-terminal deletions of the A fragment resulted in the identification of the binding site for the adherence-inhibitory mAb against epitope 4 in the deletion fragment Δ3A.

Panel A Coommassie stained proteins
Panel B Western blot with mAb 8C12
Panel C Probed with ^{125}I-GalNAc$_{20}$BSA

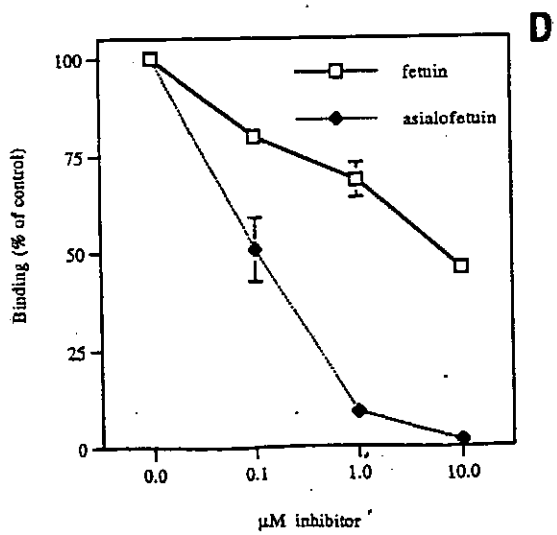


Figure 7. Carbohydrate-binding activity of the Δ3A fragment of the Gal/GalNAc lectin. The CW and the Δ3A domains of the lectin heavy subunit were expressed as His-tag fusions in *E. coli* and purified using nickel affinity chromatography. **Panel A** shows (left to right) molecular weight standards, purified CW, and purified Δ3A. **Panel B** is a Western blot of Panel A, demonstrating recognition of Δ3A but not CW by the adherence-inhibitory mAb 8C12. **Panel C** is a Western blot of Panel A probed with ^{125}I-GalNAc$_{20}$BSA, demonstrating carbohydrate binding activity of Δ3A. A low level of nonspecific binding of ^{125}I-GalNAc$_{20}$BSA to CW is also seen. **Panel D** demonstrates competition of the binding of ^{125}I-GalNAc$_{20}$BSA to Δ3A by the glycoproteins fetuin (which contains O-linked terminal Gal) and asialofetuin (which also contains N-linked terminal GalNAc).

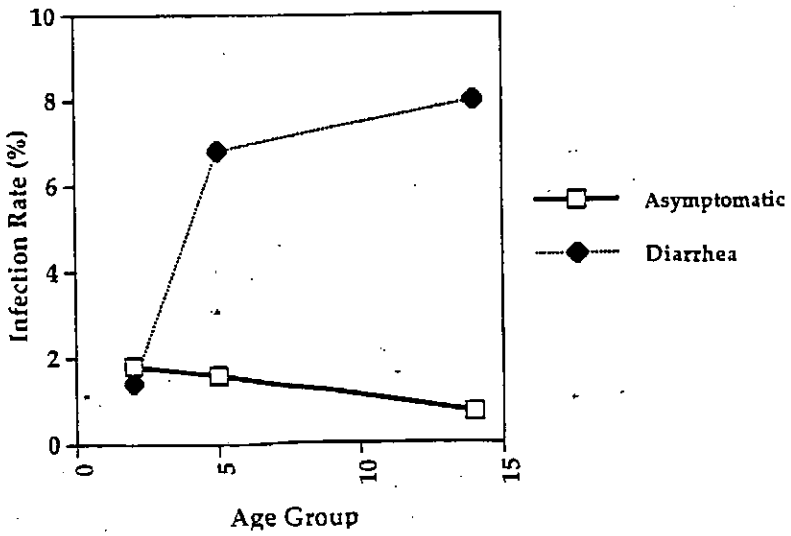
Table 3. Active and passive immunization of gerbils with the carbohydrate binding domain. Gerbils were actively immunized with 100 μg of Δ3A in complete Freund's adjuvant and boosted with 100 μg in incomplete Freund's adjuvant at 2 and 4 weeks. Animals were challenged with 500,000 trophozoites at week 6. Passive immunization consisted of transfer to naive animals of 1 ml of control or Δ3A-immune sera (half intramuscularly and half intrahepatically) on the day of challenge. Animals were sacrificed 10 days after challenge and liver abscess weights measured.

Trial	Group	Liver Abscess Weight	Abscess weight as % of Control	p value
Active Immunization	Control (Sham immunized)	7.0 +/- 1.7 g (n = 4)	-	
	Carbohydrate binding domain	1.5 +/- 0.8 g (n = 5)	21%	<0.001
Passive Immunization	Control (Sera from sham immuniz.)	4.6 +/-1.5 g (n = 4)	-	
	Immune (Sera from immuniz. gerbils)	.65 +/- 1.0 g (n = 4)	14%	<0.001

C-II. Application of diagnostic tests to study *E. histolytica* infection in Bangladesh.

C-II: 1. Prevalence of *E. histolytica* infection in Bangladeshi children. We used three well characterized diagnostic tests for amebiasis, microscopy, culture, and antigen detection, to first examine the prevalence of *E. histolytica* and *E. dispar* infection in children in Bangladesh. We compared 1,049 urban children with diarrhea seen at the ICDDR,B hospital to 987 rural children without diarrhea (Haque et al 1997). Urban children with diarrhea had a 4.2% prevalence of *E. histolytica* infection and a 6.5% prevalence of *E. dispar* infection. Rural asymptomatic children had a 1.0% prevalence of *E. histolytica* infection and a 7.0% prevalence of *E. dispar* infection. *E. histolytica* infection was more common in children ages 3-14 than in children ages 0-2 (**Figure 8**). We concluded that because of the relatively high prevalence of *E. histolytica* disease and the proximity to the ICDDR,B laboratories, that future studies of amebiasis should be conducted in an urban area of Dhaka.

Figure 8 Prevalence of E. histolytica in Asymptomatic Rural Children and in Urban Children with Diarrhea



C-II: 2. Identification of the Mirpur community in Dhaka, Bangladesh as an optimal location for the study of *E. histolytica* and *E. dispar* infection. Mirpur, a suburb of Dhaka, Bangladesh, is an urban slum. The majority of the inhabitants are of Bihari ethnic origin,

who settled in Mirpur after the war with Pakistan in 1971. The area is densely populated, and located 15 minutes away from the ICDDR,B. The population is stable with low socioeconomic conditions, with a population of approximately fifty thousand. Mirpur is especially attractive as a site for the proposed studies because (1) the prevalence of *Entamoeba* infection (as determined by microscopy) is high (**Figure 9**); (2) the population is stable; (3) Mirpur is a few minutes drive from the ICDDR,B laboratories and hospital; and (4) the co-Principal Investigator is successfully conducting another prospective study there.

The initial study estimated the prevalence of asymptomatic *E. histolytica/E. dispar* complex infection in Mirpur by microscopy of single stool specimens collected from 1,765 individuals. The prevalence of *E. histolytica/E. dispar* complex infection increased and *Giardia lamblia* decreased with increasing age (**Figure 9**), as has been observed elsewhere (Bray & Harris 1977). We have followed up on these studies by using the *E. histolytica* and *Entamoeba* antigen detection kits to specifically identify asymptomatic *E. histolytica* infection in Mirpur (**Table 4**). To date we have examined single stool specimens from 672 children. As expected antigen detection and culture identified higher prevalences of *E. histolytica* and *E. histolytica/E. dispar* complex infection than did microscopy. These studies will be expanded in Specific Aim 1, and are important to the design of Aim 2.

Figure 9

Prevalence of infection with intestinal protozoa among 1,765 people living in Mirpur, Dhaka

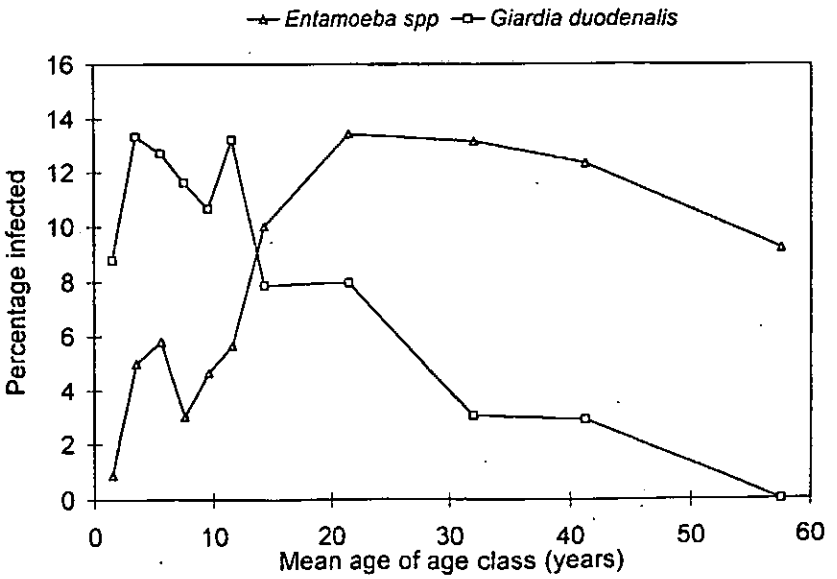


Table 4. Prevalence of Asymptomatic *E. histolytica* and *E .dispar* Infection in 672 Children Ages 2-5 Living in the Mirpur Community of Dhaka, Bangladesh

