Abstract

Hepatitis E virus (HEV) is an emerging enteric pathogen responsible for most acute hepatitis worldwide. This dissertation presents data from studies conducted from 2001-2006 in the Matlab Health Research Center population under the International Center for Diarrheal Disease Research, Bangladesh. These studies define the previously unknown burden of HEV in rural Bangladesh and address gaps in HEV epidemiology. This work begins with a literature review, focusing on the challenges presented by this virus: the unexplained high case fatality in pregnancy, the absence of HEV in children, the rapid deterioration of immunity, and recent evidence implicating some HEV genotypes as zoonoses. The first study is a cross-sectional assessment of antibody seroprevalence to three hepatitis viruses (B,C&E) in a representative random population sample (n=1134). This study revealed a 22.5% seroprevalence of anti-HEV, 35.2% anti-HBc and 1.5% anti-HCV. Anti-HEV seroprevalence peaked in the second/third decades of life, as seen in India/Nepal. Male gender and outdoor employment were significantly associated with seropositivity. The second study follows this baseline cohort longitudinally for 18 months to determine HEV infection and disease rates. From 837 person-years (P-Y) of exposure, an incidence rate of 60.3/1000P-Y was estimated. Age-specific seroincidence increased in subsequent 10-year categories, peaking at 41-50y. Although clinical illness seemed infrequent, a disease:infection ratio as high as 49 per 100 (95%CI:31–73) was estimated. Third, an exploratory nested case control study attempts to identify putative risk factors for sporadic hepatitis E disease. Over 22 months, 13 field workers used a morbidity-scoring algorithm to identify acute hepatitis-like illness in their catchment population of 23,500. Finally, 46 confirmed HEV infections were
compared to 134 sero-naïve age-matched controls. Cases were less likely to be <15y, female or use unsanitary latrines in their homes. Outdoor employment, work outside the home, and travel to a town/city emerged as risk factors. Unlike in previous studies, recent contact with a “jaundice” patient and injection exposures were significant. These studies establish that: HEV is endemic in rural Bangladesh; sporadic infections are frequent in the absence of outbreaks; and, aside from classic hygiene risk factors, there may be other pathways through which HEV is transmitted.

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My parents and in-laws have been extremely understanding in allowing me to keep their only daughter and grandson thousands of miles away in rural Bangladesh, while going through this process, learning to interpret the phrase “only one more year” with a wide confidence interval.

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I dedicate this work to the rural poor of Bangladesh, whose quiet dignity, generosity, and unflagging spirit in the face of the harsh realities of daily life inspired me to pursue a career in Public Health.
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1. Introduction

A. Research Summary

This dissertation titled “The Epidemiology of Hepatitis E Virus Infections in Rural Bangladesh” presents the data collected from a series of studies conducted between 2001 and 2006 in communities under the catchment of Health and Demographic Surveillance System (HDSS) at the Matlab Health Research Center of the International Center for Diarrheal Disease Research, Bangladesh (ICDDR,B). These studies attempt not only to address epidemiologic gaps in our understanding of HEV as an emerging enteric pathogen that contributes to significant morbidity in the developing world, but also to carefully define the burden of this disease in rural Bangladesh. There are no prior studies on the population-based distributions of antibodies to HEV in rural Bangladesh and estimates of the burden of this pathogen have relied on reports of HEV among clinical hepatitis cases, travelers to this country, or small convenience samples from urban populations or military cohorts.

This work begins with a review of the classical and recent peer-reviewed literature from this rapidly evolving field. New paradigm shifts in the epidemiology of HEV have challenged researchers to explain bewildering patterns of infection and illness. Notable among these are a) the yet unexplained high case fatality rate, over 20%, when pregnant women are infected with HEV, b) the unexpected absence of HEV infection or markers of infection in children, who are normally the first to succumb to enteric infections in conditions of poor sanitation and unclean drinking water, c) the rapid deterioration of protective immunity following infection in certain populations, and d) an
emerging body of evidence which implicates some genotypes of HEV as being largely zoonotic, with animals serving either as amplifying reservoirs or vectors (through contact or consumption), and HEV crossing from animals to humans causing sporadic or epidemic disease.

The first study presented is a cross-sectional assessment of the antibody prevalence to hepatitis viruses, namely HAV, HBV, HCV and HEV, among a representative sample of the rural Matlab population under the HDSS. Exactly 1134 individuals between the ages of 1 and 88 years were enrolled into this study, and provided a fingerstick blood specimen which was tested at the Armed Forces Research Institute of Medical Sciences (AFRIMS) in Bangkok, Thailand. AFRIMS, a recognized global leader in the field of hepatitis virus illness, is also a regional reference laboratory for HEV testing using an extremely sensitive and specific in-house enzyme immunoassay developed by the Walter Reed Army Institute of Research (WRAIR). This study revealed between a 12.9-22.5% seroprevalence of antibodies to HEV, 35.2% of antibodies to hepatitis B core antigen and 1.5% to hepatitis C. Antibody seroprevalence peaked in the second and third decade of life, confirming that the age-specific seroepidemiology of HEV in Bangladesh closely matches that seen in India, Nepal and other HEV endemic regions of south Asia. Older age, male gender and outdoor employment were the characteristics most significantly associated with anti-HEV seropositivity.

The second study described is the longitudinal follow-up, as a cohort, of the randomly selected individuals who contributed to the baseline seroprevalence assessment. At two timepoints, exactly twelve and eighteen months after their original visit, the baseline 1134 individuals were re-visited by the study teams, interviewed and tested for
antibodies to HEV. Those under the age of 10 were also tested at the subsequent timepoints for HAV antibodies, and as many subjects as possible were tested for HBV. The outcome of interest was anti-HEV seroconversion between any two follow-up points. A total of 879 baseline susceptible participants were followed for 12 months, contributing 837 person-years (P-Y) of exposure. This cohort experienced an HEV incidence rate of 60.3 per 1000 P-Y (95% CI: 44.6 – 79.7 per 1000 P-Y). Extending the surveillance to 18 months increased the incidence rate to 63.9 per 1000. An analysis of age-specific seroincidence rates revealed an increase in HEV infections rates in each subsequent 10-year age category, peaking at at 41-50. The age-specific incidence of 21-30 year olds (66.3 per 1000 P-Y) was more than twice that of 1-10 year olds (28.9 per 1000 P-Y). No significant difference between incidence rates was detectable in this study by gender, religion, household size, location of primary employment or indicators of socioeconomic status (education, income, and employment type). A low rate of overt hepatitis E disease was associated with seroconversion, but extrapolating from a subsample of the whole cohort under close disease surveillance and using self-reported scleral icterus as evidence of hepatitis E diseases, a disease to infection rate possibly as high as 49 per 100 (95%CI: 31 – 73) was estimated. This study’s careful estimate of infection rates clearly establishes this rural Bangladesh population as HEV-endemic. Despite the marked absence of HEV outbreak reports in the literature, it is evident that HEV is circulating in this population and may be contributing to substantial specific and non-specific morbidity, that may have previously unrecognized social and economic costs.

The third study described here is an exploratory nested case control study, with longitudinal community-based surveillance for acute hepatitis illness, undertaken to
identify potential risk factors for sporadic hepatitis E disease in rural Bangladesh. For a period of 22 months, a population of 23,500 individuals was under active monthly surveillance for acute hepatitis illness by a team of 13 trained Community Health Research Workers (CHRWs). (This consisted of over 286 person-months of CHRW surveillance time, covering a little over 43,000 person-years of participant exposure time.) Each family was visited monthly by a CHRW, who used a locally developed and tested hepatitis illness scoring algorithm to evaluate members of the family for morbidities consistent with acute viral hepatitis. Based on a combination of calculated illness score and time since onset of symptoms, CHRWs would notify the HEV team of a potential acute hepatitis E disease case through a network of 4 field bicycle-messengers. Over the course of the study, 279 potential cases were identified, among which 46 were likely acute HEV infections. An exploratory nested case-control study compared these cases to healthy, age-matched, sero-naïve controls to identify putative risk factors for disease (socioeconomic status, travel, water use, domestic and wild animal exposures, parenteral exposures and hygiene behaviors).

Nearly 70% of cases were over 15 years old, with few pediatric cases presenting with multiple hepatitis co-infections. Female gender was protective against hepatitis E disease, probably due to social restrictions on movement and outdoor employment. Measures of SES were not significant predictors of hepatitis E disease, although outdoor employment, work outside the home, and travel to a town or city were significant risk factors for incident hepatitis E illness. Self-reported contact with a “jaundice” patient in the 3 months before illness was statistically associated with incident hepatitis E disease, a finding not previously shown in epidemic situations. Although no increased HEV risk
was found between water use patterns or livestock / poultry exposures, the use of sanitary latrines was highly protective. An unexpected significant risk of injection exposure in the 3 months prior to hepatitis E disease was detected.

This study suggested a number of putative risk factors for incident hepatitis E disease associated with poor sanitation. Similar to tourists visiting endemic countries, susceptible individuals from within endemic communities seem to be at increased risk of hepatitis E disease when exposed to sources of HEV, especially during travel to urban areas or when doing outdoor work, in which cases eating or drinking in public establishments may be inevitable. This study also raised the possibility of parenteral and person-to-person transmission of HEV in non-epidemic, sporadic disease settings. Most interesting was a suggested association of hepatitis E disease risk and the daily consumption of large amounts of water, which implies a possible dose-response relationship necessary to cause hepatitis E disease instead of only subclinical infection.

Together, these three studies represent the several “firsts” in the detailed epidemiologic investigation of HEV in Bangladesh. In contrast to the neighboring countries of India and Nepal, from which a rich body of HEV research has emerged over the past three decades, there was a paucity of data on the nature of HEV infection and morbidity in this country. These studies have clearly established a) that HEV is endemic in Bangladesh, b) that a high rate of sporadic infections exists in rural communities despite the apparent absence of periodic HEV outbreaks and that c) aside from the classic hygiene and sanitation risk factors HEV shares with other enteric pathogens, there may be other important pathways (person to person, parenteral, or zoonotic) through which this virus is maintained. These observations are important in this apparently unique
endemic population in which there is no evidence of HEV outbreaks. Given the severe risks to pregnant women and to individuals infected with multiple hepatitis viruses, the continued study of HEV in Bangladesh is warranted, with a focus on the role of HEV in pregnancy, and the characterization of any strain-specific epidemiologic differences between the dominant HEV in Bangladesh with those in neighboring countries of South Asia.
B. Research History

These data reflect a process which began in 1999 with a search for research funding which involved the submission of 22 separate grant applications to various funding agencies over a two year period. A number of major geopolitical events resulted in dramatic changes in federal priorities and consequent cuts on available research funding. Despite competition for limited funds, our efforts culminated in the award of a ~$478,000 R01 with a priority score of 130 (0.6th percentile). These studies were made possible through a unique intersection of research interests, leading to three formal collaborations drawing upon recognized leadership in the fields of epidemiology, hepatitis virology, and community-based research. Through a core team of hepatitis researchers at Johns Hopkins University (Kenrad Nelson, Jon Ticehurst, Alain Labrique) collaborative agreements were established with the International Center for Diarrheal Disease Research, Bangladesh (ICDDR,B) in Bangladesh, and with the Armed Forces Research Institute of Medical Sciences (AFRIMS) in Bangkok, Thailand. The ICDDR,B was subcontracted to carry out the aims of the proposed studies in the Matlab field research site in southwestern Bangladesh. The AFRIMS virology lab graciously served as the hepatitis testing reference laboratory for all aims of the study.
C. Research Timeline

1999 – 2000
Academic Quarter 1: Enrollment into PhD program, Coursework and proposal writing
Academic Quarter 2: Coursework and proposal writing
Academic Quarter 3: Proposal completion and grant applications
Academic Quarter 4: Grant applications and international research ethics certification

2000 – 2001
Academic Quarter 1: Grant applications - NIH R01 written
Academic Quarter 2: NIH R01 submitted (January 23, 2001)
JHU Faculty Appointment (May 1, 2001)
Academic Quarter 3: Relocated to Bangladesh to begin Study Setup and Clearances in Country
Faculty responsibilities running the JiVitA Trials (www.jivita.org)
October: Post-9/11 Security Restrictions on all expatriate movement
October 31: NIH Announcement of R01 Score (130, 0.6 percentile)
November 1: Evacuation of expatriates due to nationwide anti-US sentiment

2002
January 5: Post-evacuation return to Bangladesh
February 4: Protocol Submission to JHU CHR
July 6: Overseas Counterpart (ICDDR,B) Subcontract Submitted
July 19: JHU CHR Approval
October 9: ICDDR,B Research Review Committee Approval obtained
October 14: ICDDR,B Ethical Review Committee Approval obtained
December 3: NIH R01 Funds Released to JHU

2003
February 13: ICDDR,B Subcontract signed with JHU
March 26: Study Supervisory Staff and Medical Officer recruited
April-May: Planning and Supervisory Staff training
June 24: Study staff hiring for HEV Study begins
June 27: JHU CHR First Annual Review of HEV Study
July 14: JHU Procured Supplies (Batch 1) arrive in Bangladesh
August 16: Study staff hiring completed
October 6: JHU Procured Supplies (Batch 2) arrive in Bangladesh
October 16: Training of HEV Study Laboratory Technicians
December 23: HEV Baseline Serosurvey - First study participant enrolled
2004
March 20: Baseline Serosurvey completed
March 24: Case Control Surveillance Pre-testing Phase I began
May 17: JHU CHR Second Annual Review of HEV Study
May 29: Case Control Surveillance Pre-testing Phase II began
June 22: ICDDR,B Subcontract Extension signed with JHU
June 29: AL/KN site visit to AFRIMS Virology Laboratory, Thailand
August 19: JiVitA Senior Management Team Visit to HEV Study
August 22: Case Control Surveillance begins (Batch 1 – Rounds 1 to 3)
September 13: Baseline Serosurvey specimens shipped to AFRIMS, Thailand
November 20: Case Control Surveillance (Batch 2 – Rounds 4 to 7)
December 23: 12-Month Follow-Up Serosurvey Round began
December 24: Baseline Serosurvey Results made available

2005
February 16: JHU Procured Supplies (Batch 4) Arrive in Bangladesh
March 19: Case Control Surveillance (Batch 3 – Rounds 8 to 11)
April 28: 12-Month Follow-Up Serosurvey completed
June 2: 12-Month Follow-Up specimens shipped to AFRIMS, Thailand
June 23: 18-Month Follow-Up began
July 20: Case Control Surveillance (Batch 4 – Rounds 12 to 15)
July 28: 100 Weeks of HEV Study
September 21: 12-Month Follow-Up Results made available
September 25: HEV Baseline findings published in ICDDR,B Science Bulletin
November 1: 1st Shipment of 18-Month Follow-Up specimens to AFRIMS, Thailand
November 1: 1st Shipment of Case Control specimens to AFRIMS, Thailand
November 19: Case Control Surveillance (Batch 5 – Rounds 16 to 19)
November 25: 18-Month Follow-Up completed
December 7: Case Control Results (1st Shipment) made available

2006
January 30: Data Entry Screens designed for Case Control and Virology Studies
February 7: HEV Presentation at 8th Commonwealth Congress (VIII CAPGAN)
March 19: Case Control Surveillance (Batch 6 – Rounds 20 to 22)
April 12: Received result of 1st shipment of 18 Months FU
May 31: Case Control Surveillance completed
June 8: 146 Weeks of HEV Study – Wrap-up Meeting, Matlab
June 27: 2nd Shipment of Case Control specimens to AFRIMS, Thailand
June 27: 2nd Shipment of 18-Month Follow-Up to AFRIMS, Thailand
August 23: 18-Month Follow-Up Surveillance Data Entry Completed
September 7: Case Control Interview Data Entry Completed
September 22: Case Control results from AFRIMS (2nd Shipment) made available
September 22: 18-Month Follow-Up results made available
November 10: Analytic Report (Draft 1) Completed
D. Collaborations

International Center for Diarrheal Disease Research, Bangladesh

The primary site for this study was the Matlab Health Research Center of the International Center for Diarrheal Disease Research, Bangladesh (ICDDR,B), within the Matlab population under ongoing demographic and health surveillance (Figure 1). The Matlab study population has participated in many longitudinal studies conducted by the ICDDR,B over the past three decades, aimed at improving the health of the local population and extrapolating findings of population-based research to the country as a whole. For the purposes of this HEV study, ICDDR,B was subcontracted as primary site for the research, under the NIH grant secured through the Johns Hopkins Bloomberg School of Public Health. The subcontract agreement covered the support of in-country investigators as well as the field teams, laboratory staff and available supplies needed.

This was the first hepatitis E study conducted by ICDDR,B, although a few small studies have been conducted in Matlab to assess the burden of hepatitis B and C infections. Access to the information in the Census, Demographic and Health, and Geographic Information System databases were provided to the principal investigators. The infrastructure of the community health workers was used to implement the viral hepatitis surveillance algorithm to identify potential cases. Matlab provided a unique epidemiologic setting to conduct population-based research on hepatitis, due to the ongoing monthly health and demographic surveillance system.
Armed Forces Research Institute of Medical Sciences

This study also involved a close collaboration with the Armed Forces Research Institute of Medical Sciences (AFRIMS) in Bangkok, Thailand. AFRIMS agreed to conduct all the serologic testing and specimen database management required for this project at no charge. The mission of AFRIMS is to support collaborative research on tropical diseases endemic to Thailand and Southeast Asia. The group functions as a Special Foreign Activity of the Walter Reed Army Institute of Research (WRAIR) in Washington, DC, and of the US Army Medical Research and Materiel Development Command. It is the largest of three overseas US Army biomedical research laboratories and emphasizes basic research on and field testing of new treatments for tropical disease threats: malaria, hepatitis, diarrhea, dengue and Japanese encephalitis. AFRIMS has played a leading role in describing the natural course of Hepatitis E, and in obtaining virus strains for research work at AFRIMS and elsewhere. The Hepatitis group at AFRIMS has been actively involved in the design and conduct of several large studies of HEV in the subcontinent and in East Asia. They have played a major role in developing and evaluating diagnostic tests for HEV, some of which were used in the proposed study.
**E. Funding**

These studies were conducted with funding from the National Institutes of Health (NIH) under an R01 grant, number AI51/31/2004. Additional support was provided in kind (HAV, HBV, HCV, and HEV antibody testing of study specimens) by the Armed Forces Research Institute of Medical Sciences (AFRIMS), a special foreign activity of the Walter Reed Army Institute of Research (WRAIR) in Bangkok, Thailand. The International Center for Diarrheal Disease Research, Bangladesh (ICDDR,B) served as the subcontracted study site for the implementation of these studies.
F. Ethical Considerations and Oversight

Review and Approval

The studies were implemented in the Matlab field research site of the ICDDR,B in Bangladesh as protocol number 2002-018. Protocols were submitted to the ICDDR,B Ethical and Research Review Committees in early 2002. Approval from ICDDR,B’s Research Review Committee was obtained on June 27, 2002, and from the Ethical Review Committee on October 9, 2002. Approval from the Johns Hopkins University Bloomberg School of Public Health’s Committee on Human Research was obtained on July 19, 2002 under CHR Protocol #H.34.02.02.04.AL. All study investigators obtained certification in Human Subjects Research from the Johns Hopkins Bloomberg School of Public Health or from the National Institutes of Health (USA), through online certification programs. Annual progress reports were filed as required by each Ethical Review Board.

Study Population Overview

Unlike most prior studies of HEV which have been based around outbreaks of disease or clinical case series, these studies were population-based, in an otherwise “healthy” rural population. The Matlab cohort represents the largest and longest-lasting population under intensive health and demographic surveillance in the developing world (1). A institutional mandate and commitment to improve the health condition of the population of Bangladesh has fostered a high level of trust and collaboration between the residents of Matlab and researchers at ICDDR,B.
For these studies, there were two principal groups of participants. For the prevalence/incidence studies, a total of 1136 randomly selected individuals (over the age of 1 year) were recruited at baseline for follow-up over an eighteen month period. For the case-control studies a population of 23,500 was followed monthly for 22 months with the intention of identifying and enrolling acute, incident hepatitis E cases. Except for infants under 1, no subpopulations were excluded. Older adults (50+) were included in the random sample, as the possibility exists that HEV immunity wanes over time, permitting reinfection.

**Children**

The epidemiology of HEV in a pediatric population is of special interest to the field of HEV research. Conflicting data exists on the infection rates and epidemiology of HEV in pediatric populations. The 2004 census of the Matlab cohort under demographic surveillance revealed that 34.5% of the population is aged 0-14 (Figure 2), similar to the distribution reflected in the sampled study population. Children under the age of 1 year were not included in the study due to cultural restrictions around the drawing of blood from healthy infants, and because of the possibility that maternal antibodies may provide some residual protection from HEV infection which is difficult to control for (2). In this population, children at the age of 15 have similar exposures to adults due to an active involvement in the agriculture or fishing familial occupations.

The ICDDR,B co-investigators and field staff have extensive experience working with pediatric populations, ranging from the field-testing of Oral Rehydration Solution (ORS) for the treatment of diarrheal diseases, as well as studies of cholera, acute respiratory infections and micronutrient deficiencies in the pediatric population of
Matlab. Several studies of infant and childhood mortality have been successfully completed at Matlab, and the HEV Study investigators have been involved in this research in varying capacities. The rules for specimen collection and assent of children established by the CHR at JHSPH and the IRB at ICDDR,B were strictly adhered to. All provisions of 45 CFR 46, as pertaining to the inclusion of children in low risk research were followed.

**Pregnant Women**

Pregnant women were included in this study primarily because this sub-population is most severely affected by HEV infections. Increasing by trimester, the mortality caused by HEV infections during pregnancy can be as high as 40%, the pathogenesis of which is poorly understood (3). One of the key foci of research at ICDDR,B and Matlab has been the investigation of causes of maternal mortality and exploring methods of reducing this death rate (4). A wide range of studies continue to investigate both demographic trends and public health interventions in maternal and child health (5).

Since the establishment of the Matlab surveillance system, the system of Community Health Research Workers (CHRWs) has been focused on maximizing access to female members of the Matlab community, and overcoming social barriers which tend to limit women's involvement in research or public outreach elsewhere in Bangladesh. The CHRWs reside in the Matlab area, and thus are considered to be members of the community at large. Past studies of diarrheal diseases and vaccine efficacy studies have been very successful in demonstrating accessibility to the female population of Matlab.
As expected, social or religious restrictions did not strongly influence the study's access to women in the community.

A review of causes of death in rural Bangladeshi women in Matlab between 1976 and 1985 identified a substantial amount of mortality among women of reproductive age attributable to infectious diseases (about 30%) (6). Of the infectious disease mortality, 20% has been attributed to some type of hepatitis illness (5). As exact etiology was not pursued in those studies, it is unclear whether these were due to HEV infections. Furthermore, with the promise of effective vaccine therapies, it is essential to identify target groups who may benefit from prophylactic treatment. If HEV is an important burden on this population, it may be interesting to pursue public health interventions to minimize the exposure of the most vulnerable groups (e.g., pregnant women) to sources of HEV in the population. Since HEV infections can have such drastic consequences in pregnant women, it was important that these individuals be included in the study.

**Biospecimens and interviews**

For each of the HEV studies, a small volume of blood (<400 μl) was collected using a minimum pain and low risk fingerstick-capillary blood collection system. Due to the minimal requirements of modern molecular assays, this low volume was sufficient for the study purposes. Participants were also administered a brief (<5-10 minute) interview at each study visit.

**Recruitment Procedures**

Participant selection and enrollment, with informed consent, of volunteers for each of these studies followed the guidelines provided by the Declaration of Helsinki, the
WHO Guidelines for Good Clinical Practice, the United States Public Health Service
Belmont Report, Public Health Service Act as Amended by the Health Research
Guidelines for Biomedical Research Involving Human Subjects issued by the Council for
International Organizations for Medical Sciences (CIOMS). Research regulations
established by Matlab and ICDDR,B committees on human research were strictly adhered
to. Individual participants were able to withdraw from any aspect of the protocol at any
time without in any way compromising their medical or obstetric care from ICDDR,B.

Consent was sought by trained Field Health Technicians, in language that is
understood by the local population. All translations were approved by the ICDDR,B
Institutional Review Board. Individuals were recruited only after witnessed verbal
(signed or thumbprinted) informed consent was obtained. Due to the high rate of
illiteracy, in many cases the consent text was read to the participants. For the inclusion of
children and infants, parents or legal guardians were responsible to give their informed
consent. Non-family and non-study team members served as witnesses to the procedure.

The informed consent forms included all the information required by the
Committee for Human Research at the Johns Hopkins School of Hygiene and Public
Health and the Institutional Review Board of the ICDDR,B. The following elements were
discussed: 1) the study involves research, 2) an overview of the purposes of the research,
3) the groups involved in the conduct of the study, 4) how and why the individual was
selected for the study (randomly, as a case, or as a healthy control), 5) the expected
duration of the subject's involvement in the study, 6) procedures to be administered and
tests performed 7) a description of the risks and benefits to the subject, 8) a statement of
voluntary participation, 9) a statement that withdrawal or nonparticipation does not influence his or her standard treatment options, 10) explanation of record confidentiality and medical disclosure to the subject's physician/medical provider, and finally, 11) all appropriate contact information for study questions and/or problems.

**Risks and Benefits**

These studies were accepted by both JHU and ICDDR,B Ethical Review Committees as ‘no more than minimal risk’. A participant was exposed to a maximum of three fingersticks or heelsticks over an 18-month period. The possibility of adverse events due to study procedures was extremely low, and none were identified during the course of the studies. All recommended infection control procedures were adhered to during blood collection and processing, for the protection of both the study participant and the staff. The fingerstick or heelstick site was cleaned thoroughly with alcohol pads and gloves were worn by the phlebotomist. Automatically retracting lancets were used to deliver a clean, measured incision and to protect the phlebotomist from needlestick injury. A bandage was placed on the site of the fingerstick or heelstick to reduce the possibility of infection.

Severely ill patients identified during the acute HEV surveillance project were visited by the study team medical officer. After a thorough assessment of the patient, appropriate prescriptions and medical treatment were provided. Recommended treatment for hepatitis E largely involves supportive management of the patient, accompanied by rest, so primarily patients were treated for fever, weakness and hyperacidity. Appropriate prophylactic health messages were also delivered to the patient or guardian.
After the baseline study results were returned to the team, the hepatitis E, B and C antibody status was made known through an IRB-approved statement of status. Care was taken to prevent unnecessary alarm, and to strongly recommend that the individual seek further care through the local health system or that of ICDDR,B.

**Data Safety and Confidentiality**

During the entire study period, all data (including questionnaire and serum results) were kept locked in filing cabinets within the office of the Study Manager or in the Data Management Center. All computers and data files were password protected and identifier data was available only to study supervisors. Consent forms were maintained separately in locked filing cabinets at the study site. Data transmittal from one study site to another was accomplished using locked transmittal cases.
2. Research Context

In neighboring countries where seasonal HEV epidemics are regularly reported, the contribution of HEV to the overall burden of infectious disease has been well established. Bangladesh is demographically and geographically similar to portions of India and Nepal, albeit culturally different due to the majority Muslim religion (1). Despite the almost predictable annual flooding in Bangladesh, which should increase the population risk of HEV epidemics, none have been documented over the past two decades.

A. Bangladesh Overview

Bangladesh is situated in the northeastern portion of the Indian subcontinent, bordered on the west, north, and east by India, on the southeast by Myanmar (formerly Burma), and on the south by the Bay of Bengal (Figure 1). It is a deltaic country traversed by rivers, which are essential for local travel, trade and irrigation. With a geographic area of 55,598 square miles, Bangladesh is one of the most densely populated countries in the world with a current population of around 125 million (7). Density estimates from 1991 calculated approximately 2000 persons per square mile. This severe population pressure on the country's resources, combined with many unfavorable socioeconomic factors, makes Bangladesh one of the poorest countries in the world. Fifty million Bangladeshi citizens live below the poverty line, measured by their consumption of less than 2122 calories a day, the minimum caloric standard for an average adult.

Bangladesh experiences anywhere from 55 to 200 inches of annual rainfall, occurring during the monsoon season from May to mid-October. In addition, cyclones
may occur from April to May and from September to November. Annual monsoon flooding helps sustain the largely agricultural and fisheries economy, but also contributes to large outbreaks of disease and accidental deaths.

Bangladesh has been a focus of international concern since its devastating war for independence in 1971. As one of the fastest growing countries in the world, Bangladesh's rapid population expansion combined with recurring natural disasters has overtaxed Bangladesh’s financial and infrastructural resources, with international aid becoming a lifeline for the country’s economy. Bangladesh is still classified as a "least developed country" by the WHO (7). Total adult literacy is a meager 38%, with education compulsory only from age 6-10 (8).

Public Health Indicators

Life expectancy at birth in Bangladesh is between 51 and 57 years (9). The age structure of the country is skewed towards the younger age groups, with 45 percent being under age 15 and 3 percent over 65 years (World and Europe Population Data Sheet, 1992). In 1995, Bangladesh had an under-5 child mortality rate of 115 deaths per 1000. Estimates of infant mortality rate are high at between 50 and 85 per 1000 live births (8). Maternal mortality in 1990 was reported as 850 per 100,000, although recent estimates have been as low as 350 per 100,000 live births in certain areas of the country (5). The trend from 1960 to 1994 has been one of slow decline in crude birth rate and a much steeper drop in crude death rate (10).

The health system is far less accessible to the general population of Bangladesh than in neighboring India (45% and 85%, respectively, have access to health services) (8). Only 15% of pregnant women have access to trained medical personnel during
pregnancy, with a mere 7-9% having these professionals attend their deliveries (8;9). The morbidity and mortality in infants and young children is primarily due to preventable infectious diseases; women of reproductive age in this population mainly die from infectious diseases and direct or indirect obstetric complications. The proportion of this mortality attributable to HEV in this population is unknown.

Access to “safe” water is reportedly high (97%) due to NGO and government sponsored rural tubewell projects (8); however a recently-discovered problem involving the contamination of groundwater by arsenic has complicated this issue. Although tubewell water may be pathogen-free, the ingestion of high levels of arsenic has many deleterious long-term sequelae. Surface water derived from ponds, lakes and rivers is often used for bathing, washing, cleaning or cooking, and pathogen-contaminated water may be inadvertently ingested. It is plausible that the use of contaminated surface water for household purposes may negate many of the health advantages of tubewell water use. The use of unsanitary methods for the disposal of human and animal feces is widespread, leading to further contamination of the surface waters near human settlements.

In spite of the many health and social drawbacks, Bangladesh has made considerable progress in recent years but still faces an enormous challenge in assuring food, energy, shelter, and health benefits to a population that could surpass 200 million by the year 2030.
B. The Matlab Health Research Center

Overview

These HEV studies were possible due to the population research infrastructure and experience of the International Center for Diarrheal Disease Research, Bangladesh (ICDDR,B) at their Matlab Health Research Center (Figure 1). Established in its current form in 1979, the ICDDR,B is globally respected as a unique population research center, with a history of population-based research going back to 1960 (10), when the Matlab research station was initially established as a test site for cholera vaccines. Elaborate demographic and health surveillance systems were established then and have been continually optimized over the past three decades. Matlab is considered the largest and longest sustained population laboratory in the developing world (5;11). The participation of this population in ICDDR,B research has been invaluable for the development and testing of interventions against health and population problems shared by developing countries, including the pioneering work on Oral Rehydration Solution (ORS).

Matlab is a deltaic subdistrict of Bangladesh approximately 45 kilometers southeast of Dhaka, the capital. This rural area has a population density of around 1500 per square mile and a total fertility rate of around six children per woman. The population engages primarily in fishing and agriculture for subsistence and seasonal monsoons ensure that much of the land is flooded for some part of the year (1;10). The population closely matches the demographic, health and cultural profile of rural Bangladesh at large, making generalizations based on Matlab quite reliable. Although exposed to a number of public health interventions over the past decades, several statistical and demographic
indicators support the assumption of generalizability; these include the age structure, adult literacy, maternal mortality and total fertility rates (10). The causes of deaths among adults in Matlab are similar to those outside the study area. However, mortality of children under five (86 per 1000 live births) is significantly lower than the rates seen at the national level (116 per 1000 live births), due to aggressive immunization strategies and active health surveillance (10).

The Matlab Health Research Center is situated in a rural area of Chandpur district in Bangladesh, and covers a population of approximately 210,000 (Figure 1), comprised of approximately 147 villages (an average of 1100 persons per village). In 1978, the Matlab study area was divided into a treatment area (70 villages) and a comparison/control area (79 villages), then of roughly equal population size (1). About 105,000 people fall within the treatment area, now known as the Maternal and Child Health and Family Planning (MCH-FP) area, where a range of public health services are being implemented and new interventions are being tested. The HEV studies were carried out in the MCH-FP area (dark gray shaded area, Figure 1). The remaining Matlab population, in the Comparison Area, receives usual government health services and was not included in these studies (Figure 1).

Each resident of the MCH-FP cohort is issued a unique census identifier at birth or entry into the cohort, and exact age is known for every individual under the age of 25. Members of this cohort have developed a formidable trust and cooperative relationship with the researchers at ICDDR,B, which is evident in the 20% or lower non-participation rate estimated for ongoing studies. This infrastructure also significantly reduces losses to
follow-up. Field surveillance, clinical and laboratory records can easily be linked using these unique identifiers.

The Matlab population experiences an average annual growth of about 1.2%, with annual fluctuations based on climate and other economic pressures (10). The population is in the middle of a demographic transition, from a situation with high birth and death rates with slow growth to one with low mortality rates and population size stability (10). At the most recent Matlab census, the proportion of the population between ages 0-14, 15-49, and over 50 were 38.4%, 47.5% and 14.1%, respectively. In absolute numbers, approximately 81,150 of the entire Matlab population are children (below 15) (1). The population pyramid, reflecting the gender-wise age distribution of this population is described in Figure 2, constructed using data from the 2004 Matlab mid-year census. Educational, health and socioeconomic conditions in Matlab are similar to those elsewhere in Bangladesh.

Importantly, the under five mortality in Matlab in 1996 was 86 per 1000 live births, which dropped to 51.9 in 2004 (significantly lower than the national rate of 116 per 1000 live births) (12). Still, one in ten children in Matlab will die before the age of five, the majority prior to their first birthday. For reasons still under investigation, female child and infant mortality tend to be higher than for males (although recently, the two figures seem to be nearing equality). In 1993-1995, life expectancy at birth was 62 for males and 63.8 for females, perhaps reflecting improvements in the social status of women in these communities (1).

**Matlab Infrastructure**
At the Matlab Health Research Station (white star, figure 1), there is a ICDDR,B-run hospital, where physicians and paramedics are always available. About ten miles from the Matlab Health Center, there is a larger district hospital in the town of Chandpur to which complex cases may be referred (5). Four Health Subcenters are evenly distributed throughout the MCH-FP (white circles in MCH-FP area, figure 1), staffed by health workers, and are easily accessible to the local population. The Matlab hospital and research staff includes trained phlebotomists and laboratory technicians, with ample experience in the collection, processing and storage of biological samples. The Matlab Hospital and Research laboratories are well equipped with standard technical equipment to process and store specimens. Round-trips from Dhaka, where ICDDR,B is located, to Matlab can easily be made within a day, initially via speedboat and (since 2005) by road. This facilitates the rapid transfer of frozen biological specimens through wet or dry ice cold chains.

**Matlab Health and Demographic Surveillance System**

One of the most important data systems maintained in the Matlab Health Research Center is the Health and Demographic Surveillance System (HDSS). This system maintains an up-to-date census of all the residents in the study area, and regularly updates individual demographic and socioeconomic changes. Vital events (births, deaths, marriages), migrations, household size and family relationships can be accurately determined using the HDSS (1). The HDSS databases can be used to generate random population samples or select appropriate controls, based on desired socio-demographic criteria.
In this Matlab cohort, trained Community Health Research Workers (CHRWs) visit every household in the research area and record vital events and migration once every month (5). Information on a limited number of morbidities is also obtained on a monthly basis for each household by a brief structured interview administered by the CHRW. In the MCH-FP area, there are presently 57 CHRWs (about 1 per 1800 people) who maintain a 26-28 day visitation schedule to each household, covering between 15 and 20 households every day. During the household visit, the health status of household members is verified, and a limited range of allopathic treatments can be provided by the CHRW, as necessary. Complex cases are referred to the local Health Subcenter or the Matlab Clinic. All demographic data is recorded twice – in a household data book, kept in the home, and in a central ledger, carried with the CHRW. All data since 1989 have been entered into a computer database, which is programmed for error and inconsistency verification. Systems of internal supervision (eg. unannounced spot checks, field visits, data review) and quality control (eg. refresher training, performance evaluation, data error correction, etc.) exist and are regularly tested.

Limited socio-economic information for each member of the Matlab MCH-FP cohort is available from existing longitudinal databases which are periodically updated. The occupation of the household head, household size, religion, maternal education, per capita dwelling area available in the household, and possession (and description) of latrine facilities near the home are among the variables collected and updated as necessary. Similarly, sources of drinking water, cooking water, bathing water and washing water are also documented.
A Geographic Information System (GIS) has been under development since the mid-1990s, mapping and identifying the location of every house, public building, tubewell and other important geographic components of public health interest in Matlab (13). The paths of rivers, ponds and canals are also described. Unlike most research settings elsewhere in Bangladesh and the developing world, finding households and identifying individuals recruited into studies is relatively straightforward with a low chance of error. The GIS can be used to assess the influence of geographic proximity to rivers, ponds or rice fields as confounders or risk factors and to look for significant disease clusters.

In summary, the Matlab HDSS represents a unique and impressive population data infrastructure that, through careful maintenance by a highly skilled field team of community health workers and supervisors, provides a reliable source of longitudinal epidemiologic data, representative of populations across rural Bangladesh.
3. Literature Review

Hepatitis E Virus – An Enigmatic Pathogen
A. Introduction

Hepatitis viruses cause substantial morbidity and mortality in the developing world, through both sporadic and epidemic disease (14-17). Two viruses have been identified as etiologic agents of enterically transmitted hepatitis: hepatitis A virus (HAV) and hepatitis E virus (HEV). HEV is now recognized as the principal cause of enterically transmitted non-A, non-B (ET-NANB) hepatitis illness, which occurs worldwide, with the greatest historical burden of disease affecting South and Southeast Asia. Concern about the emergence and spread of HEV has increased over the past two decades, as the virus continues to be implicated in periodic outbreaks in some parts of the world, and as the cause of sporadic disease, even in the most unexpected populations (17-19).

In the past four decades, many hepatitis outbreaks associated with fecal contamination of drinking water have been attributed to this virus, the earliest of which was a 1955 waterborne outbreak in Delhi, India (20). India, China, Myanmar, Pakistan and Nepal regularly report outbreaks of hepatitis E illness (16;21-27) and most acute viral hepatitis (AVH) in the Middle East and Latin America is increasingly attributed to hepatitis E (28-32). In India alone, over 2.2 million cases of hepatitis E are thought to occur annually (33;34). A series of consecutive epidemics between 1986 and 1988 of hepatitis E in southern Xinjiang Uighur Autonomous Region (XUAR), China, lasted over 20 months and included over 119,000 clinical cases (35). In 1991, a hepatitis E epidemic in Kanpur, India, caused over 79,000 cases of jaundice (18;36).

Recently, the report of large outbreaks triggered by conflict situations in Chad, Sudan, and Iraq have further raised the profile of this emerging infectious disease (37). In Darfur alone, over 2600 clinical cases were reported within 6 months, as a result of which
45 people (19 of whom were pregnant women) died, despite efforts to chlorinate the water supply (38). As assays for HEV have become commercially available, this pathogen has increasingly been identified as the etiologic agent for sporadic acute hepatitis even in developed countries where HEV is not endemic and even where patients could not be linked to HEV-endemic areas or other HEV cases, suggesting autochthonous infections (39-41). A 2006 study that tested over 5000 sera from 11 countries concluded that not only is HEV globally dispersed, but there is a greater prevalence in industrialized countries than previously thought (42). Figure 3 presents an updated picture of the global spread of HEV, including not only where the virus is endemic and causes large numbers of human cases, but also where HEV is thought to be endemic in animals such as swine, sometimes resulting in autochthonous human cases without travel to endemic areas.

Evidence for this epidemiologically-distinct virus was first reported in 1980, and some speculate that it may have been responsible for epidemics as far back as the early Middle Ages (37), yet the hepatitis E virus was only recently cloned and sequenced in 1991 (43-46). This single-stranded non-enveloped RNA virus, a member of the newly formed Hepeviridae family, is now classified into four major genotypes (47). Distinct patterns of HEV infection and disease in different populations have led to hypotheses of genotype- or subtype- specific epidemiology, ranging from epidemic to sporadic disease, and even cross-species infection of humans from homologous viruses in pigs, deer, and even cats (48-51).

Like HAV, HEV was classically believed to be mainly spread through fecal-oral transmission (52), manifesting as large waterborne outbreaks or clusters of sporadic infections. However, studies of the virus in the past decade have led to major paradigm
shifts in HEV epidemiology. Historically, the absence of early childhood infection or immune response was considered perplexing. Now adding to the puzzle is the increasing evidence that some genotypes of HEV are primarily spread or maintained as zoonoses (in both domestic and wild animals) (47). Furthermore, clear evidence of HEV infection through the consumption of infected meat (both farmed and wild game) (51;53;54) and a newly-emergent avian strain of HEV (55) have added layers of complexity to efforts to explain HEV.

Unlike other enteric viruses which tend to infect and produce immunity in early childhood in endemic areas, immunologic evidence of HEV infection begins to be seen in the second and third decades of life (17;37;56-58). In most cases (70-80% of infections), HEV infection is asymptomatic; furthermore, clinical hepatitis E disease (icterus, nausea, anorexia, etc.) is normally seen in young adults between 15 and 40 years of age. Hepatitis E mortality in the general population is low (0.5-4.0%), in contrast to the >20% case fatality rate experienced by pregnant women hospitalized with HEV infections (59-64).