Entamoeba dispar/histolytica Infection Detected By:			
Microscopy	Culture	*Entamoeba* Antigen	*E. histolytica* Antigen
28 (4.1%)	72 (11%)	123 (18%)	32 (4.8%)

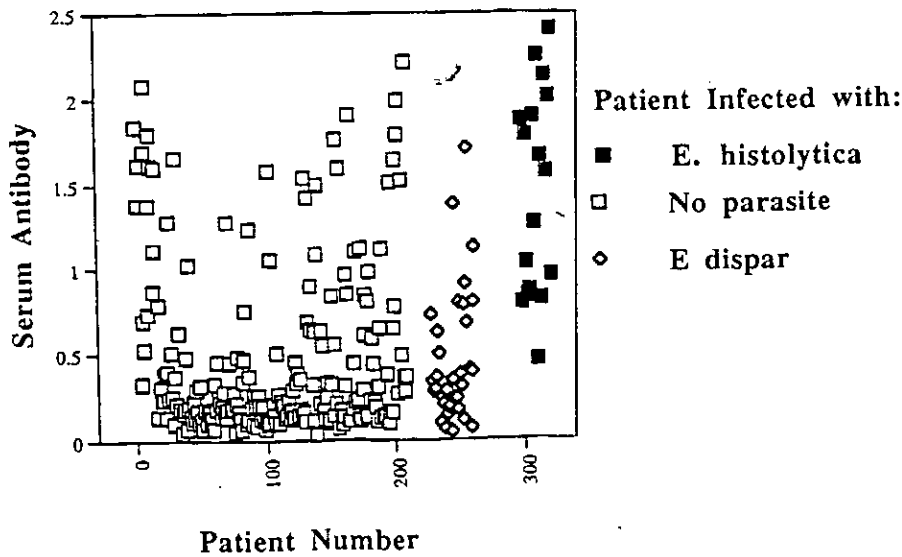
We next tested sera from 257 of the children ages 2-5 from Mirpur for antibodies against *E. histolytica* as an indicator of prior infection (see Section 3A-1 for data on use of

anti-lectin antibodies to predict prior disease). Sera were tested at a 1:1,000 dilution in a microtiter plate ELISA assay (by the method of Ravdin et al 1990). The serum antibodies were tested for reactivity with purified *E. histolytica* Gal/GalNAc lectin and purified recombinantly expressed carbohydrate binding domain (the Δ3A fragment from Figure 6). The cut-off point for positive results was determined to be 0.5 for the anti-lectin antibodies and 0.3 for the anti-carbohydrate binding domain antibodies (mean + 3 standard deviations of the results of negative sera samples).

Serum anti-Gal/GalNAc lectin antibodies were detected in 33% (86/257) of the children (**Figure 10**). Similar rates of sera positive for anti-lectin antibodies have been observed in poor individuals in Brazil (Braga et al 1996) and South Africa (Ravdin et al 1990). Anti-lectin antibodies were present in the sera of 94% (16/17) of children who were asymptomatically infected with *E. histolytica* at the time of serum collection, as has been seen previously in South Africa (Ravdin et al 1990). In contrast, children infected with *E. dispar* at the time of serum collection did not have higher rates of seropositivity than uninfected children. Other workers have also noted the lack of a systemic antibody response with *E. dispar* infection (Gathiram & Jackson 1985; 1987; Ravdin et al 1990). We conclude from these studies that (1) anti-lectin antibodies are an excellent marker of current asymptomatic *E. histolytica* infection; (2) that *E. dispar* infection does not result in serum anti-lectin antibodies that are detectable by this technique; and (3) that 33% of children ages 2-5 in Mirpur have evidence of prior *E. histolytica* infection.

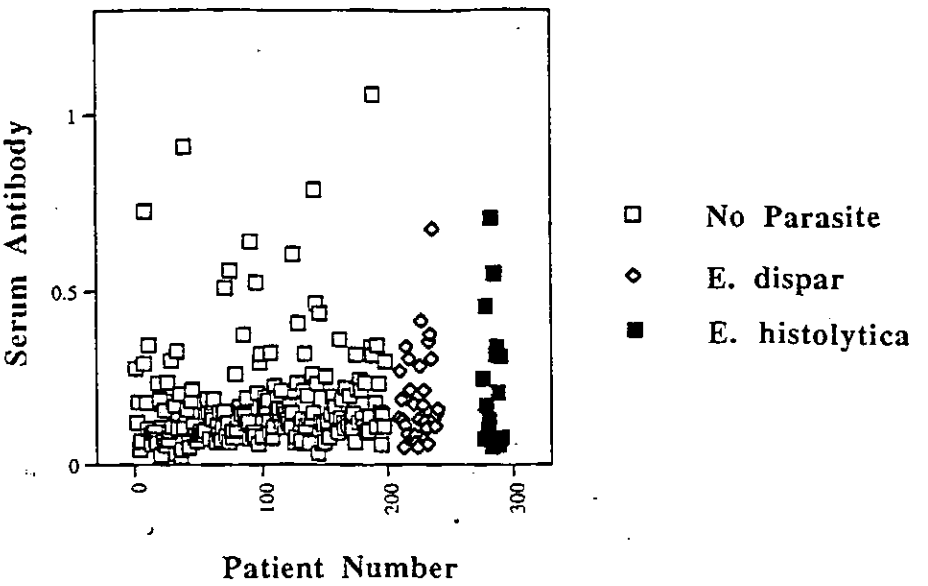
Figure 10

Serum Antibody Against *E. histolytica* Lectin in Children Ages 2-5 in Bangladesh



C-II: 3. Evidence that serum antibodies against the lectin carbohydrate binding domain are associated with clearance of *E. histolytica* infection. The sera from the 257 Mirpur children were also tested for antibodies against the lectin carbohydrate-binding domain. The antibody response against the carbohydrate-binding domain was strikingly different than that to the intact lectin: 18% of all children, and only 41% (7/17) of *E. histolytica* infected children, had a positive antibody response (**Figure 11**).

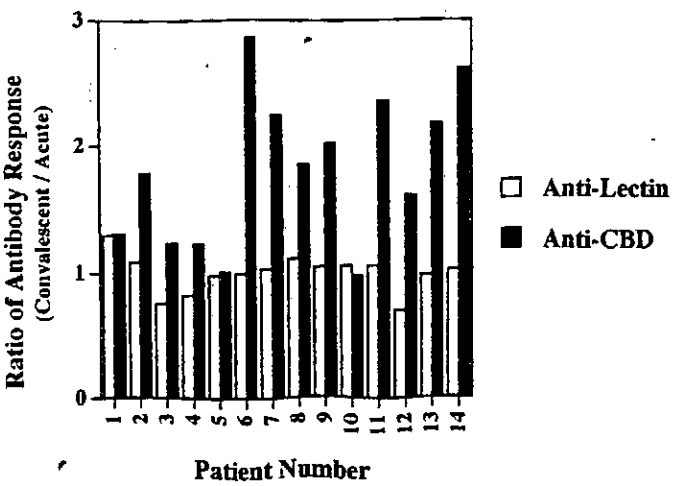
Figure 11 Serum Antibody Response Against Lectin Carbohydrate-Binding Domain



We collected follow-up stool and sera samples six months later from the children infected with *E. histolytica*. One child was lost to follow-up; *E. histolytica* infection was detected in only 2/16 children six months after the initial infection was detected. In the 14 children that had cleared their *E. histolytica* infection, the antibody response against the carbohydrate-binding domain was significantly higher at 6 months follow-up: the ratio of convalescent (6 month follow-up) to acute anti-CBD serum antibody levels was 1.8 +/- 0.17. In contrast the level of anti-lectin antibodies was unchanged (ratio of convalescent/acute antibodies of 0.99 +/- 0.04; $p<0.0001$ compared to anti-CBD). **Figure 12** shows the ratios of convalescent to acute antibody responses against the lectin and its carbohydrate binding domain for the individual patients. We conclude from these studies that clearance of *E. histolytica* infection is associated with increased antibodies against the lectin carbohydrate binding domain, but not against the intact lectin. We propose to test this association in cross-sectional and prospective studies in Specific Aims 1 and 2.

Figure 12

Clearance of *E. histolytica* Infection Correlates with Increased Anti-Carbohydrate Binding Domain (CBD) Serum Antibodies



Summary of Preliminary Studies:

- Serum anti-lectin antibodies are a sensitive marker of past/current *E. histolytica* infection
- *E. dispar* infection is not associated with detectable serum anti-lectin antibodies
- Antigen detection, PCR, and culture have been field tested. The three methods are complementary, sensitive, and specific means to detect *E. histolytica* infection
- Children in Mirpur, Bangladesh have a high prevalence of *E. histolytica* infection
- Antibodies against carbohydrate binding domain passively protect gerbils from amebiasis
- Recovery from asymptomatic *E. histolytica* infection is associated with increased anti-carbohydrate-binding domain antibodies (but not anti-lectin antibodies).

Publications by Drs. Petri & Haque Relevant to this Proposal and Included in Appendix:

(A) **Haque R**, Kress K, Wood S, Jackson TFHG, Lyerly D, Wilkins T, and **Petri WA Jr**. Diagnosis of pathogenic *Entamoeba histolytica* infection using a stool ELISA based on monoclonal antibodies to the galactose-specific adhesin. J Infect Dis 167:247-9, **1993**.

(B) **Haque R**, Lyerly D, Wood S, and **Petri WA Jr**. Detection of *Entamoeba histolytica* and *Entamoeba dispar* directly in stool. Amer J Trop Med Hyg 50:595-6, **1994**.

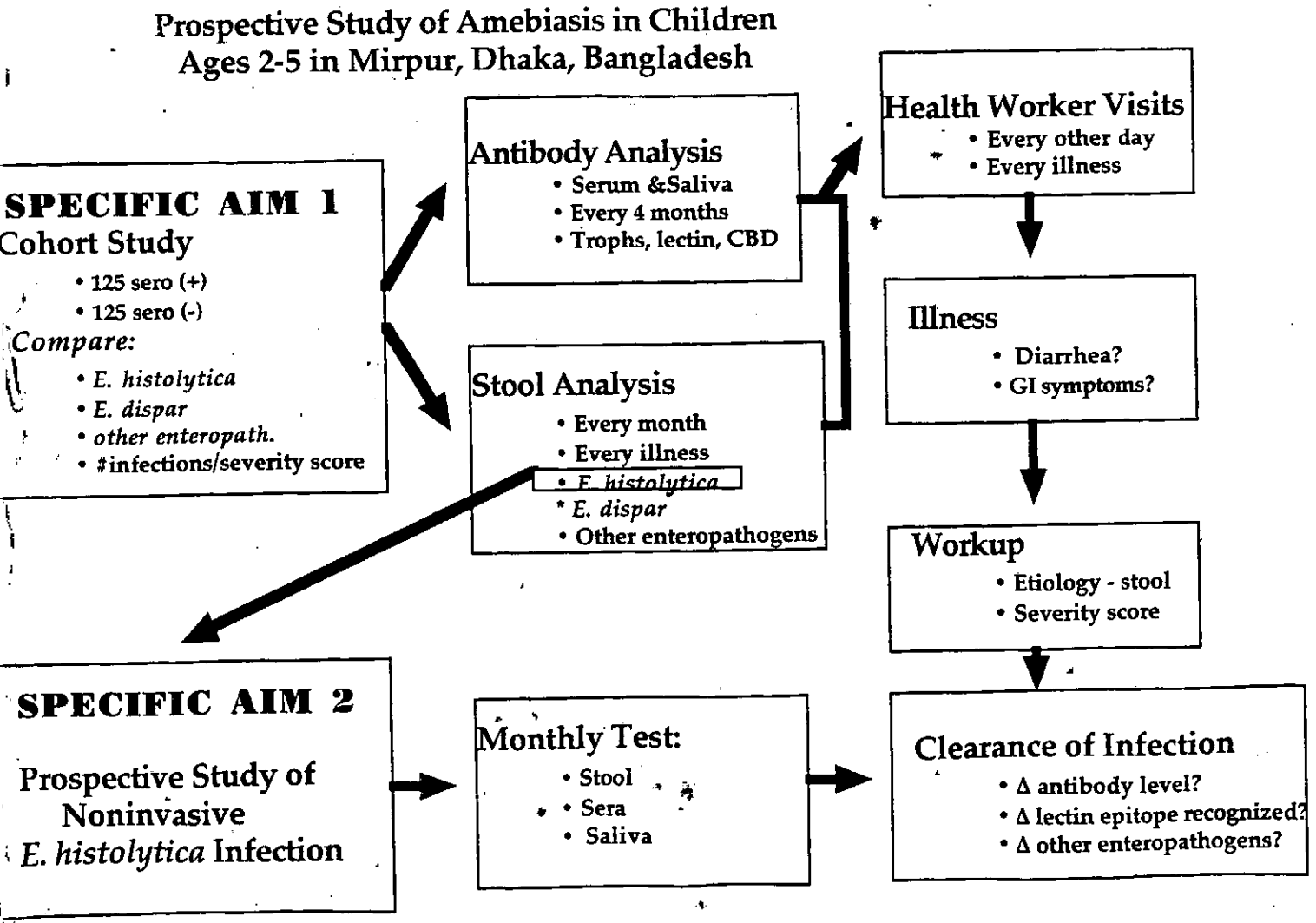
(C) **Haque R**, Neville LM, Hahn P, and **Petri WA Jr**. Rapid diagnosis of *Entamoeba* infection using the *Entamoeba* and *Entamoeba histolytica* stool antigen detection kits. J Clin Microbiology **1995**; 33:2558-61.

(D) **Haque R**, Faruque ASG, Hahn P, Lyerly DM, **Petri WA Jr**. *Entamoeba histolytica* and *Entamoeba dispar* infection in children in Bangladesh. J Infect Dis **1997**; 175:734-6.

(E) **Haque R**, Ali IK, **Petri WA**. Detection of *Entamoeba histolytica* infection: Comparison of PCR, isoenzyme analysis, and antigen detection. J Clin Microbiology **1997**; in press.

D. RESEARCH DESIGN AND METHODS

Mirpur, Bangladesh, is an ideal setting to determine if immunity is acquired to *E. histolytica* infection. We have already determined, using *E. histolytica*-specific tests, that 5% of 300 children ages 2-5 tested in Mirpur are colonized with *E. histolytica*, and 33% have serologic evidence of prior *E. histolytica* infection. We estimate, based on the 3 month average duration of *E. histolytica* colonization, that the incidence of *E. histolytica* infection in these children is 20% annually. Mirpur is a stable community in which adherence to protocols and follow-up has been excellent. In fact we have already successfully followed children with *E. histolytica* colonization in Mirpur, and Dr. Haque is currently conducting fruitful prospective studies of nutritional interventions in children there. The high prevalence of *E. histolytica* infection in Mirpur enables statistically powerful studies of immunity with manageably small numbers of study patients.



Hypothesis 1: *Immunity to invasive infection by E. histolytica is acquired from a previous (symptomatic or asymptomatic) infection.*

Specific Aim 1: A three year prospective study will measure *E. histolytica* infection and invasion in two cohorts of children ages 2-5 in the Mirpur district of Dhaka. One cohort will be composed of 125 children with serologic evidence of prior *E. histolytica* infection. The second

cohort will be composed of 125 children without evidence of prior infection. We will test if children who have had prior infection are less likely to be re-infected with *E. histolytica,* or if reinfected are less likely to have severe amebiasis. If hypothesis (1) is correct, we would predict the following outcomes: (a) children who have had prior *E. histolytica* infection will be less likely to have future episodes of invasive amebiasis; (b) it is possible that they will also be less likely to be re-infected with *E. histolytica;* and (c) if re-infected may clear infection more rapidly.

RATIONALE. The design of these experiments is simple: individuals with and without evidence of prior infection with *E. histolytica* will be compared for their susceptibility to infection with *E. histolytica.* Based on the experience with other enteropathogens such as rotavirus and *Vibrio cholera,* it is a reasonable expectation that acquired immunity exists to amebiasis. Acquired immunity could include immunity to infection or immunity only to severe disease. We have designed the study to have high statistical power to detect a 50% difference in infection or disease rates. We have chosen the 2-5 year age group as these children are approaching the age where we have observed the steepest rises in the prevalence of *E. histolytica* infection **(Fig. 8 & 9).**

Serum anti-amebic antibodies are an excellent marker for prior infection with *E. histolytica.* Because the main goal of this study is to test for the acquisition of immunity to amebiasis, we need a sensitive means of identifying individuals with prior *E.* histolytica infection. Serum anti-*E. histolytica* and anti-lectin antibodies have been detected for greater than 3 years after infection in up to 94% of patients with amebic colitis, 97% of patients with amebic liver abscess, and 95% of individuals colonized with *E. histolytica.* antibodies (Jackson et al 1985; Petri et al 1987b; Ravdin et al 1990; Stanley et al 1991; Zhang et al 1992; Lotter et al 1995; Preliminary Studies). Infection with *E. dispar* in contrast does not result in detectable anti-*E.* histolytica or anti-lectin serum antibodies (despite the antigenic relatedness of the two organisms) (Jackson et al 1985; Ravdin et al 1990). Therefor the presence of serum anti-amebic antibodies is a measure of current and prior *E. histolytica* but not *E. dispar* infection.