Two decades of intensive research on hepatitis E have produced a significant body of knowledge, but several important questions remain unanswered about the epidemiology of HEV. The natural history of protective immunity following infection is unclear (65). Specific risk factors for sporadic disease have not been characterized. The role of animals as reservoirs or vectors in the human epidemiology of the virus needs further exploration at a genetic level. Recent phylogenetic analyses of isolates of HEV have suggested genotype or even subtype-specific epidemiologic characteristics, shedding light on a) why certain populations seem to be HEV endemic, based on antibody seropositivity, in the absence of reported outbreaks, b) the absence of antibodies
in young children, and c) the potential role of zoonotic reservoirs of HEV. Better diagnostic assays suggest that this virus may be more widespread globally than originally believed. The specific virologic characteristics, human immune response and pathogenesis of HEV continue to need further exploration.

The pace of research into hepatitis E is increasing, as is interest in “emerging” infectious disease. Studies of HEV continue to draw support as evidence is uncovered supporting a worldwide zoonosis of HEV and the increasing risks (due to travel, conflict, and livestock trade) to inhabitants in non-endemic countries (66). A study of the economic costs of HEV in Nepal alone calculated a loss of 35 productive days per case, or ~19% of annual income (67). Understandably, an important proportion of HEV research is taking place in Asia, particularly in the Indian Subcontinent, where epidemic infection was first recognized. In view of current research efforts, it is likely that within this decade there will be a vaccine available to prevent HEV infection, accompanied by a greater understanding of viral reservoirs and transmission.

**B. The Discovery of HEV**

Prior to the development of convenient laboratory assays, clinical hepatitis E virus infection was implicated by the exclusion of hepatitis A and B (hence the appellation enterically transmitted non-A, non-B, or ET-NANB) (68). As the epidemiology of hepatitis A had long been characterized, it was reasonable to assume that nearly all the adult population of most South Asian countries had had childhood infection and were subsequently immune to HAV. By then excluding hepatitis B serologically, and hepatitis C through risk assessment, cases of acute hepatitis were assumed to be caused by another agent. Using this method of elimination, a substantial proportion of AVH in the Indian
Subcontinent was attributed to ET-NANB infections well before the introduction of specific diagnostic tools for HEV.

Much of our epidemiological and virologic understanding of the hepatitis E virus has been gleaned from studies of the sporadic and epidemic disease in this region. The predictable seasonal epidemics, coincident with the monsoon rains, have provided numerous opportunities to study the risk factors for HEV infection, the clinical course of hepatitis E disease, and the characteristics of the virus. Clinical and epidemiologic studies in India, Pakistan and Nepal have led researchers to identify and isolate the agent responsible for hepatitis E (34;69;70).

The Delhi epidemic of 1955-1956: A new enteric hepatitis

The Delhi, India, epidemic from December 1, 1955 to January 20, 1956 was the first reported outbreak of disease attributable to a 'novel' enterically-transmitted non-A, non-B viral hepatitis (43;44). From a population of 1.6 million, approximately 29,300 jaundice cases occurred with an estimated 67,700 nonicteric infections. (The original estimate of nonicteric cases was based on the premise that in an outbreak of infectious viral hepatitis, over 70 percent of the cases are asymptomatic. (71)) Further epidemiologic studies reported a 2.3 percent attack rate (72).

The majority of cases presented with icterus of the conjunctivae and sclerae. Yellow fever was not epidemiologically plausible due to both the season and the clinical and epidemiologic characteristics of the cases. A leptospiral etiology was eliminated by antibody testing of serum samples. The epidemic curve, based on reported cases, had a steep rise and fall, with a clear unimodal peak following a normal distribution. This was clearly a point source, common vehicle epidemic. The epidemiological characteristics
were carefully studied, including the incubation period (18 to 62 days, mean 40 days). The age-specific disease incidence was highest in the age group from 15 to 39 years (2.9 percent), lower among those older than 40 years (2.0 percent) and lowest in children under 14 (1.2 percent). Mortality among pregnant women was 10 percent (72). Both this elevated mortality and the age distribution of cases led researchers to later suspect that a novel hepatitis virus was responsible.

The common exposure among cases was the use of drinking water from the municipal supply. Examination of the Wazirabad water intake plant on the flooding Jamuna River revealed severe fecal contamination from a nearby sewage-filled stream. A subgroup analysis of two military regiments living under identical conditions in different parts of the city was conducted. An attack rate of 50 per 1,000 was documented in the regiment supplied by municipal water, compared to one per 1,000 in the other regiment, which was supplied by a different water source. The addition of high levels of chlorine to the contaminated water did not have an appreciable impact on the progression of the hepatitis epidemic (20;33;71), although the dose and timing of the chlorine addition may have reduced the efficacy of that intervention.

Over the decades that followed the Delhi epidemic, several other outbreaks of infectious hepatitis were reported in India and the USSR (73;74). In 1980, Khuroo and Wong independently concluded, from retrospective serologic analyses, that these epidemics (thought to have been caused by a variant of the hepatitis A virus) were instead due to a novel enterically-transmitted hepatitis agent (43;44). Three years later, the putative etiologic agent of ET-NANB hepatitis was identified by Balayan and colleagues through immune electron microscopy (IEM) of the feces of an infected human volunteer.
The virus was cloned and partially sequenced in 1990 (46;65), and the agent was labeled “hepatitis E virus” (46;72). The first complete nucleotide sequence of this virus was published in 1991 by Tam and colleagues (45).

Since the Delhi epidemic, the magnitude of this pathogen’s impact on the public health of South Asia and the developing world is much better understood. In the absence of population-based seroprevalence data, evidence that the hepatitis E virus is the primary cause of acute hepatitis in South Asia is convincing. Numerous outbreaks of hepatitis E have been reported from both urban and rural areas across the Indian Subcontinent (21;24) (see Figure 4). Extrapolating from reports of outbreaks and sporadic disease, approximately 2.2 million adult cases of hepatitis E are believed to occur in India annually (34). Major epidemics in Indian cities including Delhi, Ahmedabad, Kolhapur and some cities in the Kashmir Valley have been carefully studied. Northern and Western India are known to be highly endemic areas, and newer data suggests that Eastern and South India are affected as well (57;76-78).

Hepatitis E virus infection is the major cause of hospitalizations for jaundice in Nepal (79). The Kathmandu Valley of Nepal is considered one of the areas of highest risk for HEV infection, as outbreaks are documented almost every rainy season (80). Serosurveys have revealed between a 10 and 25 percent population prevalence of antibodies to HEV (anti-HEV) in Kathmandu and rural Nepal (58;81-83). A substantial amount of sporadic viral hepatitis is also seen annually in Nepal (88 percent of cases in adults and 58 percent in children are caused by HEV (83)), with seasonal increases during the monsoon months of June through August (58).
In Pakistan, HEV has been implicated as the etiologic agent in 77 percent of hospitalized jaundice patients and has caused several outbreaks (84). Studies of a 1987 HEV outbreak in Sargodha, Pakistan, helped to characterize the fecal shedding of this virus and patterns of post-infection anti-HEV IgM and IgG persistence (70;85). Although the data from Bangladesh is sparse, there is good evidence that hepatitis E virus infections may be responsible for most of the acute clinical hepatitis in adults (68;86;87). A small 2002 convenience sample of 273 apparently healthy adults in the capital city, Dhaka, reported a 60% anti-HEV IgG seroprevalence (88).

As can be seen in Table 1, a wide range of seroprevalence estimates exist for populations in the Indian Subcontinent alone, depending on the population sampled and the assays used to determine acute or historic infections. Table 1 lists prevalence estimates of HEV infections obtained by various studies of both healthy and clinically ill populations in the Indian Subcontinent.

**C. Virologic Aspects of HEV**

Hepatitis E virus is a non-enveloped, polyadenylated, single stranded, positive sense RNA virus which appears to be icosahedral in shape, based on cryo-electron microscopy and 3-D image reconstruction (89). Its diameter is approximately 27 to 34 nanometers, as determined by immune electron microscopy (IEM), comprising of a capsid, with interesting spoke-like protrusions on the surface which may be immunogenic (Figure 5)(75;90). As HEV shares certain morphologic and biophysical properties with members *Caliciviridae* family, it was initially classified as a calicivirus (45). Further genomic definition overturned this classification, leaving HEV unclassified until 2004, when it was placed in the *hepevirus* genus, in a new family, *Hepeviridae* (37;91).
The 7.2 kb genome contains three discontinuous, partially overlapping open reading frames (ORF1, ORF2, ORF3) that encode for structural and non-structural proteins (based on similar consensus motifs in caliciviruses) (47;92). The capsid polypeptide encoded by ORF2 has been the focus of most HEV vaccine efforts. At present, four distinct genotypes under a single serotype have been identified, and are discussed in greater detail section K (47). The genomic and molecular characteristics of this virus have also been extensively characterized and are described elsewhere (3;93-95).

As HEV does not proliferate well in cell culture, animal models have been pursued since the virus’s discovery. Most experiments have been conducted in non-human primate species, primarily the cynomolgus macaque, *Macaca fascicularis* ((96-98). Chimpanzees (99), tamarins (100), owl monkeys (101) and rhesus monkeys (102) have also been studied. Viral replication has been successfully demonstrated in pigs, rats and chickens, which may represent more convenient research models (96;103;104).

**D. HEV Detection**

Initial assays to detect HEV infection included immune electron microscopy (IEM) of stool samples or bile to detect HEV particles (69;75;101) and fluorescent antibody blocking assays for anti-HEV in serum and liver sections (105;106). Both these methods have very limited sensitivity to detect both acute and remote HEV infections and are impractical (94). In the absence of convenient tests, a diagnosis of ET-NANB (HEV) infection was made by excluding other viral agents serologically and by verifying the absence of risk factors such as transfusions or drug use (83).

Although commercial tests are still not available for clinical use in the United States, anti-HEV tests of varying quality are marketed globally. Research and reference
laboratories have also developed a number of techniques to detect HEV infection. An excellent, comprehensive review of HEV detection, collection and storage techniques has been published recently (95). Today, enzyme immunoassays (EIAs) are most commonly employed for the detection of IgA, IgG, and IgM anti-HEV. These immunoassays use recombinant HEV antigens or synthetic peptides corresponding to immunogenic HEV epitopes (94;95;107). In many such assays, at least two geographically distinct HEV strains are used to represent diverse antigenic domains (65). Tsarev and colleagues developed an EIA using a recombinant protein from ORF2 (HEV strain Pakistan 1987), expressed in insect cells (108). Others have recently used recombinant technology to express HEV structural proteins which self-assemble into empty virus-like particles (VLPs); these were used as the capture antigen in a new EIA (109;110). These detection assays are highly sensitive and specific (109;111). Western blot assays can also detect IgM or IgG anti-HEV in serum, although with less sensitivity (90).

Reverse-transcriptase polymerase chain reaction (RT-PCR) is used to detect HEV nucleic acids (RNA) in serum or stool samples with high sensitivity and specificity; this process is, unfortunately, labor-intensive and the timely collection of useful samples is often difficult (112;113). The use of RT-PCR to detect HEV RNA in serum is a useful addition to enzyme immunoassay anti-HEV antibody testing when diagnosing acute HEV infections (114). When the RT-PCR method is used in less-experienced laboratories, problems of nonspecificity, primarily due to specimen contamination, might complicate the interpretation of results. Methods are also available for the detection of HEV in environmental water samples using a combination of granular activated carbon (GAC) column filtration and conventional molecular biologic techniques (115).
Available assays for anti-HEV have a wide range of sensitivity between 17 and 100 percent in non-endemic areas (94). Due to highly discrepant results between some of these assays (using blood donor sera from non-HEV endemic populations), caution has been suggested when interpreting data obtained with these assays from low-prevalence populations (94). These tests may perform differently in endemic and non-endemic settings, and against different genotypes or subtypes (116). Even in endemic areas, commercial assays have been shown to vary substantially by manufacturer’s lot, and overall agreement between two laboratory-developed non-commercial assays was only moderate (117). It has also been proposed that antibodies specific to different epitopes of HEV may differ in persistence, resulting in conflicting data. The ORF2 RNA sequence of HEV has shown little variation among geographically diverse strains, when compared to the ORF3 region (94); recent field testing has upheld the greater reliability of ORF2-based assays and suggested that ORF3 tests “are of limited value for seroepidemiologic studies” (118). However, researchers at CDC (Atlanta, USA) in 2005 developed an extremely efficient single step real-time RT-PCR assay using a conserved sequence of ORF3 for detection of HEV in clinical or environmental samples (119). For the evaluation of results of sporadic cases and assessing seroprevalence in non-endemic areas or healthy populations, low test specificity may also be a problem.

As there is still no widely applicable “gold standard”, sensitivity and specificity of new assays for HEV are often evaluated in outbreaks and in small experimental studies. The lack of standardized commercial tests leads to significant variability in sensitivity and specificity among assays. Possibly, the use of RT-PCR to detect HEV RNA in conjunction with a recombinant ORF2 enzyme immunoassay to detect anti-HEV IgM
would be the ideal diagnostic protocol to yield high sensitivity and specificity for the
diagnosis of acute HEV infections.

E. Epidemiologic Aspects of HEV Infections

Geographic Distribution and Phylogenesis

The epidemiology of hepatitis E is likely more complex than that of other enteric
viral infections (120). Outbreaks and high seroprevalence of hepatitis E have been
reported from India, Pakistan, Afghanistan, Bangladesh, Nepal, Myanmar (formerly
Burma), Borneo (Indonesia), Algeria, Somalia, Sudan, Ivory Coast, Mexico, China,
Vietnam, Thailand, Egypt and some former Soviet republics (including Kazakhstan,
Tajikistan, Turkmenistan, and Uzbekistan) (27;72;121-129) (Table 1 and Figure 5).
Recent evidence suggests a higher-than-expected prevalence of antibodies to HEV in
many developed countries where outbreaks have not been reported, including the United
States, indicating that HEV may cause isolated cases of sporadic illness or inapparent
infections (130-133).

In 2006, Lu and colleagues published a thorough phylogenetic analysis of HEV
sequences from sporadic, epidemic, and zoonotic cases around the world, in which they
provide strong evidence for the existence of only four distinct genotypes under a common
serotype (47). Various degrees of intra-sequence divergence between isolates suggest a
number of subtypes exist for each genotype. Genotype I (Asia-Africa) isolates can be
divided into five highly conserved subtypes. This is consistent with the primary
circulation of this genotype in humans, primarily in South or Southeast Asia and Africa.
Limited isolates are available to investigate genotype II (Mexico), but at least 2 subtypes
have been proposed. Both genotype III (US-Japan-Swine) and IV (China-Japan-Swine) displayed a wide genetic diversity with ten and seven subtypes proposed, respectively (47). The circulation in both human and animal hosts likely contributes to the wider genetic diversity within genotypes III and IV.

The majority of HEV infections in subcontinental Asia, typically linked to waterborne outbreaks, are attributable to subtypes of genotype I, whereas sporadic cases in developed, “non-endemic” countries have been largely genotypes III and IV. Sequences of the latter two genotypes, identified in both humans and animals, were closely related, suggesting either a strong zoonotic component to the maintenance and spread of these genotypes or a common infectious source (47).

Although there have been no documented outbreaks of HEV in the United States, several patients in the US have been diagnosed with acute hepatitis E, caused by unique strains (107). These strains, initially identified as genetically divergent from known HEV genotypes, are now classified as genotype III (47;107;134;135). Interestingly, although having only a 77 and 76 percent nucleotide identity with other human strains (ORF1 of Mexican and Burmese strains, respectively) (107), the genotype III strains are 97% identical (by amino acid comparisons of ORF1 and 2) to the recently discovered Swine HEV (HEV-S), which is ubiquitous in the US swine population (135;136). HEV isolated from wild boar and deer in Japan have also been classified under genotype III (47;53). Viruses from outbreaks in Mexico have little phylogenetic relation to either of these strains and have been classified as genotype II (47).

A strain of HEV identified in Taiwan had only 79 percent identity to other strains from around the world (137). Chinese researchers have extensively studied a series of
autochthonous cases attributable to HEV, with only 74 to 83 percent identity with known human sequences. Isolates from domestic pigs in Japan, Indonesia, and China also clustered into this genotype, representing the HEV genotype IV (47;138). To date, a large number of HEV isolates have been either completely or partially sequenced, and even patients without a history of travel to HEV endemic areas invariably fall into one of these four distinct genotypes (47;139).

Clinical and epidemiologic differences have been observed between infections with HEV subtypes within genotypes (95). Different genotypes and subtypes may co-exist in a single population, some causing epidemic and others sporadic disease (140). Possibly, variations in the pathogenicity of HEV infections in one area may be due to infections by different strains, although host and environmental factors must also be considered (95;141). Interesting recent data from southwestern India, where human hepatitis E epidemics are common, revealed that the HEV strains isolated from humans over a period of 26 years (1985-2002) are genotype I, while swine samples from this same period reveal only infection by genotype IV (142). In contrast, data from Nepal suggest cross-species infection due to a strong relationship between swine HEV seropositivity and a history of human HEV infection near the swine’s enclosure (143). Recent studies in Nepal have even found evidence of genotype I circulating in rats, making it difficult to postulate that only certain subtypes (III or IV) are capable of both zoonotic and human circulation, or that a given subtype has a mainly human or mainly animal circulation (144). The continued characterization and genotypic classification of emerging HEV strains has helped to understand the likely sources of various HEV genotypes and to establish dispersion patterns.
In 2004, Shrestha and colleagues published some fascinating data from Nepal, characterizing dominant subtypes over a period of five years. They confirmed that one subtype had been predominantly (albeit not exclusively) associated with an outbreak of hepatitis E in 1997, whereas sporadic cases were attributable to a separate subtype. They also demonstrated the annual frequency of a subtype (1a-3) went from 5% of cases in 1997 to 95% of cases in 2002, whereas the dominant subtype in 1997 (1a-2) virtually disappeared from the etiologic landscape by 2002 (145;146). Despite the multiple genotypes and subtypes, there is evidence of serological cross-neutralization of HEV variants. In cross-challenge experiments, monkeys initially infected with a strain of HEV from Burma or the Soviet Union were protected from reinfection when challenged later with strains from India or Mexico (99;147).

**Transmission and Risk Factors for HEV Infection**

Hepatitis E incidence in South Asia has been characterized by marked seasonality, with outbreaks occurring during the rainy or monsoon seasons. These epidemics have been documented in April and October in Bangladesh (68) and from May to September in the Kathmandu valley of Nepal (80). A periodicity of five to ten years has been suggested for recurring epidemics of HEV in India, China, and certain central Asian republics of the former Soviet Union (52;65).

Outbreak investigations often reveal fecally contaminated drinking water supplies as the source of HEV (14;86). It appears that rain-induced flooding allows sewage to contaminate water supplies, explaining the seasonal associations (86). Dry conditions, too, seem to increase risk of HEV outbreaks, as this may prevent the dilution of HEV particles in contaminated water (122;148;149). When water is intermittently pumped
through broken or cracked piping, as observed in regions of Nepal and Pakistan, negative pressure can pull in fecally contaminated water from the surface above the pipes, thereby increasing the risk of HEV contamination (69;84). Irrespective of mechanism, communities with inadequate sewage disposal or processing arrangements are prone to recurrent HEV outbreaks (93). A low level of community sanitation is also strongly associated with both epidemic and endemic HEV. For example, in Indonesia, the use of river water for cooking and drinking, poor personal hygiene, and improper disposal of human excreta were significantly associated with increased anti-HEV IgG seroprevalence (122).

Most HEV patients from developed countries report a recent history of travel to HEV-endemic areas, and have often consumed unsanitary food and water (131;150-153). Several recent studies of hepatitis E patients in non-endemic Asian countries have suggested that the consumption of undercooked or raw pork and deer meat were significant risk factors for HEV infection (37;50;154). One study even demonstrated that the meat consumed contained viral RNA identical to the strain identified in the case-patient (51). However, large-scale foodborne hepatitis E outbreaks have not been reported.

No secondary cases of hepatitis have been reported among sexual partners of cases under conditions of epidemic or sporadic disease (97;125). These data, although sparse, suggest that sexual transmission does not play an important role in the spread of HEV. Studies of anti-HEV prevalence in non-endemic areas suggest that an O blood type may predispose individuals to HEV infection (131). (Precedence for such an association has been established in Vibrio cholera infections, where an increased risk of severe
disease has been shown in persons with blood type O (155). A single study comparing allelic and genotype variations in Indian acute hepatitis E patients to healthy controls suggested that genetic changes in interferon-g might increase likelihood of clinical disease after HEV infection (156). This hypothesis also warrants further exploration.

Compared to enterically transmitted HAV, which has a 10-20 percent secondary attack rate among household contacts, the hepatitis E virus has a relatively low infectivity (with a secondary attack rate of about 2 percent) (157;158). A detailed epidemiological study of intrafamilial transmission was conducted in 1991 after a large bimodal waterborne HEV epidemic in Kanpur, India (159). Secondary, or "later", cases (n=111) were defined as those developing illness a minimum of two weeks after the index case in a household. Only eight of these 111 (7.2 percent) "later" cases could be attributed to probable intrafamilial transmission; most of the “later” cases were exposed to the initial source of HEV. Thus, from 402 household contacts of primary cases at risk for secondary infection, the secondary attack rate in this study was only 2.0 percent (8/402). (159)

Another study of two separate HEV epidemics in Maharashtra, India, compared anti-HEV IgM seroconversion rates among family members of case-patients to family members of healthy individuals in the same communities a month after the epidemic began (160). No statistical difference in seroconversion rates was found between the two groups.

Some studies, which did not account for the incubation period of HEV to separate coprimary from secondary cases, found higher attack rates within primary case households (161). For example, a study in Myanmar found the relative risk of HEV acquisition in an index case's household to be six times greater than in control households.
(125). There is, however, a high probability of bias from differences in water supplies and other potential HEV risk factors between case and non-case households, which were not adjusted for in these studies (159).

It is not clear why HEV has a lower secondary attack rate than HAV. HEV is believed to have a lower environmental stability, which may result in decreased secondary transmission (157;161-163). Furthermore, animal HEV studies have shown a positive association between disease severity and inoculum size (164); a larger infective dose may be necessary to cause overt disease among contacts of HEV patients. It is important to realize that disease, not infection, is often measured in outbreaks of HEV. Higher rates of subclinical secondary transmission may be possible if the dose received in person-to-person transmission is lower than that required to cause disease (20). In a macaque model, some studies have demonstrated serial subclinical transmission of disease, with high viral titers in stool, even without ALT elevation (165).

A high proportion of subclinical infections was demonstrated in a 1995 outbreak near Kathmandu, Nepal, which involved 692 soldiers (25). Thirty-two clinical cases were identified and eighty-three others were anti-HEV IgM seropositive but remained asymptomatic. Serologic evidence of past infection (anti-HEV IgG in the absence of IgM) was found in 29 percent of the cohort; assuming that these individuals were not at risk for re-infection, the incidence of clinical disease was 7 percent with an overall infection rate of 24 percent (25). In studies of sporadic HEV in similar military cohorts, a 2.8 percent rate of clinical disease and an infection rate of 9.2 percent has been documented (58).
No significant difference in attack or infection rates by gender has been documented when equal exposure to the source of HEV occurred (102;125;161). However, some studies of outbreaks in Nepal, Pakistan and India have suggested that adult men may have between a two- to five-fold higher risk than women of the same age of reporting with clinical illness (26;166;167). A higher proportion of male cases may be caused by behavioral factors resulting in differential exposure to HEV sources, as in a recent outbreak studied in India, where travel outside the home increased risk of hepatitis E illness five-fold (167). Not only might social restrictions on women minimize their HEV exposure (26;72) but gender differences in health seeking behaviors may exist in these communities.

Vertical transmission of HEV from mothers to their infants has been reported, with associated morbidity and mortality (64;168). A small study was conducted in 10 pregnant women infected with HEV in their third trimester. Of eight successfully delivering women, six transmitted HEV to their infants; HEV RNA was detected in the cord blood (without maternal contamination) of five of the six infants and IgM anti-HEV in three of these infants (168). Vertical transmission of HEV has not been successful in monkey models of infection (169). Given the possibility of vertical transmission, it may be difficult to assess the risk of fecal-oral infections of newborns. The risk of infection through breast feeding is also not known.

Accidental HEV transmission in the laboratory has also been reported, including one Calcutta outbreak involving 21 people in a research facility where HEV was being studied (170). Nosocomial spread of HEV has been reported from South Africa and in refugee camp hospital settings (127;171). In 2001, an outbreak in a Pakistani
neurosurgery ward was linked to the improper sharing of intravenous kits among patients in an environment where no water-borne transmission was likely (171).

Although HEV transmission has been documented largely in outbreak situations, it is also responsible for over 50 percent of acute sporadic hepatitis in both children and adults in many countries (127). The exact mode of HEV transmission outside epidemics remains undetermined (90;107) as few studies have attempted to determine individual risk factors for the development of sporadic illness. The prolonged incubation period prior to clinical manifestations increases the probability of significant recall bias.

**F. The Natural History of HEV Infection**

**Pathogenesis**

Clinically, HEV and HAV infections are virtually indistinguishable (14). The illness occurs in two phases, the prodromal (pre-icteric) and icteric. During the first phase, patients may experience fever and nausea. During the icteric phase, the sclerae become discolored, jaundice occurs and dark urine is noted. Other general symptoms include abdominal pain, anorexia, clay-colored stools, hepatomegaly, malaise, and vomiting. Less common symptoms include arthralgia, diarrhea, pruritus and an urticarial rash (127). The disease is self-limited and most patients recover completely without complications or sequelae (137). No chronic or carrier state has been demonstrated after hepatitis E infection (150).

After infection, HEV find their way to the liver and are thought to primarily replicate in the cytoplasm of hepatocytes (3;17). Extrahepatic sites of HEV replication have been suggested in animal models, but have not yet been confirmed in humans (172).
The typical incubation period ranges from 15 to 60 days (mean 40 days) from the time of exposure (90). In humans, liver enzymes and indicators of liver inflammation (aspartate and alanine aminotransferase, alkaline phospatase, γ-glutamyl transpeptidase and bilirubin) rise dramatically four to five weeks after infection and remain elevated for 7 to 90 days (see Figure 6) (151). The trajectories of immune markers have been analyzed in animal models and human volunteer studies, and will be discussed in a later section (see Viremia and Immune Response). In many patients, biopsies may reveal cholestatic changes including intracanicular bile stasis and a gland-like transformation of parenchymal cells (63;173). HEV, however, is thought not to be cytopathogenic, although histologic changes including focal necrosis and inflammation have been observed (17). Electron microscopy has been used to detect virus-like particles in the liver of patients with HEV-attributable fulminant hepatitis (63).

G. Hepatitis E Mortality – The Pregnancy Conundrum

A low mortality rate (0.5 to 4 percent) is associated with this infection in the general population (59). Acute HEV infection was implicated as the primary cause of fulminant hepatic failure (FHF) in over 40 percent of a clinical case series of adults and children in New Delhi, India. When fulminant hepatic failure does occur, it is often fatal (174). For reasons still unclear, pregnant women, especially those in their third trimester, have a poor prognosis when infected with HEV (17;173). Some have hypothesized that liver sinusoidal cells (eg. Kupffer cells) are damaged by HEV, which affects their ability to protect hepatocytes against endotoxins from Gram-negative gut bacteria. The hepatocytes may sustain injuries directly from the endotoxins or from eicosanoids (20-carbon chain polyunsaturated fatty acids known to cause clotting (platelet aggregation),
inflammation and other effects). The release of prostaglandins can lead to the attraction of neutrophils, which subsequently cause swelling through edema and also cholestasis (17;175). This chain of events triggered by endotoxins may overwhelm the system during pregnancy, resulting in a higher mortality (3;72). Monkey studies of HEV infection have also suggested viral damage of kidney tissue during replication, precipitating eclampsia (176).

High rates of both infant and maternal mortality have been documented in many studies; this has been one of the hallmarks of even the earliest HEV outbreaks (79;177). Case fatality rates among pregnant women infected with HEV (during outbreaks or in clinical series of sporadic infections) range from 10 to 42 percent (58;69;79;83), and are primarily due to hepatic encephalopathy and disseminated intravascular coagulation (59;62;178). Other complications during pregnancy include the premature rupture of membranes and fetal distress (179). An Ethiopian study found that over 35 percent of HEV-infected hospitalized pregnant women experienced premature deliveries (180).

During a 1993-1994 Pakistani outbreak of HEV (26), attack rates of icteric hepatitis increased by trimester of pregnancy. This study also found a two-fold increased risk of clinical hepatitis E disease for pregnant women compared to non-pregnant women of reproductive age. Although fulminant hepatitis E in non-pregnant individuals is uncommon (95), both epidemic and clinical studies have shown that pregnant women have an increased likelihood of developing acute hepatitis and even fulminant hepatic failure after HEV infection (59;178;181;182). Once hepatic failure occurs, however, higher mortality rates were not observed in pregnant, HEV infected women when compared to nonpregnant women and men with FHF (181;182). It has been suggested
that the aggressive monitoring and treatment of HEV-induced fulminant hepatic failure may help reduce the high maternal mortality observed in Asia (183). In sharp contrast, severe AVH in pregnancy caused by HEV has not been reported in Egypt, despite high anti-HEV antibody seroprevalence in rural communities, suggesting potential genotype-specific differences in virulence or pathogenicity (184).

HEV infection during pregnancy also increases the rate of adverse outcomes for the newborn, as the risks of neonatal icterus and death increase (177). In a Pakistani epidemic, four of eight fatalities were infants born to HEV-infected mothers, indicating the serious consequences of this infection in neonates. (The other four fatalities were HEV infected women in their third trimester) (26). In a study of eight HEV infections among newborns in Kashmir, India, two died and one was icteric (168). Khuroo and colleagues speculated that even mild HEV infection during pregnancy may contribute to a higher rate of abortion and intrauterine death (168). The high risks of acute clinical progression in pregnant women and the severe fetal consequences are sufficient to warrant exclusion of pregnant health-care personnel from contact with HEV (95).

**H. Viremia and Immune Response**

Viremia is thought to last between 14 and 28 days in most patients with clinical disease, although it may be prolonged in some patients (90;185). Viremia or fecal shedding (or both) is detected in patients prior to liver abnormalities or even antibody evidence of HEV infection (15;17). In one report, HEV RNA was detected in serum for 112 days after the onset of clinical signs (186). The apparent uncoupling of viremia and liver damage has been suggested by some as evidence that, like other non-enveloped viruses like SV40 and poliovirus, HEV may be released from its host cells by means
other than lysis (143). The prolonged viremia of HEV even after the development of IgM and IgG antibodies suggests that factors other than acute phase immunoglobulin may be important for viral clearance (187). Viral shedding in stool has been shown to begin up to nine days prior to the icteric phase of disease (70;188). Normally, fecal shedding lasts up to 14 days after the onset of symptoms, but has been reported to continue for as long as seven weeks (90;186).

The serologic course of HEV infection has been determined using nonhuman primate models, human volunteer studies, and outbreak investigations (See Figure 6). Both IgM and IgG antibody responses are detected soon after infection, with peak antibody titers occurring two to four weeks after infection (85). The anti-HEV IgM titers seem to decline within three months after infection, during early convalescence (90;107). (As the antibody response to HEV and clinical hepatitis tend to occur simultaneously, some have suggested that hepatitis E may be an immunopathologic disease, where the host response to infected cells causes hepatitis (17;95).)

Published data on the persistence of IgG anti-HEV in populations are conflicting. IgG antibodies have been detected one to fifteen years after HEV infection (85;94;152;189), but do not persist in all subjects. Most studies indicate IgG anti-HEV titers peak about four weeks after infection, and then decline rapidly (85;187;190). For example, in Egypt, anti-HEV became undetectable in 67 percent of children 6 to 12 months after acute infection (191). In Indonesia, 28 percent of sixty incident HEV cases lost anti-HEV IgG within two years (122). A 1993 follow-up study in Kashmir (India) of a 1978 HEV outbreak cohort detected anti-HEV IgG in only 47 percent of the cases.
tested, suggesting loss of antibodies in a large proportion of the affected population (189).

However, the probability of detecting low levels of IgG anti-HEV may also depend upon the type of assay used. Mast and colleagues found that synthetic peptide-based enzyme immunoassays were less sensitive for the detection of remote infections (anti-HEV IgG) when compared to recombinant protein assays (94).

A study in Sargodha, Pakistan, which revealed that anti-HEV IgG persisted at a low to moderate titer for at least 20 months after acute illness, also suggested that anti-HEV IgG provided protection against disease (85). Some have proposed that circulating antibody levels decline significantly with age (82); however, it is not clear why attack rates and seroprevalence of anti-HEV are lower in the elderly population during waterborne outbreaks (20). Currently, our understanding of the correlation between anti-HEV IgG and immunity is incomplete (3;16;85;148). The cross-protection of antibodies against co-circulating subtypes or genotypes of HEV is also poorly understood.

In the 1955 Delhi epidemic and a 1973 outbreak in the Kathmandu Valley of Nepal, attack rates were four to eight times higher among persons of high SES (20;85). In both these examples, lower SES individuals tended to live under poor hygienic conditions, indicating that some protective immunity may exist as a result of frequent environmental exposure to HEV (83). Low levels of anti-HEV IgG could be sufficient to protect against clinical disease when the inoculum is small, such as with secondary exposure in contrast to ingestion of heavily contaminated water (20). However, with heavy exposure, disease could still occur in segments of the population who have waning or no protective immunity. Animal model studies have demonstrated that the challenge
The dose required for disease production is at least 1000-fold greater than needed for infectivity (164). The interplay of inoculum size and time-dependent decline in the infection threshold may partially explain the age distribution of HEV infections.

Melnick and colleagues suggested that this explanation might be the reason for low numbers of pediatric cases during the 1955 epidemic, because children, infected at an early age, maintained a strong protective antibody response during the first decade of life (20). However, Bryan and colleagues, studying a 1987 Pakistani epidemic, found that all clinical cases of HEV were serologically consistent with a primary infection, not a reinfection, by the detection of anti-HEV IgM in every clinical case (85).

I. Age Distribution of HEV Infections – An Epidemiologic Puzzle

Two distinct age-specific patterns of HEV seroepidemiology have become clear in recent years. Early serosurveys conducted across South Asia, where HEV is known to be endemic and the cause of large annual epidemics, revealed a perplexing pattern of most hepatitis E infections and illness occurring in the older adolescents and adults between the ages of 20 and 50 (14;85;95). This is in sharp contrast to the similarly enteric hepatitis A virus, where most infections occur in the first decade of life, and anti-HAV antibody prevalence is both ubiquitous by age 10 and protective into later adolescence and adulthood (192). The paucity of infections, noted by both clinical disease rates and the absence of antibody (68), in persons under 15 is unexpected for a fecal-oral pathogen under environmental conditions which facilitate such transmission.

More recent evidence from non-epidemic countries such as Egypt, anti-HEV seroepidemiology resembles that seen with anti-HAV in India. Anti-HEV is seen in children, and peaks around 70% seroprevalence in early adolescence (193;194). As
mentioned earlier, even the pathogenesis of HEV infection in pregnancy seems to be different in this population. Possibly, different genotypes or subtypes may produce different patterns of infection, immune response, or have varying levels of pathogenicity.

During most HEV outbreaks in India since 1956, the peak incidence of disease has been in persons aged 15-30. In Nepal, 75 percent of HEV cases in epidemic situations occurred in the population aged 15 to 34 years (58;69). Clinical observations in Bangladesh have documented that the frequency of overt hepatitis E disease is highest in those aged 21-40 and lowest in those under 10 (M.S. Hassan, Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM), Personal Communication, 1998). Repeated observations of this type have led to the suggestion that the majority of pediatric infections, if occurring, are anicteric or subclinical (116).

However, a population-based serosurvey conducted in India suggested that a substantial proportion of children under five had not been infected by HEV (56). Similarly, studies in other HEV-endemic settings have shown that less than 10 percent of children under ten had antibodies to HEV (195). In most settings, the prevalence of antibodies to HEV is seen to gradually increase with age until about age 30 (58). (Few studies have demonstrated a substantial increase in seroprevalence after the third decade, leading previous reviews of HEV epidemiology to conclude that, paradoxically, anti-HEV prevalence rates are much lower than would be expected in endemic areas (95).)

Two similar cohorts of schoolchildren in Pune, India from 1982 and 1992 were compared for their age-specific prevalence of IgG antibodies to HAV and HEV (56). By age 10 at least 95 percent of the population had antibodies to HAV, whereas in both
cohorts, the prevalence of antibodies to HEV was low (zero to nine percent) in children under age 15, increasing to 40 percent among those 16 to 25 years old (56). The absence of anti-HEV antibodies in younger children could not be explained either by a rapid decay of post-infection IgG, or by a failure to mount an antibody response. The age distribution for both infections remained stable over a decade, suggesting that the epidemiology of HEV, including risk factors for sporadic infections, also remained unchanged. The similarity between the two timepoints also suggests the absence of a cohort effect in this population.

In 1997, a very different distribution of age-specific anti-HEV IgG seroprevalence was reported in India (116). The Abbott Labs HEV immunoassay (for IgG antibodies against recombinant ORF2 and ORF3 from a Burmese HEV strain) was used to test ninety-five individuals covering four age groups (0-5 yrs, 6-10yrs, 11-18 yrs and 18+). Anti-HEV IgG seroprevalence for these groups were 64 percent, 59 percent, 64 percent and 50 percent, respectively. The high pediatric anti-HEV seroprevalence seen in this study have not been replicated by others in South Asian populations (116;196;197).

Instances of sporadic hepatitis attributable to HEV have been reported in children (2 months to 15 years) from the Sudan, Egypt, and Somalia (166;191;198-200).An Egyptian study examined the sera from a 1994 convenience sample of healthy Lower Egyptian children and adults using the Abbott immunoassay. The study revealed a 57% seroprevalence in 21 children between ages four and nine (201). Observed HEV antibody prevalence levels in this population remained constant at around 60 percent throughout adulthood (201). (Some have argued that this high HEV infection rate in young children conflicts with the results of a previous study in a neighboring Egyptian population as
sample selection bias may have inflated the observed seroprevalence (195). Nonetheless, different age distributions of HEV infection patterns between communities (regardless of their geographic proximity), would support a hypothesis that local factors, or even subtype characteristics, influence the incidence of HEV infection (202).

Other North African studies continue to support the possibility of higher pediatric susceptibility to infection than suggested by research in the Indian Subcontinent (203;204). Among hospitalized Egyptian children, 12 to 22 percent of AVH has been attributed to HEV (191;199;205). A case control study in Sudan found that 59 percent of hospitalized pediatric AVH cases were anti-HEV IgM seropositive. The age and gender matched control group in this study suggested a high (18 percent) anti-HEV IgG seroprevalence among children in the general population (198).

The Indian data suggesting a high HEV incidence in adults with a virtual absence of HEV infection among children is bewildering, assuming a transmission modality similar to that of HAV (206). No explanation for the available data satisfies all the findings. It does not seem to be a regional phenomenon since these distributions have been found on several continents and may vary within a given country. Some have hypothesized that adults are exposed with greater frequency and volume to HEV through greater mobility, access to high-risk environments and from the ingestion of larger quantities of contaminated food and water (56). Since epidemics of HEV have been reported among adolescents and adults in Nepal, India, Pakistan and other South Asian countries during the monsoon rainy season, but not in North Africa, the different patterns of rainfall could be an additional factor to consider.
It has been suggested that the second/third-decade predilection is due to peculiarities of the assays used, but this finding has been reported among clinically defined cases in outbreaks and hospitalized case series studies using the same assay. Age-specific immune response differences have also been proposed (such as a rapid loss of anti-HEV among children or the failure of early childhood exposure to confer lasting protective immunity) (116). However, this explanation would not account for the occurrence of childhood infection in some but not other areas (95;207). In non-epidemic areas, high adult seroprevalence observed in combination with lower pediatric seroprevalence may indicate a region-specific cohort effect, reflecting improved sanitary conditions over the past few decades (207;208). To date, this remains one of the more perplexing issues in the epidemiology of HEV.

**J. Interactions with other Hepatitis Viruses**

Several studies of clinical hepatitis have demonstrated, however, that children presenting with acute hepatitis E illness tend to be infected simultaneously by other hepatotropic viruses (209;210). Acute HAV-HEV coinfection seems to lead to more severe clinical progression than infection with either virus alone. Twenty two percent of cases of hepatic failure in children attending an Indian tertiary health care facility were infected with both viruses (210). (Whether these infections occurred simultaneously or were sequential is difficult to establish.) A clinical case series of 528 children in the Ukraine also found more serious disease with concurrent hepatitis A and E infections (211). Similar findings were published from a 2006 prospective study of 149 children presenting with AVH or FHF in an urban Indian hospital; HEV infection was associated with another hepatotropic viral agent in 88% of these pediatric cases (212). The biologic
basis for an interaction is unknown, but may not be unique to HAV and HEV. Superinfection with other hepatitis viruses is also associated with more severe disease as multiple hepatotropic viruses infecting a single patient may amplify liver damage.

More severe hepatitis was associated with HEV infection in chronic hepatitis B surface antigen (HBsAg) carriers in Chad (203). Sixteen patients with chronic liver disease from hepatitis B virus infection experienced acute deterioration from a superimposed HEV infection (203). No such interaction was found among chronic HBsAg carriers in India (162).

A serological study of German patients with a history of acute hepatitis found a statistical association between the presence of anti-HEV IgG and infection with more than one hepatitis virus (213). In a Turkish population, Thomas and colleagues found (after adjusting for age, sex, location and education) that HCV seropositive individuals were nine times more likely to be anti-HEV seropositive than HCV seronegative individuals (207). This association between HCV and HEV has been reported in other populations (208;214). It is possible that this association reflects an increased risk of HEV and HCV infection in persons of lower socioeconomic status.

K. Reservoirs of Hepatitis E Virus & The Zoonotic Connection

It is not clear how HEV is maintained between epidemics. Some suggest that sporadic human cases are sufficient to sustain HEV in the population. A large number of subclinical cases may also maintain the viral reservoir, as suggested in a prospective study of Nepali soldiers and police in which the HEV infection rate was 2 to 4 times higher than the disease rate (58). Since most HEV infections are inapparent (157;158), and viremia and viral shedding have been demonstrated in the absence of clinical
symptoms or even liver enzyme responses (165;187), subclinical infections may help maintain HEV transmission in endemic areas. A small study of hepatitis E patients in India even noted viremia in the absence of anti-HEV IgM (186).