RESEARCH DESIGN

Enrollment of children:

- Mirpur children ages 2-5
- Cohort of children with prior *E. histolytica* infection: prior infection defined as a positive serum antibody test for anti-lectin antibodies.
- Cohort of children without evidence of prior infection: negative serum antibody response for anti-lectin antibodies.
- Cohorts matched for age, sex, family size. The parents of each child will be questioned about the child's symptoms of intestinal and extraintestinal amebiasis, dysentery, fever, and history of drug ingestion, and the child will be examined for hepatomegaly prior to enrollment. Subjects with a recent (< 1 month) history of anti-amebic medication use will be excluded from the study.

Outcomes to be analyzed:

- Incidence of *E. histolytica* and *E. dispar* infection in the "immune" and naive cohorts of children
- Severity of *E. histolytica* or *E. dispar* associated diarrhea in the two cohorts
- Incidence of *E. histolytica* associated dysentery in the two cohorts

• Association of *Shigella dysenteriae* infection/disease or other enteropathogens with *E. histolytica* and *E. dispar* infection (based on preliminary evidence of such an assoication - see Appendix D).

Statistical evaluation:

The assumptions that underlie this aim are as follows: First we estimate that 5% of the children at any one time are infected with *E. histolytica* (Table 4). From our own data (14/16 children cleared *E. histolytica* colonization at 6 months) and that of Jackson and colleagues in South Africa, we estimate that the average duration of asymptomatic infection with *E. histolytica* is 3 months. Therefor a 5% prevalence of *E. histolytica* infection represents a yearly incidence of infection of approximately 20%. Second, we estimate that 10% of the children will be lost to follow-up each year. The power of this study to detect a 50% or a 30% difference in infection rates between naive and immune individuals over 3 years is 99.9% and 80% respectively (Bayer 1988). We have chosen the 30-50% levels of protection as a estimate based on other enteric diseases. For example prior rotavirus infection provides 77% protection from diarrhea and 38% protection from re-infection (Velazquez et al 1996) and vibriocidal serum antibody titers $\geq$ 20 are associated with 50% protection from infection and illness (Glass et al 1985). Dr. Barry Farr of the Division of Epidemiology at the University of Virginia will be assisting with statistical analyses throughout the project.

Prospective analysis:

Children will be visited every other day by health care workers who will interview the parents and child. Parents will be asked about the child's eating patterns, stool frequency, consistency and presence of blood, and fever. For all children, stool specimens will be collected every month for detection of *E. histolytica* and *E. dispar* infection by antigen capture. Serum and saliva will be collected every 4 months in all individuals, and every month in *E. histolytica* infected persons.

Work-up of children who develop diarrhea:

Children with diarrhea will be detected either by the every other day visits of the health care workers, or through the parents contacting project personnel at the field office. Parents will be instructed to contact the field office in Mirpur whenever their child has a diarrheal or gastrointestinal illness. There are no telephones available to the families in Mirpur, so the every other day visits to the families and the proximity of the field office to the subjects is key. When diarrheal disease is detected, a stool sample will be collected and examined for enteropathogens (see General Methodology, at the end of this section) and the child will be examined. Samples will be kept on ice and transported to the laboratory for processing within 2-4 hours. Parents will be instructed in the use of oral rehydration solution, and antibiotics administered or the patient seen or hospitalized at the ICDDR,B when appropriate.

Definitions:

Diarrhea: An episode of diarrhea is defined as the occurrence of three or more loose or watery stools, or one or more bloody stools, in a period of 24 hours.

Scoring of the severity of diarrhea: Severity of diarrhea will be assessed by a modification of the point system of Ruuska et al (1990) developed for rotavirus infection that will measure: (a) duration of diarrhea in days; (b) maximal number of stools per day;

(c) presence of occult or gross blood; (d) degree of dehydration/weight loss; and (e) need for rehydration/hospitalization. Greater weight will be placed on criteria (c) and (e) when the severity score is calculated.

Dysentery: Diarrheal stools containing occult or gross blood.

Subclinical *E. histolytica* Infection: Individuals will be considered to have subclinical infection if *E. histolytica* is detected in stool but diarrhea is not present and no evidence exists for extra-intestinal amebiasis.

Amebic Colitis: Diarrhea with gross or occult blood present and *E. histolytica* identified in the stool, no other intestinal pathogens identified capable of causing dysentery (*Salmonella, Shigella,* invasive/enterohemorrhagic *E. coli, Yersinia, Campylobacter*), consistent findings of colonic ulceration if sigmoidoscopy or colonoscopy with biopsy is performed (not required), and a response to therapy with anti-amebic medications.

Probable amebic colitis: Diarrhea with gross or occult blood present and pathogenic *E. histolytica* identified in the stool, and with a response to anti-amebic medications, consistent findings of colonic ulceration if sigmoidoscopy or colonoscopy with biopsy is performed, but with other intestinal pathogens capable of causing dysentery present.

Amebic Liver abscess: Demonstration of a liver abscess by ultrasound or other imaging technique in a patient with serum anti-*E. histolytica* antibodies and a clinical response to metronidazole.

Feasibility of a prospective study at Mirpur:

The study will be conducted among residents of a slum community in Mirpur, a suburb of Dhaka, Bangladesh. The prevalence of intestinal parasitic infections (including *E. histolytica*) is very high in Mirpur. The majority of the inhabitants are of Bihari ethnic origin, who settled in Mirpur after the war with Pakistan in 1971. The area is densely populated, and located 15 minutes away from the ICDDR,B. The population is stable with low socioeconomic conditions, with a population of approximately fifty thousand. Mirpur is an excellent site for this study for several reasons. First, the prevalence of infection with *E. histolytica* is approximately 5% in children ages 2-5, which should give our study the statistical power necessary to detect acquired immunity to *E. histolytica*. Second, prospective studies have been and are now being conducted successfully in Mirpur. The co-PI Dr. Haque is currently conducting a prospective study in Mirpur entitled "Field trial of beta carotene and anti-helminthic therapy to improve micronutrient nutrition among preschoolers" which is funded by the Thrasher foundation. Dr. Haque also participated in a second study in Mirpur conducted by Dr. Andrew Hall entitled "Antibody isotype response to the antigens of *Ascaris lumbricoides* in a case-control study of persistently heavily infected Bangladeshi children" (Parasitology 1995; 111:385-93).

The current field trial of beta carotene supplementation is a good example of our ability to conduct prospective studies in Mirpur. About 900 children were initially registered for the study during October 1996 by three Health Assistants. From November 1996 Dr. Haque started collection of stool samples from the targeted children for quantitative stool examination of intestinal helminths. Stool samples from 858 children were examined during November and December 1996. In early January 1997 he started to recruit children into the study for intervention, with recruitment completed at the end of February 1997. A total of 244 children were assigned randomly into four groups for intervention according to the study design. Blood samples were collected from all of those 244 children for estimation of serum retinol and beta carotene. All 244 children were followed according to the study plan after recruitment for daily beta carotene or placebo capsule

supplementation. Health Assistants have visited every alternate day to the households of the children. Children were again treated with albendazole or placebo during May and June 1997 according to study plan. The second set of blood samples and anthropometric measurements of the children were completed during July and August 1997 from 227 children. There were 17 children dropped from the study due to several reasons including migration out of Mirpur and non-cooperation with the study. Therefor the loss of subjects in the first year of the study has been only 7.5%.

Hypothesis 2: *Clearance of asymptomatic infection by E. histolytica is associated with production of adherence-inhibitory antibodies against the carbohydrate-binding domain of the Gal/GalNAc adherence lectin.*

Specific Aim 2: Children with asymptomatic, noninvasive, *E. histolytica* infection (identified in Aim 1) will be prospectively followed from the onset of infection to its clearance. The children will be monitored for serum and salivary antibody responses against (a) the native Gal/GalNAc lectin, (b) the lectin carbohydrate-binding domain, and (c) lectin adherence-inhibitory, and (d) lectin adherence-enhancing epitopes.

If the hypothesis (2) is correct, we would predict that clearance of *E. histolytica* infection will be associated with the development of an adherence-inhibitory antibody response against the carbohydrate- binding domain of the lectin.

RATIONALE:

- (1) We have demonstrated through paired sera analyses an association of increased levels of serum anti-lectin carbohydrate-binding domain antibodies with clearance of asymptomatic *E. histolytica* infection (**Fig. 12**).
- (2) Additionally, in preliminary experiments we have shown that antibodies against the carbohydrate-binding domain of the *E. histolytica* Gal/GalNAc lectin passively transfer protection against amebiasis to non-immune animals (**Table 3**).