Some subtypes of HEV may also have a zoonotic epidemiology, a characteristic which sets this virus apart from other enterically transmitted diseases. Wild-caught, non-human primates have occasionally had anti-HEV IgG before laboratory inoculation. Antibodies to HEV have since been documented in domestic swine, cows, water buffaloes, sheep, goats, chickens, mongoose, rats and even domestic cats (47;48;215-217). In Somalia, Tajikistan and Turkmenistan, countries with a high endemicity of human HEV, 29-62 percent of the animals possessed anti-HEV (216). In the Ukraine, where no human HEV has been reported, the prevalence of anti-HEV in domestic 1-2 year old cattle was almost 12 percent (216). The serum of 44 to 94 percent of wild rats captured from different regions of the US contained antibodies to a human strain of HEV (218).

These animal findings are not limited to antibody reactivity; in Nepal, HEV RNA and anti-HEV were found to be highly prevalent in domestic swine populations (216;219). Wild rodents captured in the Kathmandu Valley of Nepal were also found to harbor HEV RNA with nucleotide sequence similarity to human strains from that area (220). In addition, HEV-like virus has also been isolated from Sika deer (53) and chickens (221). Some have proposed that animals may serve as reservoirs, amplifying the virus to create the environmental conditions (fecal contamination of drinking water) necessary for human infection (47). Consumption of infected animals (pigs, boar, deer) has also recently been demonstrated as a means of human infection (51;54). The list of
possible animal hosts or vectors of HEV continues to grow, and some have suggested that even insect vectors may contribute to the spread of HEV, although this has not yet been proven (33;143;188). As people in many South Asian and African settings live in close proximity to and have frequent contact with animals and use animal dung for fuel, these remain critical areas for future study.

**Swine HEV – The “Missing” Zoonotic Link**

Of particular interest as possible reservoirs of HEV, in both developed and developing countries, are domestic swine (222). A purportedly “novel” virus, identified in 1997 in the domestic swine population of the United States, was designated swine hepatitis E virus (swine HEV or HEV-S) (136). This virus, commonly present in domestic swine older than 3 months, has now been found to have identical nucleotide sequences (79-85 percent) and amino acid sequences (77-92 percent) with human HEV strains in genotype III. Early evidence prior to the separation of HEV into four genotypes, suspected a broad HEV zoonosis, as the US-1 strain isolated from an apparently autochthonous case-patient was genetically more similar to the swine HEV (>97 percent nucleotide identity in ORF1 and 2) than to other “human” HEV genotypes (134;135). HEV-S infected young pigs do not have clinically-demonstrable hepatitis, although some microscopic evidence of liver inflammation is seen (136).

In 2002, 54% of the pig herds sampled from 37 different swine farms across the US tested positive by RT-PCR for swine HEV RNA, again with a high degree of sequence homology to the two US strains of HEV, in genotype III (223). Recent studies on European swine populations have identified varying antibody prevalence to HEV genotype III (by ELISA to ORF2) in Canada (0 to 90%), the UK (85.5%), Sweden
(58%), Spain (25%) and the Netherlands (23.5%) (224;225). A 2001 New Zealand study found 91% of 22 tested pig herds were infected with HEV (226). Other isolates from enzoonotic swine populations in China (40% antibody prevalence (227)), Thailand (20-90% antibody prevalence (227)), Taiwan, Indonesia and India have clustered as subtypes of either genotype III or IV (142;228).

HEV isolated in swine is serologically crossreactive with human HEV (136), and can be detected by most assays for human HEV. Inoculation of non-human primates with swine HEV caused infection (135), but laboratory attempts to infect swine with human HEV have been unsuccessful except when GT III strains were used (229;230). As early as 1990, Balayan and colleagues were able to infect four domestic pigs (Sus scrofa domestica) with human HEV and cause jaundice, although it is unclear what human genotype was used (231). A similar finding was published nearly a decade later, comparing the pathogenesis of HEV-S and HEV Genotype III (US-2 strain) – where the human strain resulted in more severe hepatic lesions in pigs than infection with HEV-S (230).

In 2005, swine populations were studied in HEV-endemic Thailand and Mexico, where epidemic and sporadic human HEV is commonly attributed to GT I or II, respectively. The HEV isolates from 44 pigs clustered only to GT III (228). A few years earlier, Arankalle and colleagues demonstrated that in western India, where GT I outbreaks are common (Figure 4), the only circulating genotype in tested swine was GT IV (142). If all genotypes have an equal capacity for cross-species infection, one would expect some distribution of the prevalent human genotypes in tested swine. However, this evidence suggests that only GT III and IV have zoonotic potential, where pigs can serve
as a reservoir, amplifying medium and occasionally, as a source of sporadic HEV infection and illness.

**New frontiers: Avian HEV**

As recently as 2002, Huang and colleagues identified a novel virus prevalent in chickens suffering from hepatitis-splenomegaly syndrome (HS syndrome) (55). Over 35% of adult chickens tested antibody positive for this virus, across five states of the US, sampling from 76 different chicken flocks. Analyses of this new virus, designated avian hepatitis E virus (avian HEV) have revealed a 56-61% identity with known human and swine HEV (232), but it has not yet been classified under any of the four recognized HEV genotypes (233). A small prospective study of seronegative chickens in a healthy farm environment revealed ubiquitous, asymptomatic seroconversion between the ages of 12 and 21 weeks of age (233). Experimental attempts to infect nonhuman primates with avian HEV have not yet been successful (221).

More research is warranted in endemic populations to completely understand the epidemiologic role of swine or avian hosts in the cycle of HEV infections or disease in humans (47). Some researchers have suggested that in the agrarian, rural communities in South Asia and Africa where HEV is endemic, less virulent genotypes (eg. GT III) may be maintained as a zoonosis between animals and humans, resulting in ubiquitous human exposures to HEV through daily contact with virus-carrying livestock (cattle, swine) or pests (rats, cats) (194). These latter exposures may also result in a certain level of immune cross-protection from infection, or disease, from other non-zoonotic HEV genotypes (I, II), although this remains to be studied.
The zoonotic reservoir of HEV also may be important in potential iatrogenic infections occurring through xenotransplantation (95;134). If swine organs are ever used for human transplantation, the clinical implications of infecting humans with animal HEV subtypes will have to be evaluated (234). New Zealand researchers developing protocols to monitor pig herds and tissues prior to xenotransplantation have identified the need to screen for HEV as one of five critical zoonotic pathogens (235).

The presence and persistence of HEV in the environment has also not been evaluated completely. Studies have shown that in developing settings where public sanitation is inadequate, both untreated and treated water supplies may be contaminated with HEV. Two of twenty four randomly selected water sources from New Delhi, India, and three of twenty three samples from Chennai City, India revealed the presence of HEV using molecular methods (115;236). Recently, infectious HEV was transmitted to animals from raw sewage in Barcelona, Spain, a non-endemic area (237). A 2005 study found HEV to be moderately resistant to heat inactivation, requiring a temperature over 60°C for inactivation (163). Additional studies of the environmental reservoir of HEV during inter-epidemic periods would be useful.

**L. Prevention and Control**

As specific risk factors for sporadic cases are undetermined, it is difficult to identify prevention and control measures in non-epidemic situations. It is clear, though, that epidemic disease attributable to HEV is closely linked with fecal contamination of drinking water (17;70). Current data on viral shedding in stool has implications for the control of both epidemic and sporadic infection. In outbreak settings, the handling and disposal of human wastes must follow strict sanitary guidelines. In sporadic cases, the
patient's excreta must be disposed of hygienically with care taken to avoid contact with these waste products. Such measures have been successful in preventing secondary cases in outbreak situations (86). Improvements in drinking water storage, treatment and distribution should be encouraged to reduce HEV transmission (178;238). Better community sanitation and sewage management will also reduce HEV infections worldwide, especially in flood-prone areas (86). Health education about personal and environmental hygiene in high risk communities may be used to reduce the likelihood of HEV outbreaks (239). It is unclear what measures, other than careful observance of sanitary practices when handling animal feces, fomites, or raw flesh, could be proposed in light of the evidence of animal reservoirs / vectors of HEV.

It is also feasible to establish surveillance for hepatitis E, to identify outbreaks early and recommend prophylactic measures. For example, hospitals can monitor admissions of pregnant women presenting with acute hepatitis as an indicator of a potential HEV outbreak (68). It is probable that outbreaks may be prevented by adequate chlorination of water supplies that are not severely contaminated (95;161). However, when an HEV epidemic is suspected, all drinking water should be boiled or imported since chlorination alone may be unsuccessful in controlling epidemics (20;122;126). To reduce the risk of HEV infections, travelers to endemic areas are advised to practice prudent hygienic practices, including avoidance of untreated drinking water and iced beverages of unknown quality (90). The consumption of uncooked or undercooked pork, deer and other wild game, shellfish, fruits or vegetables should also be avoided (90;163). In laboratory settings, the use of iodinated disinfectants or autoclaving is believed to destroy HEV (95;163).
It remains controversial whether immune globulin (IG) prophylaxis is efficacious, even if prepared from HEV endemic populations (95). Passive immunization was not successful in preventing infection (although disease was attenuated) in two non-human primate studies (one used IG from a human volunteer with historical HEV infection, the other used late convalescent plasma from a previously infected cynomolgus monkey) (240;241). The use of IG in an HEV outbreak in India did not reduce disease rates in a controlled study (242). Furthermore, treatment of pregnant women with IG did not demonstrate a significant reduction of adverse events (243). Since there is no specific treatment for hepatitis E infections, patient management is primarily supportive (173).

**An HEV Vaccine?**

Vaccine development has been difficult due to the lack of a cell culture system for *in vitro* HEV manipulation. However, despite four identified genotypes, the existence of a single recognized serotype suggests that a broadly protective vaccine might be possible. HEV vaccine research was advanced when recombinant DNA technology was used to produce immunogenic ORF2-encoded capsid proteins in insect cells (65). In rhesus monkey experiments, recombinant protein vaccines (based on ORF2) were highly effective in preventing infection and disease from a wild virus challenge four weeks after immunization (241;244). Protection against genetically distinct strains was provided by this vaccine (244). Multiple doses of vaccine increased the protective effect of the vaccination against an HEV challenge (241). Other HEV vaccine strategies include testing recombinant, empty hepatitis E virus-like particles (rHEV VLPs), *ORF2* expression in transgenic plant systems for a potential orally ingested vaccine, and even a DNA vaccine (3;89;245).
Two candidate vaccines were simultaneously revealed by the National Institutes of Health (Bethesda, MD, USA) and by Genelabs Technologies (Redwood City, CA, USA). The Belgium-based pharmaceutical company SmithKline/Beecham licensed these vaccines against hepatitis E (80) based on baculovirus-expressed ORF2 protein from a Pakistani strain (244). In 1998, phase I trials of the NIH vaccine were successful in Nepalese and US volunteers (246;247). The results of the ensuing phase II/III trials of the vaccine have not yet been published, and have raised some controversy as they were completed in 2003 in a cohort of the Royal Nepalese Army (RNA) (248). However, preliminary results presented by Shrestha and colleagues in 2005 suggest the vaccine was well tolerated, with an efficacy of 96% (95% CI: 86-99%) after three doses (247). Questions about the duration of vaccine protection still remain, and may be answered by this long-term follow-up of vaccine trial participants. Post-exposure vaccination has not been protective against disease (244).

**M. Global Spread of HEV**

Not only is HEV an important cause of sporadic and epidemic disease in the developing world, but the widespread global mobility we enjoy today makes this infectious agent a concern for developed nations as well. Travel has increased the propensity for the rapid spread of infectious diseases; pathogens can be imported into susceptible populations, where they may easily become established and spread (86). As mentioned earlier, incident hepatitis E in persons from non-endemic countries is usually associated with a history of recent travel to endemic areas. Travelers to Bangladesh, Pakistan, Nepal, Mexico and other endemic countries have acquired HEV infections (123;150;151;231;249-252).
A 1998 molecular and epidemiological study examined HEV infections occurring in Taiwan, a non-endemic area (153). Individual histories of travel to specific HEV endemic areas (Burma, India or China) were confirmed by nucleotide sequencing and phylogenetic matching of the patients' strains to known or published sequences from those areas. This represents a novel way to conduct surveillance for new, imported strains and to better understand the global epidemiology of HEV.

As an exported disease, HEV has been carried by migrant workers to host nations. A retrospective analysis of a 1981 epidemic of viral hepatitis in Qatar found 76 percent of the 126 cases of ET-NANB to be attributable to HEV. Most were imported from the Indian Subcontinent (253). The use of multinational cohorts for international military missions has created new concerns about the spread of communicable diseases (254). It is vital to consider the consequences of importing HEV into non-endemic nations where conditions may be ideal for the spread and proliferation of an enterically transmitted pathogen (86).

The development and commercial availability of rapid, sensitive assays to detect HEV will enable physicians to ascertain the etiology of clinical hepatitis when enteric transmission is suspected (95). Recommendations have been made by many researchers for the testing of icteric patients for hepatitis E infection, when indicated by specific risk factors or travel to endemic areas (249;250). As a result of the many recent exported cases, physicians in the US and Europe as well as in developing countries are now being advised to consider HEV in the differential diagnosis of acute hepatitis (255). Guidelines for HEV sample collection and for the interpretation and reporting of clinical tests have been published (95). As the international exchange of large groups of workers,
professionals and travelers continues to occur, it is inevitable that hepatitis E disease will be seen more commonly in non-endemic areas.

Over the past decade, a number of studies have found seroreactivity to HEV among individuals without clear HEV risk factors in what are regarded as non-endemic areas (95;130;131;250;256). Among Californian blood donors, a 1.2 to 1.4 percent seroprevalence of anti-HEV antibodies was found (131). A Baltimore, Maryland, study evaluated the HEV seroprevalence in several local populations (130). An unexpectedly high prevalence of anti-HEV was found among homosexual men (16 percent), intravenous drug users (23 percent), and blood donors (21 percent). Seropositivity was not associated with either high risk sexual or drug using behavior in these populations (130). In Switzerland, researchers found anti-HEV seropositivity among 7 percent of drug users. Further research is warranted in similar populations to define issues of test specificity in these non-epidemic areas and to identify cross-reactions with other antigens (130).

**N. Conclusion**

Hepatitis E is a public health problem of global importance. Despite our discovery of the etiologic agent of enterically-transmitted NANB hepatitis, and many advances in understanding the clinical, virologic and epidemiological characteristics of HEV, a number of important issues remain unresolved. For a variety of reasons, few well-designed, age-stratified, population-based studies of HEV have been conducted to date and much of our understanding about the epidemiology of hepatitis E is based on outbreak investigations and clinical observations (58). In addition to the fact that in early
published studies, serologic confirmation of HEV infection was not available, the variety of assays used over the past decade has produced inconsistent and sometimes discrepant results.

The major epidemiologic issues to be resolved include: a) the reasons for the apparent increased morbidity and mortality among pregnant women (168), b) the reported male predominance among clinical cases, c) the higher clinical attack rates among adults in outbreaks, d) the factors which determine the persistence of protective antibodies in children and adults, e) whether minor genetic changes or subtype-specific characteristics influence HEV virulence and epidemic capacity, and f) the role of domestic and wild animals as reservoirs and intermediate hosts for human HEV. Hopefully, the epidemiologic features of this important hepatitis agent will be clarified further and the development of treatment and the release of a protective vaccine will take place in this decade as research intensifies on this important emerging infectious disease.
4. HEV in Bangladesh

A. Background

Over the past four decades, there have been numerous reports of HEV outbreaks nearly each year from Nepal and North/Western India. Despite the apparent endemicity of outbreak-prone HEV in these countries neighboring Bangladesh, there is a paucity of literature on HEV in that country, and rarely are HEV epidemics reported in Bangladesh. In the past five years, there have been only two reports of jaundice outbreaks, only one of which was a serologically confirmed hepatitis E outbreak among dormitory residents of a large national university in Dhaka, the capital city (257).

The first indication of disease attributable to an enterically transmitted non-A, non-B hepatitis virus in Bangladesh occurred in 1987 with a case series of 19 patients with acute hepatitis. Exclusion of HBV and HCV by serology and risk factors, respectively, implicated a non-A, non-B, non-C hepatitis virus as the cause. HEV was confirmed in one patient using a fluorescent antibody-blocking assay (258). In addition, subclinical HAV infections are ubiquitous among Bangladeshi children, conferring immunity from disease in adulthood. Immunoassays were not available to confirm HEV infection serologically.

In 1995, an outbreak of hepatitis E occurred in a cohort of United Nations Mission in Haiti (UNMIH) peacekeepers (86). Four Bangladeshi soldiers in their early 30's developed icteric hepatitis within a few weeks of entering Haiti. The probability that they were infected in Haiti is low, as the soldiers developed disease within four weeks of
entry into Haiti, less than the standard incubation period of HEV, suggesting exposure occurred in Bangladesh. Seven others were found to have anti-HEV IgM (86).

This outbreak (see above) precipitated a serosurvey of the entire UNMIH cohort. Approximately 100 soldiers were selected from the Pakistani, Indian, Nepali, and Bangladeshi units. The prevalence of anti-HEV IgG antibodies was among Bangladeshi troops was 27% (254). Selection bias makes extrapolation from these data difficult.

Travelers from developed countries to endemic areas have often been infected with either HAV or HEV. There are several reports of travelers to Bangladesh developing hepatitis E illness shortly after their visit (16;150). These patients have often fallen ill days after their return, with most recovering completely.

Sequential testing for anti-HEV IgM of 500 clinical jaundice patients in Dhaka found 57.6% seropositivity (n= 288). HEV was responsible for 31.4% of acute viral hepatitis patients aged 21-40, and 2.7% of patients under age 10. (MS Hassan, Personal Communication, 1999). It is difficult to evaluate these data, as a denominator of susceptibles/exposed is impossible to calculate. The age distribution matches similar case series from India and Nepal. We may only conclude that in specialized hepatitis reference clinics in Dhaka, the proportion of acute viral hepatitis attributable to incident HEV infections is high.

In early 2000, data from an advanced testing laboratory in Dhaka were shared with us. The Popular Diagnostics Center is a private fee-for-service center, used by physicians from across Dhaka. For June 1999, 32% of persons tested for anti-HEV IgM were seropositive. As in the previous example, the denominator is not clear. (Paul Law, Personal Communication, 2000). Also in 2000, a Japanese research team investigated the
etiology of 113 cases of acute hepatitis and 22 cases with fulminant hepatitis occurring in an urban population. Anti-HEV IgM was found in 35.1% of the acute cases and in 59.1% of the fulminant cases (88). Another clinical study in Dhaka found a 53% prevalence of anti-HEV IgM among individuals presenting with acute hepatitis (259).

The most recent HEV data from Bangladesh comes from two small studies published in 2002 from a large national teaching hospital in Dhaka (88). The first found a 64% HEV etiology in a series of 22 fulminant hepatitis patients presenting to the gastroenterology ward of the hospital. The second study attempted to estimate the seroprevalence of historic and acute anti-HEV antibodies in apparently healthy adult volunteers. Overall, 60% of the 273 individuals tested were anti-HEV IgG seropositive, and about 7% were subclinically anti-HEV IgM seropositive (88).

Given that Bangladesh is subjected to dramatic annual flooding and the overall sanitation conditions in both rural and urban populations are not dramatically different from neighboring villages and cities of India or Nepal, it is intriguing that the scientific literature has not reported any HEV epidemics in the past two decades.

**B. Maternal Mortality**

Although HEV data for the Matlab area is not available, a longitudinal investigation of maternal mortality from 1976 to 1985 found a high maternal mortality ratio of 5.5 per 1000 live births (4). Thirty-four percent of deaths among women of reproductive age (in the MCH-FP area) were attributed to infectious disease, 17% of which were reported as "hepatitis" (1), although the exact etiology was not determined. A 2000 study in Matlab of the association between antenatal markers and labor/delivery complications found an odds ratio of 2.16 (95% C.I. 1.24-3.75) associating maternal jaundice with delivery
complications, adjusted for other known obstetric risk factors (260). There have also been some unpublished reports of small outbreaks of jaundice, associated with elevated maternal mortality in this cohort (C. Ronsmans, Personal Communication, 1999).

C. Unresolved Questions

Population-based Epidemiology of HEV in Bangladesh

Despite the number of aforementioned studies which have suggested either directly or indirectly that HEV is an endemic pathogen in Bangladesh, no population-based studies have been done to clearly define the burden of this disease. Although it has experienced substantial improvements in many sectors over the past two decades, Bangladesh still struggles with many unresolved public health problems. The lack of resources and infrastructure sometimes exacerbates these problems or simply delays the effective deployment of solutions in rural, remote and resource-poor areas of the country. To help maximize the effective use of limited resources, it is essential to clearly define the range and population burden of infections, including those attributable to viral hepatitis.

These studies represent the first efforts to address these gaps in HEV epidemiology in Bangladesh. The initial studies presented in this series are the first to describe the burden of HEV infections in a rural population in this country, as well as population estimates of the prevalence of hepatitis B and C. This work also estimates the annual incidence of infections in a typical rural community, which helps to understand the maintenance and transmission of the virus, the rates and burden of hepatitis E illness, and also to gauge the potential for large HEV outbreaks in Bangladesh. Finally, these
studies also afforded the opportunity to explore potential risk factors for HEV infection, through a careful examination of the most severe infections – those who become ill. An understanding of the potential pathways for HEV transmission in these non-epidemic situations provides insights into how this infection and disease might be prevented not only in Bangladesh, but in much of rural South Asia.