It has long been suspected that antibodies against *E. histolytica* and its galactose-specific lectin could be protective against future infection, by blocking adherence (by binding to lectin epitopes 4-6), cytotoxicity, or serum resistance, or by opsonizing the trophozoite (Saxena et al 1986; Petri et al 1987a). Conversely these antibodies could be disease-enhancing by increasing trophozoite adherence (by binding to lectin epitopes 1 or 2) (Petri et al 1990b). In fact we demonstrated the presence in human immune sera of antibodies against both adherence-enhancing and inhibiting epitopes (Petri et al 1990b). Experiments in a SCID mouse model of amebic liver abscess have demonstrated anti-*E. histolytica* antibody-mediated protection when antibodies against the trophozoite, the serine rich protein, and cysteine-rich domain of the lectin (encompassing the Δ3A carbohydrate-binding domain of the lectin) are passively transferred (Cieslak et al 1992; Zhang et al 1994a; Lotter et al 1997). Enhanced formation of liver abscess was observed when antibodies against the aminoterminal domain of the lectin were transferred (Lotter et al 1997). It therefore may be important to measure not only the total *E. histolytica* antibody response but also the antibody response against specific proteins and their domains.

- (3) Additionally it is important to measure serum antibodies, as amebiasis is a systemic as well as an enteric disease. Serum anti-amebic antibodies are also roughly correlated with secreted antibody responses in amebiasis.

• (4) In cholera and in rotaviral disease serum antibodies have been demonstrated to correlate with protection, perhaps because serum antibodies reflect not only the systemic immune response but also part of the mucosal antibody response to infection.

EXPERIMENTAL DESIGN: Whole blood (100-200 μl) will be obtained by finger prick and sera isolated by conventional procedures. Microtiter wells will be coated with total *E. histolytica* proteins, or 0.1 μg of affinity-purified *E. histolytica* galactose-specific adherence lectin, or 0.1 μg of the purified Δ3' lectin fragment in 100 μl of pH 9.6 bicarbonate buffer overnight. Wells will be washed and blocked in 1% BSA-PBS-0.1% Tween for 1 hour and then human sera (serial dilution from 1:250 - 1:4,000) added to the wells and incubated for 4 hours. After washing, the absorbed human anti-lectin antibodies are detected with an anti-human IgG peroxidase-conjugated secondary antibody. An anti-IgA peroxidase-conjugated secondary antibody will be used to detect IgA antibodies against *E. histolytica*. To determine the epitope-specificity of the human immune response against the lectin, human sera will be used to compete the binding of mAb against the seven defined epitopes on the lectin heavy subunit. For additional details, see Petri et al (1990b). Results are expressed as the reciprocal of the last dilution of sera for which the optical density exceeded the mean optical density values for reference non-immune sera by 3 standard deviations.

Analysis of Salivary Anti-lectin IgA Antibodies:

RATIONALE: IgA is the most abundant of the immunoglobulins and the predominant antibody present in secretions (Mestecky & McGhee 1987). IgA is also present in serum, although the subclass of IgA in serum is predominantly IgA1, while IgA2 predominates in secretions. This is a potentially important reason to test both secreted and serum IgA, as IgA1 antibodies are more likely to be directed against protein antigens and IgA2 against carbohydrate antigens (Mestecky et al 1989). Measurement of salivary sIgA is thought to reflect intestinal sIgA because of the interconnections of the mucosal immune system. The role of secreted IgA in protection from enteric infections is not clear: most people with a deficiency in IgA, the most common disorder of antibody production (1:300-600 in the normal population), are not predisposed to enteric infections (Brown et al 1972; Heinzel 1995). However passive protection mediated by IgA has been demonstrated in several infections (Ogra et al 1968; Bessen & Fischetti 1988), and induction of an intestinal sIgA response by oral poliovirus vaccine has been correlated with protection from colonization (Ogra & Karzon 1971).

There are several approaches to measuring secretory IgA. The most common sense approach, direct measurements of intestinal IgA, is fraught with technical difficulties, not the least of which is the difficulty in obtaining intestinal secretions and tissue. Detection of circulating antigen-specific IgA-secreting cells by ELISPOT is another approach, but correlations of this test with protection from infection have yet to be conclusively demonstrated (Quiding et al 1991). Salivary IgA levels are less labor-intensive to determine than ELISPOT, and have been shown to correlate with IgA (+) immunocytes in rectal mucosa biopsies of patients with amebic liver abscess. IgA antibodies against *E. histolytica* (and specifically against the galactose lectin) present in saliva of patients with intestinal amebiasis have been demonstrated to block adherence of the trophozoite, and therefore could be important in protection from infection (Carrero et al 1994).

EXPERIMENTAL DESIGN: Saliva will be collected from the subjects and stored at 4° C for transport to the laboratory where it will be either immediately tested or stored at -70° C for future assay. Saliva will be serially diluted in PBS (from 1:2 - 1:128), and anti-lectin IgA antibodies detected using the same microtiter well procedure detailed above for serum antibody analysis, with the exception that a rabbit anti-human IgA peroxidase-conjugated secondary antibody will be used to detect. Results will be standardized to the total IgA content of the saliva sample.

General Experimental Methods

EVALUATION OF STOOLS FOR THE PRESENCE OF *E. HISTOLYTICA* AND *E. DISPAR*

Examination of Stool Specimens for the Presence of *E. histolytica/E. dispar* complex by Microscopy and Culture: A single stool specimen will be collected from each subject. Stool samples will be transported to the laboratory at 4° C where a direct smear will be prepared for microscopical examination for *E. histolytica.* Other protozoans or helminths, if present, will be recorded as well, and the stool sample will then be cultured and cultured amebae analyzed by isoenzyme analysis to identify *E. histolytica* and *E. dispar.*

Stool Antigen Detection ELISA for *E. histolytica*:

Polystyrene 96 well microtiter plates (Corning Glass Works, Corning, NY) will be coated with 100 ml/well of a 1:2500 dilution of protein A-purified rabbit polyclonal antibodies against the galactose lectin in 0.1 M bicarbonate buffer pH 9.6 overnight at 4° C. The plates after washing 3 times in PBS containing 0.05% Tween 20 (Sigma, St. Louis MO) (PBS-Tween) will be blocked in 1% BSA (Sigma)-PBS-Tween for 1 hour at room temperature. Stool samples to be tested will be suspended 1:1 in PBS containing 2 mM phenylmethylsulfonylfluoride and 2 mM *p*-hydroxymercuribenzoate (Sigma) and the stool suspension in 100 ml final volume added to the microtiter well. The microtiter plates will be incubated with the stool suspension overnight at 4° C or for 1-2 hours at room temperature. The plates will then be washed 3 times with PBS-Tween and incubated 1-2 hours at room temperature with a 1:1500 dilution of a 4 mg/ml solution of peroxidase-conjugated anti-lectin monoclonal antibodies 7F4 and 8C12 (directed against *E. histolytica*-specific epitopes 3 and 4 respectively of the lectin 170 kDa subunit). The wells will be washed 3 times in PBS-Tween, followed by addition of substrate. Absorbance at 445 nm of the microtiter wells will be measured with an ELISA plate reader (Titertek Multiskan, Flow Laboratories, VA) after 10 - 45 min of development.

This assay has been applied to single stool specimens from over a thousand patients with diarrhea or dysentery in Dhaka, Bangladesh (Haque et al 1993, 1994, 1995, 1997a). Compared to culture plus zymodeme analysis, differentiation of *E. dispar* from *E. histolytica* using this antigen capture assay was 95% sensitive and 93% specific. This *E. histolytica*-specific stool antigen capture assay has been approved by the FDA for use in humans.

Stool Antigen Detection ELISA for the *E. histolytica/E. dispar* Complex

In order to detect the *E. histolytica/E. dispar* complex in stool samples the ELISA uses the same polyclonal antisera to capture the lectin, but is modified at the detection stage by use of a cocktail of mAb 3F4 and 8A3 (which bind to the lectins of both *E. histolytica* and *E. dispar*). The assay is otherwise the same as the *E. histolytica* -specific assay. This *E. histolytica/E.* dispar complex stool antigen capture assay has been approved by the FDA for use in humans.

PCR Detection of *E. histolytica* and *E. dispar* in stool.

The PCR test that we have developed is a sensitive and specific means to identify *E. histolytica* and *E. dispar* in stool specimens. The main drawback of the test is that it is labor-intensive. For that reason we will reserve use of the PCR test to the subset of samples that are positive for *E. histolytica* and/or *E. dispar* infection by some other technique (microscopy, antigen detection or culture). A number of samples that are negative for *E. histolytica* and *E. dispar* (by microscopy, antigen detection and culture) will also be tested by PCR to confirm the specificity of the PCR reaction and to ensure that the screening tests are not missing infections. Our initial experience with this PCR test has been quite positive: the correlation of antigen detection with PCR for identification of *E. histolytica* in stool was 93% (45/48). PCR and isoenzyme identification *of E. histolytica* in cultures agreed in 96% (51/53) of samples (Haque et al 1997).

Extraction of DNA from stool samples. 0.2 g of stool sample will be diluted with PBS to 1.5 ml in microcentrifuge tubes, and 33.3 µl of 1 M KOH and 9.3 µl of 1 M dithiothreitol added. The samples will be mixed thoroughly by stirring with a pipette tip, followed by brief shaking. After incubation at 65° C for 15 min, the samples will be neutralized with 4.3 µl of 25% HCl, buffered with 80 µl of 2M Tris-HCl (pH 8.3) and the suspension mixed again. The DNA will be extracted by shaking with 250 µl of phenol:chloroform:isoamyl alcohol (PCI, 25:24:1) saturated with 10 mM Tris (pH 8.0) and 1 mM EDTA. The phases will be separated by a 4-min spin in a microcentrifuge. The aqueous phase will be transferred to a new tube and the DNA further purified by adsorption to 5 µl of glassmax matrix suspension (Gibco-BRL). The DNA will be eluted in 39 µl of deionized water.

PCR Method. Primer construction is based on sequences from the small subunit ribosomal RNA gene of *E. histolytica* and *E. dispar*. The PCR reaction products are digested with restriction enzymes to arrive at the specific identification of *E. histolytica* and *E. dispar*. In our hands we have found this technique to be 87% sensitive and 96% specific for identifying *E. histolytica* in stool (Haque et al 1997). For the first PCR, the primer pair E-1 (TTT GTA TTA GTA CAA A) and E-2 (GTA (A,G)TA TTG ATA TAC T), which amplify a 0.9 kB fragment of the rRNA gene will be used. The primer pair E-1 and E-2 is complementary to both *E. histolytica* and *E. dispar* sequences, with the E-2 primer constructed two-fold degenerately, i.e., as a mixture with half corresponding to the *E. histolytica* sequence and the other half corresponding to the *E. dispar* sequence. The first PCR amplification with E-1 and E-2 will be followed by two additional PCRs, each of which is specific for either the *E. histolytica* or the *E. dispar* sequence. The primers used for these reactions are located downstream of E-1 and E-2, making this a nested PCR. For the second (nested) PCR, two different primer pairs specific for *E. histolytica* (EH-1 AAT GGC CAA TTC ATT CAA TG and EH-2: TTT AGA AAC AAT GCT TCT CT) or *E. dispar* (ED-1: AGT GGC CAA TTT ATG TAA GT and ED-2: TTT AGA AAC AAT GTT TCT TC) will be used. Both of the PCRs will be done using a hot start technique. In the first PCR, 18.4 µl of the DNA extracts are denatured at 96° C for 2 min after the addition of 0.6 µl each of 40 µM solutions of the primers (E-1 and E-2) & one drop of mineral oil. After cooling to 80° C, 5.4 µl of freshly prepared "mastermix" [2.5 µl of 10X PCR buffer (Gibco BRL, cat no. 18038), 2 µl of 50 mM $MgCl_2$, 0.64 µl of dNTP mix (10 mM each; Perkin Elmer, USA), & 0.25 µl (5 I.U./µl) of *Taq* polymerase (Gibco BRL)] will be added. Fifty cycles will be performed with denaturation at 92° C for 60 sec, annealing at 43° C for 60 sec, and extension at 72° C for 90

sec. In the second (nested) PCR, 3 μl of the first PCR product will be taken in 26 μl of water and denatured at 96° C for 2 min after addition of 1 μl each of 40 μM solutions of the primers (EH-1 and EH-2 for *E. histolytica*, ED-1 and ED-2 for *E. dispar*) and two drops of mineral oil. After cooling to 80° C, 8.6 μl of freshly prepared "mastermix" (10X PCR buffer, 3.2 μl of 50 mM $MgCl_2$, 1 μl of dNTP mix and 0.4 μl of *Taq* polymerase) will be added and the PCR performed as above except that the annealing temperature will be 62° C. PCR amplifications will be performed using a Bio-Rad gene-cycler. Products will be visualized on a 1.3% agarose gel containing 0.2 μg/ml of ethidium bromide (Sigma). Bands excised from the agarose gel will be silica gel-purified as described above, eluted in 9.6 μl of buffer, and digested with 0.8 μl (10 U/μl) of *Dra* I (Gibco BRL) for 60 min at 37° C, followed by the addition of 0.4 μl (10 U/μl) of *Sau* 96 I (Amersham) and further incubation at the same temperature for another 90 min.

IDENTIFICATION OF OTHER ENTEROPATHOGENS IN STOOL

Our initial studies demonstrated that *Shigella dysenteriae* infection is more common in children with *E. histolytica* and *E. dispar* infection (Haque et al 1997). This is a potentially important observation as both shigellosis and amebiasis can cause dysentery but the treatment is completely different. It is also an interesting observation, as *Shigella* can survive within *E. histolytica* trophozoites (Verdon et al 1992) raising the possibility that *E. histolytica/E. dispar* could be a vector for *S. dysenteriae*. We will attempt to confirm this observation, as well as re-examine the presence of other enteropathogens.

Bacterial causes of diarrhea that will specifically be excluded include *Salmonella spp, Shigella spp, Campylobacter spp, Aeromonas spp, Plesiomonas spp, C. difficile, B. fragilis,* and *Vibrio spp* all by standard methodology of the ICDDR,B lab (Albert et al 1993 & 1995; Unicomb et al 1996). *E. coli* strains will be picked from MacConkey agar and stored separately in vials containing trypticase soy broth with 15% glycerol at -20°C. These *E. coli* colonies will be later probed for diarrheagenic properties (including Shiga-like toxin) with specific DNA probes by standard ICDDR,B methodology (Faruque et al 1992). Parasites to be excluded will include *Giardia, Strongyloides, Cryptosporidia,* and *Isospora* (Baxby & Blundell 1983). MIF concentration method will be used for the cysts of *Giardia* and other gut protozoa, Bearmann method for *Strongyloides* and modified Ziehl-Neelsen stain for *Cryptosporidium, Cyclospora,* and *Isospora* will be used. In addition to this, an ELISA for detection of Cryptosporidium and Giardia stool antigen will also be used. Rotavirus, adenovirus and astrovirus will be determined by ICDDR,B methods (Moe et al 1991; Jarecki-Khan et al 1993).

E. HUMAN SUBJECTS

The subject population involved in the study of intestinal colonization by *E. histolytica* is the inhabitants of the urban slum Mirpur in Dhaka, Bangladesh. The criteria for inclusion into the study are residence in Mirpur and age of 2-5 years. The consent forms for enrollment and blood drawing have been approved by the Human Investigation Committee of the University of Virginia, and will be reviewed and approved by the ICDDR,B IRB and translated prior to use in Bangladesh. Since much of the study population is illiterate, the consent form will be read to them. Enrollment will be done by Dr. Haque in conjunction with the visiting nurses and field research assistants. Informed consent will be obtained from the children and their guardians prior to entry into the study and prior to any intervention (institution of treatment for diarrhea, for example). Each child will have stool and blood samples taken during the study. It is not anticipated that the study will pose any adverse risks to the study population (besides the minimal risks of hematoma or vasovagal reaction which can be managed at the site by the co-investigators or visiting nurses). Data sheets will be handled confidentially and the reporting of data from this study will not lead to the identification of the individuals involved.

Children in the prospective study identified as being infected with *E. histolytica* will be followed closely. A field office will be set up in the community where a physician will work 6 days a week. Parents will be instructed to contact the field office when their children have diarrhea or are otherwise ill. Field workers will also visit each household on alternate days. Upon detection of any diarrheal, gastrointestinal, or other illness the child will be brought to the project field office where a history and physical exam will be performed and a stool sample taken for detection of enteropathogens. Anti-amebic treatment will be instituted if invasive amebiasis occurs. Studies by Jackson and colleagues from South Africa have estimated the risk of *E. histolytica* -colonized individuals developing invasive amebiasis to be 10-14% for one year of follow-up. Participants in the prospective study will not be paid; however medical treatment for fever, diarrhea and other illness will be provided at no cost for all the family members of the study participants. Transportation to the ICDDR,B hospital and clinics will also be provided free of charge. It is anticipated that the successful completion of this study will provide a basis for rational vaccine design against this disease, so we believe that the potential benefits of this study outweigh its risks.

F. VERTEBRATE ANIMALS

None

G. LITERATURE CITED

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H. CONSORTIUM/CONTRACTUAL ARRANGEMENTS

"The following signatures attest to the awareness of PHS consortium grant policy and the willingness of the 2 institutions involved in this grant application to establish the necessary inter-institutional agreements consistent with the policies of the PHS, the University of Virginia, and the International Centre for Diarrhoeal Disease Research."

William A Petri

William A. Petri, Jr., M.D., Ph.D.
Principal Investigator
University of Virginia

Rashidul Haque

Rashidul Haque, M.B., Ph.D.
Co-Principal Investigator
ICDDR,B

Norma S Miller

Norma S. Miller
Manager, Grants & Contracts Admin.
University of Virginia

Director
ICDDR,B Acting
21/10/97

I. CONSULTANTS/COLLABORATORS

Rashidul Haque, M.B., Ph.D., Associate Scientist, International Centre for Diarrhoeal Disease Research, Dhaka, Bangladesh. Letter attached.

Barry M. Farr, M.D., M. Sc., Professor of Medicine and Epidemiology, University of Virginia. Letter attached.