**Virologic Characterization of HEV-Bangladesh**

With much effort, it was possible to successfully isolate HEV from a small number of viremic cases enrolled in this study. The genomic and virologic analysis is still ongoing but should enable us to phylogenetically define the HEV circulating in Bangladesh, and to examine its similarity to other South Asian strains. Given the epidemiologic differences observed between HEV in Bangladesh and in neighboring countries, the identification of strain-level differences would open new avenues for study. One could pursue the hypothesis of whether here too, more than 1 genotype, or subtypes of varying degrees of virulence circulate in this population, causing mostly sporadic and, sometimes, epidemic disease, independently (140). The genotyping of emerging HEV strains can also help track origins and establish dispersion patterns (47), all of which leads to a better understanding of how HEV has spread over much of the globe in the past two decades, and presently seems to be emerging in swine and avian populations. A better understanding of enzoonotic infections with potential for cross-species infection and disease is both timely and relevant amidst rising global concerns about zoonotic pathogens such as avian influenza.
Zoonotic HEV in a Non-outbreak, Endemic Population

In contrast to the US and Taiwan where endogenous human and swine HEV share a common genotype (GT IV), a 2002 Indian study demonstrated that two distinct genotypes co-circulate in human (GT I) and swine (GT IV) populations in western India (GT IV) (142). The US and Taiwan reported rare, sporadic autochthonous cases attributable to GT IV, whereas India frequently reports large epidemics of GT I - HEV in both urban and rural areas. Another study recently published findings from Indonesia comparing anti-HEV seroprevalence in the Muslim-majority islands of Lombok and Surabaya with the Hindu-majority island of Bali. Only the non-Muslim Balinese raise swine and eat pork, a practice prohibited by Islam. Interestingly, the anti-HEV IgG seroprevalence rates in Bali were ~20%, in sharp contrast to the 4% and 0.5% seen in Lombok and Surabaya, respectively (261). Interestingly, although Indonesia is considered to be HEV-endemic, relatively few outbreaks have been reported from this archipelago. Specimens from domestic pigs in Bali identified the HEV as GT IV, raising the possibility that zoonotic GT IV - HEV, and consequent human disease, is not restricted to developed countries such as the US, Korea or Japan.

These findings have specific relevance to Bangladesh, which although predominantly Muslim, has several indigenous tribes and non-Muslim groups which raise pigs and consume pork. These populations are largely clustered in the northern regions of the country, but in these rural areas pig farmers often roam across the countryside herding large groups of 50-100 pigs. The importance of potential swine reservoirs in the maintenance and spread of HEV in Bangladesh needs to be further explored, and the question of whether animals can serve as reservoirs for GT I needs to be resolved.
**Vaccine Effectiveness**

As mentioned earlier, a candidate vaccine has been developed and tested in an adult Nepalese population (247). The population effectiveness of such a vaccine in high-risk target groups remains to be determined. The groups which would most benefit from protection against HEV are most likely pregnant women living in endemic areas, followed by travelers to endemic areas. It is also possible that individuals entering into professions where intimate handling of livestock (eg. swine, cattle, etc) is inevitable may benefit from vaccination coverage. Of course, efforts to improve access to drinking water and sanitation must also be stressed, but the political and economic realities, combined with the many logistic challenges developing countries face make the use of a vaccine a worthwhile public health intervention.
5. Study 1 -
Seroprevalence of antibodies to Hepatitis E Virus in a representative population of rural Bangladesh
A. Abstract

Hepatitis E virus (HEV) causes a substantial burden of sporadic and epidemic disease worldwide. HEV infections result in serious morbidity and mortality, especially among pregnant women; they also cause significant economic losses in affected populations. Few population-based studies have been conducted to characterize the epidemiology of HEV. A study was conducted in a rural Bangladeshi population in Matlab to determine the age- and gender-specific population seroprevalence of antibodies to HEV. Of 1,134 specimens tested from a representative population random sample, 146 (12.9%) had an anti-HEV IgG titer above cutoff of 40 WRAIR units/ml, implying a definite past infection. Seroprevalence was lower among women (11.1%) than among men (15.0%). We found anti-HBc (hepatitis B core) in 380 of 1080 (35.2%) tested individuals, anti-HCV (hepatitis C) in 14 of 917 (1.5%) tested individuals, and anti-HAV (hepatitis A) in 116 of 124 (93.5%) tested individuals. The results suggest that viral hepatitis, especially HEV, remains an unrecognized and significant public health problem in rural Bangladeshi populations which warrants further attention.

B. Keywords

Hepatitis E, HEV, HAV, HBV, HCV, Epidemiology, Prevalence, Bangladesh, South Asia, hepatitis E virus, antibody, anti-HEV, anti-HBV core, anti-HBc, anti-HCV, anti-HAV
**C. Introduction**

Hepatitis E virus (HEV) is an emerging infectious agent, recognized globally as the leading cause of enterically transmitted hepatitis illness both in epidemics and sporadic cases (16;17). Identified as a distinct viral agent in 1991, the molecular biology and some epidemiology of HEV has been extensively characterized and described in the past two decades (47;93;95;262). Infections are primarily subclinical, with only 20-30% of cases experiencing signs and symptoms of acute viral hepatitis (including jaundice). Although the disease is self-limiting and no chronic sequelae or carrier state has been documented (17;58), the feature of hepatitis E which has drawn the most attention is the poorly understood case fatality rate (>20%) in infected pregnant women, especially in their 2nd and 3rd trimesters (60;62;263). Membrane rupture, spontaneous abortion and stillbirth, as well as increased neonatal mortality (up to 25%) are associated with HEV infections in pregnancy (62;64;168). This pathogenesis remains poorly understood.

The epidemiology of HEV is complex, and unlike other enterically transmitted pathogens such as hepatitis A virus (HAV), evidence of HEV infection only begins to appear in the second and third decade of life in endemic South Asian populations, while African studies have demonstrated antibody prevalence in young children (16;17). A wide range of seroprevalence estimates exist for the Indian subcontinent, depending on the population sampled and the assay used (Table 1). Recent evidence has implicated some HEV genotypes as primarily zoonotic, occasionally causing infection in humans through either enteric or foodborne routes (47;53;135). HEV antibodies have been found in a wide variety of domestic and wild animals, and virus has been isolated from swine, deer, and recently, chickens (53;55;223).
Although the greatest historical burden of disease has been confined to South and Southeast Asia and seasonal outbreaks of HEV are documented with predictable regularity in India and Nepal, few studies have characterized the burden of HEV infections in Bangladesh. In the early 1990’s, reports were published of travelers to Bangladesh acquiring HEV infections (150;151); this was followed by an documented outbreak of hepatitis E in a contingent of Bangladeshi UN peacekeepers (254) which confirmed that HEV was circulating in the country. Several hospital-based studies have suggested that much of the acute viral hepatitis (30-60%) in Bangladesh has an HEV etiology (88;259). Despite the absence of seasonal outbreaks as in Nepal and India, Bangladesh is considered to be HEV endemic, with seroprevalence estimates ranging from 27 to 60% (88;254).

This study represents the first ever rural community-based seroprevalence survey of HEV in Bangladesh, in a representative, random population sample. The primary purpose of the study was to quantify the burden of HEV infections by age and gender in rural Bangladesh. The data confirms that Bangladesh is indeed HEV endemic, and raises questions of whether seasonal epidemics occur, but are undetected, or whether population risk factor or HEV genotypic differences contribute to a different epidemiology from that experienced in neighboring countries.

D. Materials and Methods

Study site and Subjects

The objective of this study was to determine the age-specific population seroprevalence of antibodies to HEV in a rural population of southern Bangladesh. The study was a random population serosurvey, using a cross-sectional design. Participants
were selected from the Maternal and Child Health / Family Planning (MCH-FP) cohort of the Matlab Health Research Program of the International Center for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). The population from which the representative sample was drawn consists of 110,000 people inhabiting 67 villages, under the Matlab Health and Demographic Surveillance System (MHDSS) (10). This is a largely agrarian, homogenous rural population, representative of rural Bangladesh in general.

A random list of 1300 individuals was generated from the updated MHDSS census lists, excluding children <1 year of age, due to cultural restrictions on drawing blood from infants in this population. Two teams of field workers, each consisting of an interviewer and a phlebotomist, visited listed persons to invite their participation in the survey. Individuals not found during the first attempt were visited as many as four times to maximize enrollment. Consenting subjects (or their guardians in the case of minors) were interviewed to collect socioeconomic, basic enteric risk factors and recent morbidities. The 3½ month enrollment of participants began on December 23, 2003 and ended April 8, 2004.

**Specimen Collection**

A fingerstick blood specimen (~350μl) was collected using a specialized capillary blood collection and separation microtube system (Safe-T-Fill, RAM Scientific, Needham, MA, USA) following procedures established by the manufacturer. In brief, the technique is as follows: the fingerstick or heelstick site is warmed by gentle rubbing. The finger or heel site is cleaned using an alcohol pad. A spring-loaded 1.5mm wide Microtainer Safety Flow Lancet (Becton Dickinson, NJ, USA) is used to puncture
the skin to a depth of 2.0mm and the first drop of blood is wiped with sterile gauze. Blood is then collected by capillary action into a microtube by holding the capillary perpendicular to the blood drop. The capillary continues to draw blood, until the required volume is reached or the finger/heel stick stops bleeding. Microtubes were immediately labeled with preprinted stickers and placed in portable field coolers containing ice. Within 4 hours of collection, the microtubes were centrifuged at 4000g for 10 minutes and the supernatant serum was aliquoted into two sterile 200μl mini-Eppendorf tubes for testing and archival. Serum aliquots were stored in -20°C freezers until shipped on solid CO₂ (dry ice) to the Armed Forces Research Research Institute of Medical Sciences (AFRIMS, Bangkok, Thailand), a regional hepatitis reference laboratory.

**Laboratory Analysis**

Due to extremely limited serum volumes, specimens were tested using commercial and in-house enzyme immunoassays (EIA) for antibodies in the following priority: anti-hepatitis E virus total Ig (anti-HEV Ig), anti-hepatitis B virus core antigen total Ig (anti-HBc Ig), anti-hepatitis C IgG (anti-HCV IgG), and anti-hepatitis A total Ig (anti-HAV Ig). (Previous studies have clearly established ubiquitous anti-HAV seroprevalence by age 5 in rural Bangladesh, making this test a lower priority for the present study.) The anti-HAV assay (Abbott/Murex, Dartford, UK) is 100% sensitive and specific in identifying prior exposure to hepatitis A virus. The anti-HBc Ig assay (Abbott/Murex, Dartford, UK) identifies both past exposure to HBV and acute/chronic infection with >99% sensitivity and specificity. The anti-HCV, version 4.0, (Abbott/Murex, Dartford, UK) is a third-generation anti-HCV assay with a reported sensitivity and specificity >99% in detecting exposure to HCV. Initially reactive
specimens were tested in duplicate. Positive and negative controls were included in every EIA run.

Although commercial anti-HEV assays are available, the appropriateness of their use for epidemiologic studies is sometimes questioned (94;118). The Walter Reed Army Institute of Research (WRAIR, Bethesda, MD, USA) has developed a quantitative anti-HEV total Ig EIA which is recognized as a highly sensitive (96%) and specific (98%) assay to identify HEV infections in populations (264;264;265). The assay uses a quantitative sandwich approach to capture and label human Ig antibodies to recombinant HEV ORF2 proteins (rHEV) of the Pakistani strain expressed in the baculovirus system, and the photometric result is compared to positive controls from a positive reference pool of Nepali HEV patients, to report antibody concentrations in WRAIR units / milliliter (WRAIR U/ml). A cutoff of ≥40U/ml is used by WRAIR to classify subjects with ‘definite past infections’, and ≥500U/ml as definite acute clinical infection (K.S.A. Myint, Personal Communication, January 2005). A recent study of post-infection antibody kinetics in Nepalese patients suggests as few as 20 WRAIR U/ml can provide reliable evidence of prior infection (190), so all analyses were repeated using this newer cutoff as well.

In brief, the assay procedure involves the following steps: coating 96-well plates with rHEV antigen, incubations with blocking solution (0.5% casein and 0.5% bovine serum albumin in phosphate buffered saline, pH 7.4), test serum, goat anti-human Ig conjugated to horseradish peroxidase (HRP) and tetramethylbenzidine. There are washing steps in between each incubation. The reaction is stopped with sulfuric acid and the plates are read at 450/650 nm in a SpectraMax 340 EIA reader set for four-parameter analysis.
The instrument’s software fit a four-parameter dose-response curve to the OD results of the standards, calculate the correlation ($R^2$) between the diluted antibody standard’s measured and expected results, and calculate the antibody content of unknowns from the standard curve equation. (264;265)(K.S.A. Myint, Personal Communication, October 2006)

**Statistical Methods**

All data were entered using custom-designed data entry screens, with appropriate field range and data consistency checks, built using Visual FoxPro (Microsoft Corp., Seattle, WA, USA). All statistical analyses were performed using Intercooled Stata Version 9.0 for Windows (Stata Corporation, College Station, TX). The age distribution of the sample was verified against the mid-year Matlab HDSS census data from 2004, to determine representativeness across age categories. Seroprevalence rates were calculated based on the number of specimens meeting the defined optical density cutoff values for the commercial assays, and the WRAIR recommended cutoff of total Ig ≤ 40U/ml described above. The 5 year age category-specific seroprevalence was determined and plotted, showing the number sampled from each age group, as a reference. The 95% confidence intervals were calculated using an exact method based on the binomial distribution. The characteristics of enrolled participants were described using median and ranges for continuous variables and proportions for categorical values. Due to non-normal distributions of continuous variables, a nonparametric equality-of-medians k-sample test was used to compare age, household size and MUAC, by gender. Univariate comparison of proportions between groups was done using the Chi-squared and Fisher’s exact tests where appropriate. To estimate strength of association, odds ratios (OR) were
determined using both ≥20U/ml and ≥40U/ml cutoffs. Variables with significant univariate associations were entered in a multivariate logistic regression model. Analysis of marital status, employment, and education excluded individuals older than 15, students or children, and those under 7 years old, respectively. Age acted as an important confounder for these three variables, due to the strong negative association between young age and anti-HEV seropositivity. The combined antibody seropositivity between hepatitides was examined by $\chi^2$ analysis, examining whether there was greater occurrence of combined anti-HAV, anti-HBc, or anti-HEV than expected by chance. A $p$-value of $<0.05$ was considered statistically significant for all analyses.

All study procedures were reviewed and approved by the Committee on Human Research at the Johns Hopkins Bloomberg School of Public Health and by the ICDDR,B Ethical and Research Review Committees. Informed consent was obtained from all adult and emancipated participants; for children, the consent of the parent or guardian was sought with assent from the children.

**E. Results**

**Participation**

Of the 1300 randomly selected subjects, 1136 (87.4%) were successfully enrolled into the study. At the end of recruitment, 57 (4.4%) were not met, 70 (5.4%) migrated out of the study area, 31 (2.4%) refused to participate and 6 (0.5%) died. Only 2 specimens were inadequate for analysis. Migration out of the study area was mostly in 16-30 year olds (n=28), representing 8.3% of the number selected within that age category. Refusals were higher in number in <15 year olds (n=17), but only 3.8% of those <15 selected. Refusals were proportionately greater in the 61-75 age category (n=6 out of 89, 6.7%).
The age distribution of the final sample closely matched that of the mid-2001 Matlab population (data not shown).

**Seroprevalence**

Among the 1134 specimens, 146 (12.9%) met the criteria for definite prior HEV infection, using a cutoff of $\geq 40$ WRAIR U/ml. Using the $\geq 20$ U/ml cutoff, 225 were reactive, suggesting an anti-HEV population prevalence possibly as high as 22.5%. We found anti-HBc antibodies in 380 of 1080 (35.2%) tested individuals, anti-HCV in 14 of 917 (1.5%) tested individuals. Due to limited specimen volumes, only 124 specimens could be tested for anti-HAV, of which 116 (93.5%) were reactive (see Table 2). Among anti-HEV seropositive individuals (n=146), the median antibody titer was 81.25, and five individuals had titers exceeding 500U/ml (0.44% population prevalence), a cutoff used to identify definite acute and clinical hepatitis (data not shown) (K.S.A. Myint, Personal Communication, January 2005). These individuals were 9, 16, 16, 50 and 69 years old.

The age-specific seroprevalence increases dramatically in adolescence well into the second and third decades of life, with a peak 24% seroprevalence in the 31-35 age group (see Table 3, Figure 7). This is an unusual epidemiologic distribution for what is thought to be an enterically transmitted virus, yet has been consistently described in similar studies in Nepal and India (57;58;266). The 33% peak in seroprevalence in the 81-85 year age category is likely an artifact of the small sample (n=6) for this age category. Population seroprevalence in this sample was lower among women (11.1%) than men (15.0%); this phenomenon is largely consistent across age categories (data not shown).
As shown in Table 4, anti-HEV seropositive individuals were significantly older (mean 34.3 years, 95%CI: 31.5 – 37.2) than their non-reactive counterparts (mean 26.6 years, 95%CI: 25.4 – 27.9). In univariate analyses, detailed in Table 4, only male gender was significantly associated with seropositivity (p<0.05). Although income was initially not significantly associated with seropositivity, stratifying by gender revealed that women in the highest income class had a decreased likelihood of exposure compared to the lowest income class, adjusting for age (OR 0.37, 95%CI: 0.19-0.72).

This data did not provide sufficient evidence of an association (p>0.05, CI overlapping 1.0) between anti-HEV seropositivity and primary employment, household size, education level of the household head, average monthly income or in the previous 3 months: travel to a town or city, contact with “jaundiced” or sick individuals, or exposure to injections. Mid-upper arm circumference, a gross indicator of adult nutritional status, was not different in this sample. Among females >12 years, current pregnancy was not associated with anti-HEV, noting that there were only 16 pregnant women in the sample (8.9% vs. 4.6% pregnant among HEV seropositive and seronegative women, respectively, p>0.2).

Table 5 examines the most significant overall characteristics associated with anti-HEV seropositivity in this random cross-section of the Matlab population. Confirming the age distribution shown in Figure 7, individuals under 15 were much less likely (OR 0.22, 95%CI: 0.13-0.37) to have antibodies to HEV. The likelihood of past seropositivity increased with each age category, dropping down slightly in the oldest group. Females were less likely to be antibody seropositive, and significantly so in both the adjusted model and when a cutoff of ≤ 20 WRAIR U/ml was used to determine seropositivity.
Outdoor workers seemed to be less likely to have a history of HEV infection (OR 0.67, 95%CI:0.47-0.95). These remained significant in the adjusted models using a 40 U/ml cutoff, but the latter association was dropped when a 20 U/ml cutoff was used.

Finally, analyses of antibodies to multiple hepatitis viruses found that there was a significant likelihood of individuals having had prior exposures to both HEV and HBV, or HEV and HCV (Table 6). This dataset was not able to demonstrate any increased likelihood of individuals having antibodies to both HCV and HBV.

Interestingly, although not statistically different by seropositivity, there is a considerable amount of recent (past 3 months) underlying morbidity in this population. Over 25% of respondents reported at least one episode of high fever (45%), anorexia (35%), extreme weakness (29%), liver (or upper right quadrant) pain (29%), nausea or vomiting (26%). A smaller proportion of respondents claimed to have dark urine (14%), yellow eyes (7%) or ash-colored stools (6%) in the past three months. Women were ~30% more likely to report weakness and anorexia, and 86% more likely to report liver (upper right quadrant) pain.

F. Discussion

The data suggests a previously undescribed substantial burden of hepatitis virus infections within a rural community of southern Bangladesh, which is felt to be representative of other communities across the country. Clearly, HBV and now HEV, especially, is an unrecognized problem of national significance in Bangladesh, which likely contributes to population morbidity as well as to the high level of maternal and neonatal mortality documented in Bangladesh. The overall prevalence of antibodies to HEV using the well-established >40 WRAIR U/ml cutoff suggesting definite past
exposure was surprisingly lower than expected at 12.9%. Using the recently proposed lower cutoff of >20U/ml, the overall seroprevalence increased to 22.5%.

With the exception of a few excellent large population studies conducted in Egypt and Nepal, population-based HEV antibody seroprevalence studies of this size are rare (193). These findings confirm the findings of previous clinical studies identifying HEV as an etiologic agent contributing to substantial hepatitis morbidity in Bangladesh (86;259). The rural population prevalence is lower than the 60.1% proposed by a recent study of 273 apparently healthy adults, but similar to the 27% estimates of anti-HEV IgG prevalence in a study of 105 Bangladeshi peacekeepers participating in the United Nations Mission in Haiti (UNMIH) (254). A 1992 population serosurvey in southern India found an overall anti-HEV seroprevalence of 26% (56), whereas other India estimates range from 4% to 64%, with varying sample sizes and age ranges. Studies in Nepal estimate population seroprevalence of anti-HEV to be between 10 to 37%. These studies also contrast sharply from the much higher seroprevalence rates (>60%) demonstrated in rural Egyptian populations (193). Interestingly, despite the marked absence of large seasonal outbreaks of HEV as reported in Nepal and India each year, the population antibody seroprevalence estimates seem remarkably similar.