R. Bradley Sack, M.D., Sc.D., Professor, Department of International Health, Johns Hopkins University. Letter attached.

25 Years of ORS
INTERNATIONAL CENTRE FOR DIARRHOEAL DISEASE RESEARCH, BANGLADESH

Phone : 600171-73
Telex : 675612 ICDD BJ
Fax : 880-2-883116, 9001-9668050
Cable : Cholera Dhaka
Mail : GPO Box 128, Dhaka-1000 Bangladesh

September 24, 1997

Dr. William A. Petri, Jr., M.D., Ph.D
Professor of Medicine , Pathology, and
Microbiology
Department of Medicine
Room 2115 MR4 Bldg
University of Virginia
Charlottesville, Virginia 22908
USA.

Fax: 804 924 0075

Dear Dr. Petri,

This letter is to confirm my readiness and enthusiasm for our continued collaboration on *Entamoeba histolytica*. The grant application that you and I submitting to the NIH for funding is of great importance in the field of amebiasis and our Centre has given high priority of research in this field. The work that we are now proposing will give us an opportunity to determine if there is protective immunity to *E. histolytica* infection, and if immunity exists, whether it protects against both colonization and invasion. I am very excited to do the work that we have proposed in the grant.

I sincerely hope that our collaboration in the field of amebiasis will bring more fruitful results in the future as in the past. Your interest in the Centre, and specifically in our work on amebiasis is most appreciated.

With best wishes and regards,

Yours sincerely,

Rashidul Haque

Dr. Rashidul Haque, M.B., Ph.D.
Head, Parasitology Laboratory
Laboratory Sciences Division
ICDDR,B

JOHNS HOPKINS
UNIVERSITY

School of Hygiene and Public Health

Department of International Health
550 N. Broadway, Suite 1001
Baltimore, MD 21205
410-955-0053 / FAX 410-614-9483
Internet: dsack@phnet.sph.jhu.edu

Vaccine Testing Unit

September 29, 1997 FAX 804-924-0075

Dr. William A. Petri
Department of Medicine
University of Virginia
MR4 Room 2115
HSC
Charlottesville, VA 22908

Dear Bill:

I have reviewed your Grant proposal involving immunological responses to, and protection against, infection and disease caused by E. histolytica, and enthusiastically support the project. I will be pleased to be a consultant on this project, primarily in the area of epidemiology.

As you know I have previously spent several years working in Dhaka, Bangladesh; during this time I have had the privilege of working with Dr. Rashidul Haque, and I am familiar with the area in which the study will be performed.

Please let me know if there is anything else you need at this time.

With best regards,

Sincerely,

Brad Sack

R. Bradley Sack, M.D., Sc.D.
Professor of International Health
and Medicine

Hospital Epidemiology

University of Virginia HEALTH SYSTEM

November 4, 1997

William Petri, MD, PhD
Infectious Disease
Box 385

Dear Bill:

I am writing to indicate my support for your grant application to study the relationship between immunity and subsequent infection with *Entamoeba histolytica*. I will be available to assist with epidemiologic and statistical analysis during the study.

Yours sincerely,

Barry-Farr

Barry M. Farr, MD, MSc
Hospital Epidemiologist
The William S. Jordan Jr. Professor of
Medicine and Epidemiology

UVa Health System, # 473, Charlottesville, Virginia 22908
Telephone: (804) 924-2777

CHECKLIST

TYPE OF APPLICATION *(Check all that apply.)*

[X] NEW application. *(This application is being submitted to the PHS for the first time.)*

[ ] REVISION of application number: _____
(This application replaces a prior unfunded version of a new, competing continuation, or supplemental application.)

[ ] COMPETING CONTINUATION of grant number: _____
(This application is to extend a funded grant beyond its current project period.)

INVENTIONS AND PATENTS *(Competing continuation appl.only)*
[ ] No [ ] Previously reported
[ ] Yes. If "Yes," [ ] Not previously reported

[ ] SUPPLEMENT to grant number: _____
(This application is for additional funds to supplement a currently funded grant.)

[ ] CHANGE of principal investigator/program director.
Name of former principal investigator/program director: _____

[ ] FOREIGN application or significant foreign component.

1. ASSURANCES/CERTIFICATIONS

The following assurances/certifications are made and verified by the signature of the Official Signing for Applicant Organization on the Face Page of the application. Descriptions of individual assurances/certifications begin on page 27 of Section III. If unable to certify compliance where applicable, provide an explanation and place it after this page.

•Human Subjects; •Vertebrate Animals; •Debarment and Suspension; •Drug-Free Workplace *(applicable to new [Type 1] or revised [Type 1] applications only)*; •Lobbying; •Delinquent Federal Debt; •Research Misconduct; •Civil Rights (Form HHS 441 or HHS 690); •Handicapped Individuals (Form HHS 641 or HHS 690); •Sex Discrimination (Form HHS 639-A or HHS 690); •Age Discrimination (Form HHS 680 or HHS 690); •Financial Conflict of Interest.

2. PROGRAM INCOME *(See instructions, page 20.)*

All applications must indicate whether program income is anticipated during the period(s) for which grant support is requested. If program income is anticipated, use the format below to reflect the amount and source(s).

Budget Period	Anticipated Amount	Source(s)

3. INDIRECT COSTS

Indicate the applicant organization's most recent indirect cost rate established with the appropriate DHHS Regional Office, or, in the case of forprofit organizations, the rate established with the appropriate PHS Agency Cost Advisory Office. If the applicant organization is in the process of initially developing or renegotiating a rate, or has established a rate with another Federal agency, it should, immediately upon notification that an award will be made, develop a tentative indirect cost rate proposal. This is to be based on its most recently completed fiscal year in accordance with the principles set forth in the pertinent *DHHS Guide for Establishing Indirect Cost Rates*, and submitted to the appropriate DHHS Regional Office or PHS Agency Cost Advisory Office. Indirect costs will *not* be paid on foreign grants, construction grants, grants to Federal organizations, grants to individuals, and conference grants. Follow any additional instructions provided for Research Career Awards, Institutional National Research Service Awards, and specialized grant applications.

[X] DHHS Agreement dated: 11/26/96 [ ] No Indirect Costs Requested.

[ ] DHHS Agreement being negotiated with _____ Regional Office.

[ ] No DHHS Agreement, but rate established with _____ Date _____

CALCULATION* *(The entire grant application, including the Checklist, will be reproduced and provided to peer reviewers as confidential information. Supplying the following information on indirect costs is optional for forprofit organizations.)*

a. Initial budget period: Amount of base $ 67,352 x Rate applied 51 % = Indirect costs (1) $ 34,350

b. Entire proposed project period: Amount of base $ 281,780* x Rate applied 51 % = Indirect costs (2) $ 143,708

(1) Add to total direct costs from form page 4 and enter new total on Face Page, Item 7b.
(2) Add to total direct costs from form page 5 and enter new total on Face Page, Item 8b.

*Check appropriate box(es):

[ ] Salary and wages base [X] Modified total direct cost base [ ] Other base *(Explain)*

[ ] Off-site, other special rate, or more than one rate involved *(Explain)* * excluded consortium/contractual costs

Explanation *(Attach separate sheet, if necessary.):*

SMOKE-FREE WORKPLACE

Does your organization currently provide a smoke-free workplace and/or promote the nonuse of tobacco products or have plans to do so?

[X] Yes [ ] No *(The response to this question has no impact on the review or funding of this application.)*

CRITIQUE 1

SIGNIFICANCE: Entameba histolytica is a major health risk in many tropical countries such as Bangladesh. This proposal seeks to determine the factors that determine the course of infection in young children. Given the enormity of the problem of the infection in this area of the world these studies are highly relevant.

INNOVATIVENESS: The proposal is not particularly innovative in that it is a straightforward field study, driven by two reasonable, but not particularly innovative, hypotheses. Thus, it appears not unreasonable to propose that previous infection can give rise to protective immunity. The innovative aspect of this study, however, is the choice of the research material, namely, young children in the field, where incidence of amebiasis is high. Similarly, given the rather extensive work and the importance of the lectin as a virulence molecule in the organism, the possibility that antibodies against this entity will be found in the course of infection and may be associated with clearance, is a reasonable hypothesis. Overall , despite the lack of innovation however, this proposal is based on solid hypotheses.

APPROACH: This is a straightforward field approach where children will be followed by standard serological analyses for the existence of infection. The detection of infection by stool examination, as well as serum antibodies, is well founded. Thus, serum antibodies to E. histolytica have been demonstrated in greater than 80% of patients with amebic infection localized to the large intestine and 90-99% of patients with invasive disease, including amebic liver abscess. Furthermore, these antibodies persist for several years after infection, confirming the prior experience of infectious disease. Demographic studies in Bangladesh by the investigator's colleagues have clearly demonstrated that the incidence of amebiasis is quite high in this region, and that young children are particularly prone to get infected. The focus on the adherence lectin is also well justified by extensive data in the literature that suggest that the presence of invasive disease is associated with significant levels of antibodies against this lectin. These data are demonstrated on page 26, where it can be clearly seen that patients with amebic liver abscess almost universally have antibodies against the GalNAc. The investigators also have developed and deployed a rapid simple antigen-detection ELIZA based on the antigenic differences in the lectins of the non-invasive E. dispar and E. histolytica.

The current proposal is relative straightforward. The investigator will identify 125 children who are seropositive for amebiasis indicating past infection and 125 children, in the age group 2 to 5, who are currently not infected and have not been infected in the past, as demonstrated by the lack of antibodies against E. histolytica. Both cohorts will be followed prospectively by antibody analysis in serum and saliva every four months for titers of antibodies against total ameba antigens and

against the lectin. Stools will be analyzed every month for the presence of E. histolytica cysts and trophozoites. In the event that there are episodes of illness between these monthly examinations, the children will be examined for the possibility of infection. By using this relatively large cohort, the investigators have insured themselves of statistical power. They can follow whether or not there is a difference in the incidence of E. histolytica infection among the sero-positive and sero-negative individuals. The prospective study for the importance of antilectin antibody in the course of E. histolytica infection will also be relatively straightforward and will identify all children who become positive for E. histolytica during the course of the study. Monthly tests will be performed to look at their parasite burden, as well as the titer of antilectin antibody in both serum and in saliva. Correlations will be attempted between the clearance of infection and a rise in the antibodies against a specific domain of the GalNAc lectin.

INVESTIGATOR: Dr. William Petri has a long history of productivity and meaningful contribution to our understanding of E. histolytica biology and infection. The collaborators that he has recruited for the performance of this field study are optimal, since Dr. Rashidul Haque, who is based in Bangladesh at the International Center for Diarrhoeal Disease Research, has in fact, also a considerable reputation as an important epidemiological researcher in diarrhoeal disease, particularly amebiasis.

Overall Evaluation: In summary, this is a proposal to perform field studies on the incidence of E. histolytica infection over a three year prospective period, using cohorts of children, aged two to five years, in Bangladesh. The hypothesis underlying this proposal is reasonable. The proposal is clearly written, well thought out and given the previous track record of the investigator, as well as of his collaborators, very likely that it will be performed suitably, even in the somewhat exacting field conditions in Bangladesh. Support is recommended with high enthusiasm in the excellent range,

CRITIQUE 2

Significance: The study will provide information relevant to our understanding of the immune response that develops to natural infections with E. histolytica. The PI convincingly argues that this information will be useful in considering how to proceed with the development of a vaccine against amebiasis.

Approach: There are two specific aims in this proposal which address two hypotheses. The first aim directly addresses whether immunity is acquired during infection by studying two cohorts of children in Bangladesh. One group that has evidence of a previous infection, and the other with no evidence of previous infection, will be followed for 3 years to identify infections with E. histolytica (as well as E. dispar and other enteropathogens). There are several aspects of this study that suggest that it has a good chance of success. First of all, for these studies to be successful patients will need to be characterized as to their past and present exposure (infection) to E. histolytica. The PI has provided critical data that he and his colleagues can distinguish between unexposed and exposed individuals based upon antibody responses to a galactose-specific adherence lectin from E. histolytica, which are present during, and for a substantial period of time after, infection. Furthermore, the PI shows data demonstrating a sensitive and rapid test for diagnosing E. histolytica infection which is critical for these studies. Secondly, these studies will be done with a population exhibiting a high incidence of infection (20%). The site in an urban area of Dhaka, Bangladesh is reported to contain a relatively stable population which increases the chance of success for this project. Finally, and most importantly, the proposal involves a collaboration between Dr. Petri and Dr. Haque who already have an ongoing, and successful collaboration (evidenced by 5 peer-reviewed publications on amebiasis in Bangladesh). Dr. Haque is a Scientist at the International Centre for Diarrhoeal Disease Research in Dhaka, a facility that is close to the field site to be used for these studies.

The second aim will address the hypothesis that immunity will be associated with antibodies to E. histolytica lectins. Dr. Petri has been the leader in the field identifying and characterizing galactose binding lectins in Entamoeba. Preliminary data is presented that: 1) immunization with the carbohydrate binding domain, as well as passive transfer of antibodies to the carbohydrate binding domain, can protect gerbils from disease (Table 3); 2) that there is a correlation between clearance of E. histolytica and increased levels of antibodies to the carbohydrate binding domain (Figs 10,11,12). In this aim, Dr. Petri and colleagues will characterize the antibody responses of children with asymptomatic, noninvasive E. histolytica infection. The prediction is that clearance of E. histolytica infection will be associated with the development of antibodies to epitopes of the carbohydrate binding domain that inhibit adherence. This is a reasonable hypothesis, and should be easily tested with the patients that they will identify as part of aim 1.

Innovation: The ability of the PI to distinguish E. histolytica and E. dispar infections is an important and novel aspect to this study. As pointed out by the PI, there is little known about the development of natural resistance to E. histolytica following a primary infection, and these investigators have the tools and patient population to examine this issue.

Investigators: A major strength of this application is the long-standing collaboration between Drs. Haque and Petri, as well as the expertise of each of these investigators in amebiasis.

Environment: The environment for studies in Dhaka appears to be adequate for these studies. The success of previous and ongoing studies by Dr. Haque some of which are published, suggests that this environment is suitable.

Overall evaluation: This new application presents a well-designed and straightforward approach to a addressing important questions concerning the development of immunity to amebiasis. Because of the strong ongoing collaboration between Dr. Haque and Petri their project has a high probability of success, and that success will mean a better understanding of human amebiasis.

Review - 3

CRITIQUE 3

Significance: Amebiasis is a common worldwide parasitic infection, responsible annually for 50 million cases of amebic colitis and liver abscess and approximately 100,000 deaths. The major hypotheses of the study are that immunity to E. histolytica is acquired from previous infection and that clearance of asymptomatic infection by E. histolytica is associated with production of adherence-inhibitory antibodies against the carbohydrate-binding domain of the Gal/GalNAc adherence lectin. Successful completion of the proposed studies should provide a foundation for understanding the mechanisms of protective immunity to amebiasis, and provide a baseline for evaluating the efficacy of vaccines to be used in clinical trials.

Approach: The conceptual framework and design are appropriate to the aims of the project. The proposed study will be conducted in Dhaka, Bangladesh, a very high incidence region for amebiasis. A number of questions about the design and analyses need to be addressed. What is the time frame for development of amebiasis after infection with E. histolytica in 2-5 year olds? Will there be sufficient time to show a difference between children with and those without prior infection? Is the 10-14% development of invasive amebiasis after 1 year of infection that was observed in the South African study appropriate for the age group in the current study?

Children in the current study will be visited every other day by health care workers. Pilot studies have demonstrated the willingness of families to undergo every other day interviews and to adhere to the study protocol. The stability of the population is also an asset. Details about the selection of study subjects are not provided. How will children be ascertained? That is, how will the prospective members of the two cohorts be identified and selected? What is the age distribution and numbers of inhabitants 2-5 years of age in Mirpur?

Serum and saliva will be collected every 4 months in all individuals, and every month in E. histolyica infected persons. Why are different approaches being used? What is the probability that a member of the uninfected cohort may become infected during the study period?

Although there appears to be sufficient power for aim 1, few details about the statistical analyses are provided. The collaborating statistician/ epidemiologist is named but without details about the statistical analyses. For Specific Aim 2, children with asymptomatic, noninvasive, E. histolytica infection (defined in Aim 1) will be prospectively followed from the onset of infection to its clearance. The predicted outcome is that clearance of infection will be associated with the development of an adherence-inhibitory antibody response against the carbohydrate-binding domain of the lectin. How will the association be evaluated? Few details about the statistical assessment are provided.

Innovation: The study uses a unique and stable population. The project lays the groundwork for development of new technologies such as providing data to evaluate the efficacy of vaccines.

Investigator: The investigators are appropriately trained and well suited to carry out this work. The investigators have the appropriate expertise in the required areas for this study. The proposed work is appropriate to the experience levels of the investigators.

Environment: The proposed study takes advantage of a unique and highly compliant population. It also employs useful collaborative arrangements between the University of Virginia and the International Centre for Diarrheal Disease Research, Bangladesh. The institutional support for the project should help in the conduct of the study.

Overall Evaluation: The proposal describes previous work that has been completed to set the stage for the current study. In general, it is well-written. The proposal addresses an important question regarding E. histolytica infection, its relationship to immunity, clearance, and re-infection. Successful completion of the study should provide much needed information for understanding protective immunity to amebiasis and for evaluating vaccines. The approach uses a unique and stable study population. Additional details are required to fully evaluate how successful the design and analyses will be to answer the specific aims. In particular, more details about the statistical analyses and ascertainment of the study subjects is needed. The investigators appear very well qualified to carry out this project. The successful collaborative relationship between the investigator and the International Centre for Diarrheal Disease Research, Bangladesh is an asset to the study.

Human Subjects: No exemptions. The risks to subjects are reasonable in relation to the anticipated benefits to the subjects and in relation to the importance of the knowledge that may be expected to result from this research.

Budget: The budget appears adequate and appropriate.