The age-specific seroprevalence estimates, shown in Figure 7, reflect the same population distribution (low prevalence in childhood which increases in the second and third decades of life before dropping off slightly in older adults) noted by many others in South Asian populations. The perplexing paucity of infections (in infancy and childhood) is again reflected in our sample, despite a large representation of participants ≤ 15 years of age (18/402, 4.5%). The highest age-specific seroprevalence was 23.6% in the 31-35
year category, dropping off to below 10% in some older categories. A small number of subjects in the higher age groups renders the estimation of exact seroprevalence among older adults difficult. Stratification by gender reveals a similar pattern in both male and female participants, peaking between ages age 16-40 (see Figure 8). As mentioned earlier, this epidemiologic pattern contrasts sharply with those of other enteric pathogens in developing countries, such as HAV, where antibody seropositivity appears early in life, and, by the end of childhood, most individuals have antibody evidence of exposure to HAV (56). This phenomenon has been a characteristic of both sporadic and epidemic HEV in South Asia (16;37;95;166).

The cross-sectional nature of this study predictably made it difficult to identify clear “risk factors” or associations between subject characteristics and anti-HEV seropositivity. None of the expected proxies for socio-economic status, type of employment or education (Table 4) were clearly associated with increased likelihood of seropositivity. However, as expected, strong significant associations emerged when comparing the odds of infection subjects in any 15-year age category above 16 with those aged 15 or less. The association peaked in the 30-44 age group (adjusted OR between 5.1 to 9.0, depending on which antibody cutoff was used). When the association between income and previous exposure was stratified by gender, increased

Female participants also were up to 61% less likely to have antibody seropositivity (Table 4, model 1 – adjusted ORs). A similar female-gender “protection” has been reported from HEV outbreaks in Nepal, Pakistan and India, where women were two-fold less likely to report clinical illness in outbreak situations (26;166). The opposite finding was reported from a study in Borneo, Indonesia where anti-HEV IgG
seroprevalence was significantly lower in men (47%) than in women (55%) (267). In much of rural Bangladesh, conservative norms often restrict the movement of women outside of the home or neighborhood, which may, in turn, limit the range of exposures to potential sources of HEV infection. Higher SES women (as proxied by income) were also less likely to have had evidence of prior HEV exposure, possibly indicative of improved sanitary conditions and limited exposure to environmental sources of HEV. More difficult to explain is the fact that individuals reporting their main place of work as outdoors were less likely to have HEV antibody seropositivity (Table 4, model 1, adjusted OR 0.51 - 95%CI 0.32-0.83). Perhaps the ubiquitous use of tubewells for drinking water by individuals working outdoors reduced the risk of HEV exposure, whereas indoor work may require water storage in potentially contaminated vessels. Possibly, indoor workers may access piped water in rural office settings. However, we had no data on water consumption behavior to analyze this for the baseline seroprevalence survey.

Limited access to clean drinking water, exacerbated by annual floods and combined with generally poor sanitary conditions in rural Bangladesh should all contribute to high levels of risk for enteric infections such as HEV. Although only a small number (n=124) were tested for HAV, the 93.5% anti-HAV seroprevalence (95% CI: 87.7 – 97.2%), provides strong evidence that this population is exposed to commonly understood risk factors for enteric viral infections. As many others have shown, HAV infections in developing countries are ubiquitous by age 10, and provide strong, long-term immunity (56;268). This discrepancy between the age-specific distributions of the
two enteric hepatitides has been noted in a recent HEV study of healthy urban residents of the capital city of Bangladesh (Dhaka) (88).

The sudden increase in evidence of exposure around age 15, peaking roughly around 30 is additionally difficult to explain. Other infectious agents with similar epidemiologic patterns have clear sexual risk factors, which has not been demonstrated with HEV (130;269). The role of hormonal changes in adolescence and the possibility that biochemical characteristics of the host may affect the immune susceptibility to this hepatotropic virus needs further examination. There may also be behavioral characteristics of early adulthood, such as travel outside the home or increased contact with infective livestock, which result in either increased exposure to sources of HEV or in larger infective doses being consumed. Others have demonstrated the increased propensity for clinical disease when individuals are acutely co-infected with multiple hepatotropic viruses (88;212). This finding is supported by the analysis (Table 4) showing that there are more individuals than expected by chance with prior infection by HEV and either HBV or HCV. This raises the question of whether a common set of risk factors or host characteristics exists for either infection, or whether physiologic changes induced by one hepatotropic viral insult increases susceptibility to another virus infection. Both of these require further investigation.

Like neighboring India and parts of Nepal, Bangladesh is networked with rivers which flood annually. General sanitary conditions are comparable in both urban and rural areas. Religion is one major difference between Bangladesh and its adjacent neighbors, which may be important in light of the increasing evidence that some HEV genotypes are strongly linked to animal exposures, especially pigs (47;270). As it is a predominantly
Muslim country, pigs are rare in Bangladesh except in cloistered indigenous tribal and non-Muslim communities. This is in sharp contrast to Nepal and many parts of India where pigs are bred as domestic livestock. The virtual absence of this potential HEV reservoir and possible amplifying vector in Bangladesh may be playing a role in keeping the environmental exposures low, and making it difficult to contaminate the water supply to a sufficient scale to cause large outbreaks.

We are presently exploring more data from this population, to identify rates of HEV seroconversion over a 12 month period, and also trying to identify risk factors for clinical hepatitis E illness in rural Bangladesh. The phylogenetic identity of the HEV circulating in this population is also being analyzed, as the genotype and geographic origins of HEV-Bangladesh may uncover important clues as to its epidemiology. Given the high infant and maternal mortality rates in Bangladesh, it is possible that HEV may be an appreciable contributor to poor pregnancy outcomes, deaths in late pregnancy and infant mortality in early life (4;11). As an HEV vaccine becomes available, it will be important to devise viable vaccine strategies, targeting vulnerable and high-risk groups in endemic countries.

The anti-HBc population prevalence of 35.2% represents a high rate of individuals in this rural community with historical exposure to HBV. This study, primarily focused on HEV, did not distinguish between acute, chronic or resolved infections. Previous studies of hepatitis B in Bangladesh have focused on high-risk or vulnerable populations such as drug users, pregnant women, professional blood donors and hospitalized patients (271-274). This study represents one of the first population-based estimates of hepatitis B exposure rates in rural Bangladesh. A 2002 study of
healthy adults and children reporting to a clinic for pre-vaccine or pre-employment testing reported a 21.1% seroprevalence of anti-HBc. Bangladesh is clearly highly endemic for HBV, and although ~90% of infected older children and adults are able to successfully clear infection, this represents a clear problem of public health concern. The Government of Bangladesh began phasing in the HBV vaccine into the national expanded program of immunization (EPI) in 2004, aiming to reach national coverage by 2007.

**G. Conflicts of Interest**

The authors have no known conflicts of interest concerning the work reported in this paper.

**H. Acknowledgements**

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6. Study 2 -
Incidence of Hepatitis E Virus (HEV) infections and antibody kinetics in rural Bangladeshi community
A. Abstract

Hepatitis E virus (HEV) is the most common cause of enterically transmitted viral hepatitis in the world. Most countries of South Asia have been shown to be endemic for HEV, causing either large seasonal epidemics of disease, continuous sporadic cases, or both. Although many reports suggest that Bangladesh is HEV endemic, there have been few population-based studies of the burden of HEV in this country. There have also been no prospective studies of HEV infection rates in any population in Bangladesh. A randomly selected representative cohort of 1134 healthy people from a rural community, between the age of 1 and 88 were enrolled and followed for 18 months to determine HEV antibody seroprevalence and infection rates over the period. The Walter Reed Army Institute of Research (WRAIR) immunoassay was used to establish the baseline seroprevalence of 22.5%. With only a 10.8% loss to follow-up, overall seroincidence between baseline and 12 months was determined to be 60.3 per 1000 person-years, peaking in those aged 30-60, and between baseline and 18 months, 72.4 per 1000 person years. The overall population incidence rate was calculated at 64 per 1000 person-years in this rural Bangladesh population. No significant associations were found between anti-HEV incidence and demographic or socioeconomic factors available and none of the seroconverting subjects reported illness consistent with viral hepatitis. Among seropositives at baseline, a phenomenon of seroreversion to non-reactive status was observed in 8.3% of baseline participants, 18 months after the baseline visit. This is the first study to document annual HEV infection rates in a rural Bangladeshi population of healthy participants from all age groups.
B. Keywords

Hepatitis E, HEV, Epidemiology, Incidence, Prevalence, Bangladesh, South Asia, hepatitis E virus, antibody, anti-HEV, seroreversion

C. Introduction

Hepatitis E virus (HEV) is an emerging infectious pathogen, identified less than 3 decades ago and first cloned as recently as 1991 (46;75). Every year, HEV causes significant morbidity in endemic countries and many studies have since identified it as the leading cause of enterically transmitted viral hepatitis illness globally (16). Large epidemics as far back as 1955 have now been attributed to infections by this agent (16;20). Hospital-based studies in Nepal, India and Bangladesh have suggested that HEV infections are etiologically responsible for between 10 and 95% of admitted cases of hepatitis (76;77;83;88;162;187;275).

Since its discovery, there has been considerable research on the epidemiology of HEV attempting to explain several perplexing phenomena. First among these is the apparent low rate of infection or illness in persons under 15, highly unusual for an enteric pathogen in an endemic environment where conditions for fecal-oral pathogen spread are ideal (58;81). Second is the unusually high mortality rate (up to 20%) seen in pregnant women infected by HEV, especially in their third trimester (3;17). Finally, there remain many questions regarding the human immune response to HEV infection, and what kind of immunity or even protection from infection or illness occurs in those infected by HEV (99;190).
Numerous surveys have been conducted in endemic and non-endemic countries to establish the prevalence of antibodies to HEV (16;131;276). These have uncovered varying rates of anti-HEV prevalence by region, population and endemic genotype of HEV (42). Many have also demonstrated the rapid decline of anti-HEV IgM class antibodies soon after infection, and the subsequent steady decline of anti-HEV IgG over a number of years (122;265). Studies of HEV outbreaks and acute infections in Egypt, India, and Indonesia showed that anti-HEV IgG levels dropped below seroreactive thresholds in 28-67% of cases within 6 to 24 months after infection (122;189;191). For most viral pathogens, antibody seropositivity is generally a marker of immunity to future infection and illness; in the case of HEV, the frequent, seasonal occurrence of outbreaks in supposedly endemic populations suggests otherwise.

These are compelling reasons for further study of HEV incidence and antibody kinetics in populations, in addition to the more common seroprevalence studies. However, given the difficulty of following large populations over time to quantify infection rates, there have been few longitudinal studies of HEV. Two noteworthy longitudinal HEV studies have been reported; first, a Nepalese police / army cohort of 757 individuals was followed for 12 to 19 months, from which a population incidence rate of 64 per 1000 person-years was estimated, with about 31 hepatitis E illnesses per 100 cases (58). In 2006, Stoszek and colleagues published an ~11 month study of a rural Egyptian cohort of 1919 villagers, revealing an estimated incidence of 42 per 1000 person-years in that population, accompanied by a notable absence of hepatitis illness (194).
This study follows a previously described, randomly selected, representative cohort of rural Bangladeshi volunteers for a total of eighteen months to determine the incidence rates of HEV infections, and begins to describe the persistence of anti-HEV among HEV seropositive individuals at baseline. The cohort is also followed to identify the burden of clinical illness in those who are infected by HEV.

**D. Materials and Methods**

**Study site and Subjects**

The objective of this study is to determine the age-specific population incidence of HEV infections in a rural population of southern Bangladesh. A randomly selected population sample over the age of 1 was recruited and followed at 12 and 18 months after baseline assessments. Participants were selected from the Maternal and Child Health / Family Planning (MCH-FP) cohort of the Matlab Health Research Program of the International Center for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). The population from which the representative sample was drawn consists of 110,000 people inhabiting 67 villages, under the Matlab Health and Demographic Surveillance System (MHDSS) (10). This is a largely agrarian, homogenous rural population, representative of rural Bangladesh in general.

**Baseline Census**

A random list of 1300 individuals was generated from the 2003 MHDSS census lists, excluding children <1 year of age, due to cultural restrictions on drawing blood from infants in this population. Two teams of field workers, each consisting of an interviewer and a phlebotomist, visited listed persons to invite their participation in the
survey. Individuals not found during the first attempt were visited as many as four times to maximize enrollment. Consenting subjects (or their guardians in the case of minors) were interviewed to collect socioeconomic, basic enteric risk factors and recent morbidities. The 3½ month enrollment of participants began on December 23, 2003 and ended April 8, 2004. A total of 1134 participants were successfully enrolled, interviewed and tested for antibodies to HEV.

**Follow-up of Study Participants**

At twelve and eighteen months from the baseline, the study teams again visited the 1134 baseline participants, up to five separate times each to minimize attrition. Follow-up visits were scheduled carefully to ensure that each participant visited during the four month baseline survey was visited exactly 12 months later. The same staggered scheduling was followed at 18 months, visiting individuals in the order of their baseline enrollment. Field team performance and refusal rates were tracked on a daily and weekly basis; subjects refusing follow-up participation were visited by a study supervisor to discuss questions or issues which the field teams may have been unable to address.

At these timepoints, in addition to collecting blood specimens as described below, a short questionnaire was administered to determine any changes in small number of exposures as well as record any self-reported recent morbidities. Prior to follow-up, the field teams conducted extensive visits to the communities to conduct pre-study advocacy and respond to any questions from the community. This helped significantly reduce attrition and lower refusal rates, by “priming” the community prior to the follow-up visits.
**Specimen Collection**

A fingerstick blood specimen (~350μl) was collected using a specialized capillary blood collection and separation microtube system (Safe-T-Fill, RAM Scientific, Needham, MA, USA) following procedures established by the manufacturer. In brief, the technique is as follows: the fingerstick or heelstick site is warmed by gentle rubbing. The finger or heel site is cleaned using an alcohol pad. A spring-loaded 1.5mm wide Microtainer Safety Flow Lancet (Becton Dickinson, NJ, USA) is used to puncture the skin to a depth of 2.0mm and the first drop of blood is wiped with sterile gauze. Blood is then collected by capillary action into a microtube by holding the capillary perpendicular to the blood drop. The capillary continues to draw blood, until the required volume is reached or the finger/heel stick stops bleeding. Microtubes were immediately labeled with preprinted stickers and placed in portable field coolers containing ice. Within 4 hours of collection, the microtubes were centrifuged at 4000g for 10 minutes and the supernatant serum was aliquoted into two sterile 200μl mini-Eppendorf tubes for testing and archival. Serum aliquots were stored in -20°C freezers until shipped on solid CO₂ (dry ice) to the Armed Forces Research Institute of Medical Sciences (AFRIMS, Bangkok, Thailand), a regional hepatitis reference laboratory.

**Laboratory Analysis**

Due to extremely limited serum volumes, baseline specimens were tested using commercial and in-house enzyme immunoassays (EIA) for antibodies in the following priority: anti-hepatitis E virus total Ig (anti-HEV Ig), anti-hepatitis B virus core antigen total Ig (anti-HBc Ig), anti-hepatitis C IgG (anti-HCV IgG), and anti-hepatitis A total Ig
(anti-HAV Ig). (Previous studies have clearly established ubiquitous anti-HAV seroprevalence by age 5 in rural Bangladesh, making this test a lower priority for the present study.) The anti-HAV assay (Abbott/Murex, Dartford, UK) is 100% sensitive and specific in identifying prior exposure to hepatitis A virus. The anti-HBe Ig assay (Abbott/Murex, Dartford, UK) identifies both past exposure to HBV and acute/chronic infection with >99% sensitivity and specificity. The anti-HCV, version 4.0, (Abbott/Murex, Dartford, UK) is a third-generation anti-HCV assay with a reported sensitivity and specificity >99% in detecting exposure to HCV. Initially reactive specimens were tested in duplicate. Positive and negative controls were included in every EIA run.

Although commercial anti-HEV assays are available, the appropriateness of their use for epidemiologic studies is still questioned (94). The Walter Reed Army Institute of Research (WRAIR, Bethesda, MD, USA) has developed a quantitative anti-HEV total Ig EIA which is recognized as a highly sensitive (96%) and specific (98%) assay to identify HEV infections in populations (264,264,265). The assay uses a quantitative sandwich approach to capture and label human Ig antibodies to recombinant HEV ORF2 proteins (rHEV) of the Pakistani strain expressed in the baculovirus system, and the photometric result is compared to positive controls from a positive reference pool of Nepali HEV patients, to report antibody concentrations in WRAIR units / milliliter (WRAIR U/ml). A cutoff of ≥20U/ml is used by WRAIR to classify subjects with ‘definite past infections’, and >500U/ml as definite acute clinical infection (190).

In brief, the assay procedure involves the following steps: coating 96-well plates with rHEV antigen, incubations with blocking solution (0.5% casein and 0.5% bovine
serum albumin in phosphate buffered saline, pH 7.4), test serum, goat anti-human Ig conjugated to horseradish peroxidase (HRP) and tetramethylbenzidine. There are washing steps in between each incubation. The reaction is stopped with sulfuric acid and the plates are read at 450/650 nm in a SpectraMax 340 EIA reader set for four-parameter analysis. The instrument’s software fit a four-parameter dose-response curve to the OD results of the standards, calculate the correlation ($R^2$) between the diluted antibody standard’s measured and expected results, and calculate the antibody content of unknowns from the standard curve equation. (264;265) (K.S.A. Myint, Personal Communication, October 2006)

**Statistical Methods**

All data were entered using custom-designed data entry screens, with appropriate field range and data consistency checks, built using Visual FoxPro (Microsoft Corp., Seattle, WA, USA). All statistical analyses were performed using Intercooled Stata Version 9.0 for Windows (Stata Corporation, College Station, TX). Seroprevalence rates were calculated based on the number of specimens meeting the defined optical density cutoff values for the commercial assays, and for anti-HEV, the WRAIR-recommended cutoff of ≥20U/ml was used, as described above. The age distribution of the sample was verified against the mid-year Matlab HDSS census data from 2004, to determine representativeness across age categories.

A cohort diagram of the population under surveillance, through the 12 and 18 month follow-up timepoints was produced to demonstrate cause-specific attrition at each timepoint (Figure 9). The specific seroconversion or seroreversion events experienced by
susceptible and seropositive individuals identified at baseline through to the 18 month follow-up timepoint is described in a second cohort diagram (Figure 10).

Individuals either not met or refusing participation at the 12-month follow up timepoint were contacted again at 18 months after baseline, and invited to participate, if they could be found within a maximum of 5 repeat visits to their household. Individuals were permitted to re-join the cohort, to contribute to the incidence calculations between baseline and 18 months follow up. The baseline characteristics of those lost to follow-up were compared to those remaining in the cohort and compared by $X^2$ tests or student’s t-tests, where appropriate.

Two periods of surveillance for seroincidence were defined: 1) from baseline to 12 months, and 2) from 12 to 18 months. Individuals with an anti-HEV titer below the cutoff for seropositivity (total Ig <20 WRAIR U/ml) at the beginning of either period were eligible for seroincidence follow-up. At the end of each period, individuals who were seronegative at baseline and found to have a titer $\geq 20U/ml$ were considered seroconverters. Individuals who either seroconverted or were lost to follow-up between baseline and twelve months were allowed to contribute 6 months of follow-up, assuming that they dropped out of the cohort or seroconverted, on average, at the midpoint of the study. Incidence density rates, or anti-HEV seroconversion rates, were calculated by dividing the number of seroconversions by the number of person-years observed in a given interval, and converted to rates per 1000 person-years for each surveillance period, by gender and by 10-year age category. To maintain consistency with other published literature on HEV incidence, the age-specific seroincidence was reported for 10-year age
intervals. Confidence intervals (95%) for the incidence density estimates were calculated using an exact approach based on the Poisson distribution.

Characteristics of the baseline susceptible pool were compared to seroconverters in either of the two surveillance periods using univariate \( X^2 \) or logrank tests. Differences between age-specific incidence rates were explored by logrank analysis, and the relative risk between the lowest age category and individual subsequent age categories was assessed using a Cox proportional hazards model. A survival “failure” event was defined as an HEV infection, or increase in anti-HEV total Ig \( \geq 20 \) WRAIR U/ml.

To explore other possible characteristics influencing incidence, logrank tests were used to detect significant differences between categories (religion, gender, household size, employment type, place of work, education, household head education, income, recent morbidity, recent contact with sick or jaundiced individuals, history of recent travel, history of injection use, traditional healer use, adult malnutrition (mid-upper arm circumference <22.5cm), injectable contraceptive use (women only), and pregnancy status at baseline or follow-up (women only). Relative risks (hazard ratios) were calculated using univariate and multivariate Cox proportional hazard models, adjusting for age when necessary. Analyses were limited to individuals eligible for a covariate or exposure, for example, the influence of marital status excluded individuals over 15 years old and employment excluded students and young children, and those under 7 years old were excluded from analyses involving level of education. In these three cases, the large number of young participants in the sample could easily confound the analysis by spuriously contributing to the ‘single’ marital category, ‘unemployed’ employment category or the ‘uneducated’ education category, respectively. A \( p \)-value of < 0.05 was
considered as statistically significant for all analyses and the width and proximity of the 95% confidence interval to 1.0 was evaluated for public health importance.

Seroreversion was defined as the movement of any individual from a seropositive status (anti-HEV total Ig ≥ 20U/ml) to a seronegative status (< 20U/ml). Seroreverters between baseline and the 12 month follow-up, 12 and 18 months, and baseline to 18 months were identified. Seroreversion was documented on the flow diagram of outcomes (Figure 10) and the total Ig titer value of anti-HEV was plotted by timepoint to demonstrate the gross total anti-HEV trajectory between timepoints (Figures 11 & 12). Paired t-tests were performed to examine the difference in mean anti-HEV titers among the pool of seroreverters at baseline, 12 months and 18 months.

**Disease incidence**

From August 2004 to April 2006, the population of an entire research block (block C) of the Matlab area was under active household surveillance for symptoms of acute viral hepatitis for another HEV study. Thirteen trained community health workers (CHRWs) of this block visited each family on a monthly basis and administered a detailed questionnaire to identify symptoms consistent with acute viral hepatitis. Exactly 20% of the baseline susceptible population was under this surveillance network.

At each longitudinal follow-up timepoint, participants were asked about a history of signs of hepatitis illness (severe weakness, yellow eyes or skin, dark urine, clay/ash colored stools, upper-right quadrant abdominal pain, anorexia, fever, nausea/vomiting) in the past 3 months. Due to issues of recall of these extremely nonspecific symptoms and the problem of identifying symptoms occurring together over a longer observation time with limited surveillance, this limited period was selected under the assumption of
continuous exposure and infection over the year. An earlier study in this same cohort
demonstrated a high rate of most of the above-mentioned nonspecific morbidities.
Information from both the CHRW surveillance and the participant interview was
combined to estimate possible clinical illness among seroconverters.

The proportion of individuals reporting each morbidity was compared between
seroconverters and non-seroconverters using $X^2$ tests for significance. Only morbidities
found to occur more frequently in the seroconverter group were used to define clinical
illness unique to seroconverters. The apparent to inapparent infection ratio (disease ratio)
was determined as the proportion of clinical cases per 100 seroconversions in this subset
of the total seroconversion cohort.

Baseline seroprevalence of antibodies to HAV were found to be nearly universal,
so the possible confounding of illness with hepatitis A is unlikely. Whenever sufficient
specimen was available at a given timepoint, it was also tested for hepatitis B core
antigen, to account for possible illness caused by HBV infection.

All study procedures were reviewed and approved by the Committee on Human
Research at the Johns Hopkins Bloomberg School of Public Health and by the ICDDR,B
Ethical and Research Review Committees. Informed consent was obtained from all adult
and emancipated participants; for children, the consent of the parent or guardian was
sought with assent from the children. Participants with positive antibody status to HEV,
HBV or HCV were informed about their status using ethical review committee approved
messages.
E. Results

Baseline Enrollment

Before baseline enrollment began, a total of 1300 individuals were randomly selected from the Matlab HDSS database. Between December 23, 2003 to April 23, 2004, 1136 (87.4%) were successfully enrolled into a study of viral hepatitis antibody seroprevalence. Of the 164 not enrolled in the baseline cohort, 57 (4.4%) were not met, 70 (5.4%) had migrated out of the study area, 31 (2.4%) refused to participate and 6 (0.5%) had died. Only 2 collected specimens were inadequate for analysis (see Figure 9).

Follow-Up and Attrition

At 12 months after baseline, a follow-up round was conducted between December 23, 2004 and April 28, 2005. Of the initial 1134 enrolled, 1025 (90.4%) were successfully visited 12 months later. Among the 109 subjects lost to follow-up at the 12-month timepoint, 36 (3.2%) were not met, 26 (2.3%) migrated out, 39 (3.4%) refused, and 8 (0.7%) died. Eighteen months after the baseline survey, all original 1134 participants were revisited in the order of their enrollment between June 23, 2005 and November 25, 2005. A noteworthy 1011 (89.2%) participated in the 18 month visit. Individuals who did not contribute to the 12 month follow-up (due to not being met after four repeat attempts, or having moved away from the study area, or refusing to participate) were allowed to re-enter the cohort at the 18 month timepoint. Only 975 (86.0%), however, contributed an interview and blood specimen at both follow-up timepoints. Thirty six subjects thought lost at the 12-month timepoint were either met or newly consented to participate at the 18 month visit (see Figure 9, dashed lines). Among
these 36, 7 were seropositive at baseline, and only 1 seroconverted between baseline and 18 months. These 36 individuals were allowed to return into the cohort for follow-up of their baseline serostatus, but did not contribute experience to the incidence calculations.

**Hepatitis E Antibody Incidence (Baseline to 12 Months)**

At baseline, 255 individuals were found to have an anti-HEV total Ig titer ≥ 20 WRAIR U/ml (22.5% seroprevalence of antibodies) (see Table 7). By the same criterion, a total of 879 individuals were seronegative at the beginning of the study. After 12 months of follow-up of the baseline 879 susceptibles, 24 (2.7%) were not met, 32 (3.6%) refused, 24 (2.7%) moved out of the study area, and 4 (0.5%) died. Losses to follow-up were not significantly different in age to those who remained in the sample ($\chi^2$ test, $p>0.2$), but were more likely to be male ($\chi^2$ test, $p<0.05$). Among the 84 losses, 57.1% were male, and 42.9% were female. As might also be expected, the losses to follow-up (primarily through refusals or moves out of the study area) were also significantly more likely to be working in farming or fishing, and less likely to be unemployed, or working at home.

Among the 795 on whom we had complete twelve-month follow-up, we observed a total of 49 seroconversions. With the 84 losses to follow-up and seroconverters contributing a half-year of person-time, 837 person-years (P-Y) of follow-up time were accrued between baseline and 12 months. This yielded an overall incidence density of 60.3 infections per 1000 P-Y (95% CI: 44.6 – 79.7 per 1000 P-Y) (see Table 8).
Hepatitis E Antibody Incidence (12 to 18 Months)

The 746 individuals still seronegative (anti-HEV total Ig < 20 WRAIR U/ml) at 12 months were considered susceptible, and followed for an additional 6 months. Among these, 10 (1.3%) were not met, 10 (1.3%) refused, 7 (0.9%) moved out of the study area, 2 (0.3%) died and 1 (0.1%) specimen collected had insufficient volume for analysis. This left 716 individuals with an 18-month follow-up visit. Within this cohort, collected during the monsoon season, we observed a total of 26 seroconversions over a period of 359 person-years, yielding an incidence density of HEV infection during the 12-18 month period of 72.4 per 1000 P-Y (95%CI: 47.3-106.1/1000P-Y)(see Table 8). Adding the number of events and total person-time experience between 0-12 and 12-18 months, an overall incidence density of 63.9 per 1000P-Y (95%CI:50.3-80.1/1000P-Y) was calculated (see Table 8). Individuals who reverted back to “non-reactive” status after baseline seropositivity, referred to as seroreverters, were not permitted to contribute experience to the follow-up study and are described separately, below.

Age-Specific HEV Antibody Incidence

The age-stratified analysis investigated the incidence density between baseline and 12 months by 10-year age category. This revealed a clear increase from 12.6 per 1000 person-years in those aged 1-10 to a peak 213.3 per 1000P-Y in those aged 41-50, before going back down to 97.6 per 1000P-Y among those aged 61 or older (see Table 8). This age-specific pattern, although similar in the follow-up between 12 and 18-months follow-up, was not significant, likely due to the lower number of infections (n=26), and the absence of any infections in the 41-50 age category.
An initial logrank test of HEV incidence between 10 year age categories, between baseline and 12 months was highly significant (p<0.001). A further quantification of the hazard ratio of infection by age strata, comparing each stratum to the youngest age category (0-10 years), found significantly higher risks of infection in each age category, with the hazard ratios (not shown) peaking at 15.81 (95% CI: 4.61 – 54.27) in those aged 41-50 at baseline (Table 9).

**Hepatitis E Antibody Incidence (Alternative Cutoff)**

When seroprevalence in this cohort was tested using an increased cutoff for seropositivity of ≥ 40 WRAIR U/ml, a total of 146 individuals were classified as anti-HEV seropositive at baseline. Of the 988 susceptibles remaining, a total of 896 were followed successfully for 12 months (92 lost to follow-up). Among these, 47 individuals would be classified as infections (total anti-HEV titer increased above 40U/ml) at the 12-month timepoint, yielding an incidence density estimate of 51.2 per 1000P-Y (95%CI: 37.6-68.0/1000P-Y). Between 12 and 18 months, a further 806 individuals susceptible at 12 months were followed, amongst whom only 9 infections were detected—an incidence density of 21.9/1000P-Y (95%CI: 10.0-41.5).

**Characteristics of Seroconverters and Predictors of Incidence**

Stratified analysis by gender found no significant difference in seroconversion rates between males and females between baseline and 12 months, even after age-adjustment. The incidence density among male participants (21 events over 351.5 P-Y) was 59.7 per 1000P-Y (95%CI: 37.0-91.3/1000P-Y), whereas for female participants (28 events over 461 P-Y) was 60.7 per 1000PY (95%CI: 40.4-87.8/1000P-Y).
No significant difference between incidence rates was detectable in this study by gender, religion, household size, location of primary employment or indicators of socioeconomic status (education, income, and employment type). Recent (three months prior to survey) travel to a town, or self-reported contact with a hepatitis / jaundice patient was also not significant. Malnutrition among individuals over 15, proxied by mid-upper arm circumference (MUAC, a robust estimator of gross nutritional status in adults), was not associated with increased seroconversion. Among women, incidence was not different by history of injectable contraception use or pregnancy at either baseline or 12 months.

When comparing the incidence among individuals whose secondary employment was farm work to those who worked at home or were unemployed, a significant 3.13-fold increased hazard ratio was found (95%CI: 1.40-7.00). Interestingly, individuals complaining of a recent (past 3 month) history of yellow eyes or skin were significantly associated with infection (hazard ratio 2.38, 95%CI: 1.01-5.60, p<0.05). Surprisingly, only one of the 6 seroconverters complaining of recent yellow eyes or skin had an anti-HEV Ig titer greater than 40U/ml, at 1486U/ml, consistent with recent acute HEV infection (defined by WRAIR as anti-HEV Ig titer > 500U/ml).

**Clinical Disease Surveillance**

Of the 49 seroconverters between baseline and 12 months follow-up, 33 individuals reported experiencing at least one self-reported symptom possibly consistent with viral hepatitis illness. Only one case of nonspecific illness (nausea/vomiting, fever and anorexia) was identified by the CHRW surveillance network. Many of these nonspecific symptoms (anorexia, weakness, nausea, fever, upper right quadrant / liver pain)
are commonly reported in this population (see Table 10). In both seroconverters and non-seroconverters, at least 40% complained of anorexia, 27% of weakness, over 28% of nausea, over 26% of fever, and over 27% of upper right quadrant pain; none of these symptoms were significantly different by seroconversion status (see Table 10). Dark urine and clay/ash-colored stools were less commonly reported, but also not significantly different between seroconverters and non-seroconverters. Only scleral icterus or jaundice was reported more often in non-seroconverters (p<0.05), a symptom consistent with acute viral hepatitis illness, with a total of 6 cases in the 3 month recall period. Assuming constant rates of illness over the whole year, we can estimate as many as 24 cases among the 49 seroconversions, yielding an disease to infection rate as high as 49.0 per 100 infections (95%CI:31.4-72.9).

Over the course of the 18 months, only 25 out of 577 successfully followed baseline HBV-susceptible individuals were infected by HBV. Over 18 months, only one person out of 573 tested for both antibodies was identified as having seroconverted to having both anti-HBc and anti-HEV during the period.

“Seroreversion” Phenomenon

Using a cutoff of ≥ 20 WRAIR U/ml of anti-HEV total Ig, 255 individuals were seropositive at baseline. After 12 months of follow-up, 230 were successfully revisited and 25 were lost to follow up (figure 10). At the 12 month visit, 9 (3.9%) of the 230 baseline seropositive individuals’ serum Ig titer fell below the cutoff for seropositivity. This phenomenon is referred to here as “seroreversion”. By the 18 month timepoint, although 13 more baseline seropositives were lost to follow-up, an additional 11 (5.3% of 209) formerly baseline seropositive individuals “seroreverted” (see gray boxes in figure
The mean baseline Ig titer of the first 9 seroreverters was 22 ±1.9 U/ml, with a range of 20.1 to 25.7 U/ml (see figure 11). The 18-month seroreverters had a mean baseline titer of 26.0±5.0 U/ml, ranging from 20.2 to 33.6. As shown in figure 11, most of the individuals who seroreverted after 18 months of follow-up had a significantly higher titer, on average, at 12 months compared to baseline (mean 12 month titer 32.7±2.4 U/ml, paired t-test of means: p<0.05). This is, therefore, not consistent with a steady decline in titer over time.

Among the 49 anti-HEV seroconverters identified at the 12 month timepoint, 7 (14.3%) were lost to follow-up by 18 months. Of the 42 remaining individuals in this branch of the cohort, 28 (66.7%) individuals’ anti-HEV titer dropped below the WRAIR cutoff, or seroreverted to anti-HEV negative at 18 months (see figure 12). At baseline, these individuals were considered ‘susceptible’ due to their low anti-HEV titer (mean 12.1±5.4 U/ml, range 0.1 – 19.2). At the 12 month visit, when they were considered seroconverters, their mean antibody titer was 29.7±9.4 U/ml, ranging from 20 to 54.6.

“Seroreversion” Phenomenon (Alternate Cutoff)

Using an alternative cutoff of > 40 WRAIR U/ml of anti-HEV total Ig, we decrease the sensitivity of our test to identify low-grade seroconverters, but increase specificity by excluding low-titer individuals exposed in the distant past whose present titer is fluctuating near the 20U/ml cutoff. Using this definition, we identify 146 baseline seropositives. After 12 months, 129 were still in the cohort of seropositives, in which 5 individuals (3.9%) seroreverted to a titer below 40U/ml. By the 18 month timepoint, 7 of the 124 remaining 12-month seropositives were lost to follow-up, but an additional 12 (10.3% of 117) baseline seropositive individuals “seroreverted”.
Using the 40U/ml cutoff, among the 47 anti-HEV seroconverters identified at the 12 month timepoint, only 1 (2.1%) person was lost to follow-up by 18 months. Similar to what was seen using the 20U/ml cutoff, of the 46 remaining, 33 (71.7%) individuals’ anti-HEV titer dropped below 40U/ml, or seroreverted to anti-HEV negative at 18 months.

**F. Discussion**

The baseline 22.5% seroprevalence of total Ig antibodies to HEV may seem low in this rural, enteric-disease prone population of rural Bangladesh. However, in comparison to the rates of HEV seroprevalence in neighboring South Asian countries, this overall seroprevalence is quite similar. Cross-sectional studies from Nepal and India have proposed seroprevalence rates between 4 and 64%, with an average near 20% (16;56), with the caveat that many of these samples were not representative of the population they were drawn from.

This study represents one of the largest longitudinal population cohorts followed for HEV infection over an 18-month period. The low attrition rate of the overall cohort is noteworthy, as over 89% of those enrolled at baseline were met at the 18 month visit. Among the 9.6% lost from the incidence cohort between baseline and 12 months, the significant loss of males, and those working in farming and fishing is worth consideration. Previous studies of the seroprevalence of HEV in this cohort have also identified an increased likelihood of anti-HEV in males than females, across all age categories. The HEV incidence in certain age categories or by gender might be underestimated if these individuals, due to exposures linked to their age, gender, or professions were at increased risk for infection. We may also have been less able to
identify “risk factors” for incidence due to these losses. However, as the role of age remains of critical interest in HEV epidemiology, it is important that this cohort’s age distribution is not different from that of the Matlab population, with over 40% under 20, enabling a confident reporting of infections in younger age categories in this population (Table 8) (1).

The incidence density over the first year of 60.3 per 1000P-Y represents a high rate of infection, nearly identical to the results of the Nepalese cohort followed between 1992 and 1993 (64/1000P-Y) (58). This is somewhat unexpected, given the sharp contrast between the profile of HEV in Nepal, where near-annual epidemics attributable to HEV are reported, and in Bangladesh, where HEV is only just being recognized as an etiologic agent for sporadic or hospital-based cases (81). As the paucity of HEV data from Bangladesh may be due to underreporting or surveillance bias, the additional 12 to 18 month surveillance round was conducted to capture a second monsoon rainy season, in the event of a potential rainy-season HEV outbreak, as frequently seen in Nepal (58). Although the estimate of seroincidence was slightly higher in this period, at 72.4/1000P-Y (Table 8), this was not significant.

Ten of the 26 “seroconversion” events in the 12-18 month period, identified using the 20U/ml cutoff, had 12-month anti-HEV Ig titers over 15U/ml. Possibly, these individuals may not have been true seroconverters, but rather previously infected individuals who experienced an antibody decline to below the WRAIR-recommended “protective” level of 20U/ml (190). The seroincidence analysis was repeated using a higher cutoff (40U/ml) to examine this phenomenon more closely. This revealed a baseline to 12 month seroincidence (51.2/1000P-Y) quite similar to that estimated using
the 20U/ml cutoff. The 12-18 month estimate, in contrast, was only 21.9/1000P-Y, with only 9 seroconversions detected.

Studies of HEV antibody seroepidemiology in HEV endemic populations, especially in South Asia, report a perplexing infrequency of antibodies in children (17;120). This contrasts sharply with the pattern observed for antibodies to hepatitis A in rural populations of this region, where exposure and subsequent immunity to HAV infections is often nearly ubiquitous by age 10 (17;88). Despite the large number of studies which have examined seroprevalence in pediatric and adult populations, there is little data available on the age-specific incidence of HEV infections, especially in younger children. The observation of low numbers of pediatric cases seen during large HEV outbreaks further strengthened the suggestion that children were less likely to be infected than adults in endemic communities (18). The age-specific HEV seroincidence seen in this cohort confirms the proposed predilection of HEV to infect proportionately more individuals beginning in the third decade of life. The low incidence rate estimates in children nearly doubles in the 11-20 and 21-30 age groups (Table 8). Among those individuals aged 31-60, nearly 10-20% of susceptibles were infected over the one-year period. Regardless of which anti-HEV cutoff was used to determine seropositivity (20 or 40 U/ml) HEV incidence increased by age strata (data not shown).

Except for the important influence of age on incidence, no other individual characteristics were found to be significantly predictive of HEV infection (Table 9). This is not surprising given the limited number of infections observed in this cohort and subsequent low statistical power to detect any existing differences. The household-level monthly surveillance of 20% of the cohort was not able to capture any clear hepatitis-like
illness in the interim period between baseline and 18 months. Among the self-reported recent illnesses, only yellow eyes emerged as clearly associated with seroconversion (Table 10), leading to an estimated disease to infection rate between 31 to 72 per 100 infections (~49 / 100 infections).

We tried to limit the potential for recall bias discussed by others in limiting the recall period to 3 months prior to the interview with subsequent extrapolation, but in doing so we may have overestimated the disease rate (58). The assumption that infections are constant throughout the year, and the fact that at least 5% of non-seroconverters also reported scleral icterus/jaundice may also contribute to disease rate overestimation. There was, however, no evidence of any seasonal outbreak of hepatitis illness despite the careful community-based hepatitis surveillance of over 23,500 individuals during the year in which this seroincidence study was conducted (data not shown). The lower end of this study’s estimate (31/100) is identical to the disease to infection rate reported in the Nepal cohort (58). Stoszek and colleagues reported a slightly lower incidence in rural Egypt (~42/1000P-Y), but were unable to identify any cases of clinical illness among the 34 seroconverters followed for nearly a year (194). As reported in a previous study of this cohort, there is a substantial burden of underlying illness in this population ranging from high rates of fever and anorexia to weakness and nausea (Table 10). This is not surprising, given the ~20% rate of malnutrition (Table 9) and known infectious disease burden common to rural Bangladesh. It is possible, then, that the underlying morbidity in this population resulted in our overestimating the disease rate, the profile in this community may also be one of subclinical infections without illness, as seen in Egypt.
The most puzzling observation in this study remains the phenomenon of “seroreversion” observed. Although WRAIR now recommends a cutoff of 20U/ml (previously 40U/ml) to determine past history of HEV exposure and immunity, it seems that the kinetics of anti-HEV in humans still deserves further exploration. Given that the exact time of initial infection is unknown in the cohort of baseline seropositives, it is difficult to explain how 4-6% of seropositive individuals can, within only 12-18 months, fall to a total Ig titer below the cutoff for seropositivity. This phenomenon is not novel, as others have described anti-HEV IgG becoming undetectable in 28-67% of newly infected individuals within 6 months to 15 years, in populations from Egypt, Indonesia and India (122;189;191). A recent study in 8 southern Chinese communities followed over 3,400 people for 12 months to document seroconversion; the authors also noted a similar phenomenon (they referred to as “negative seroconversion”) occurring at an overall rate of 1.4% -- as high as 18.8% among children under 5 and between 0 and 3.6% per year among adults, depending on the age category (2).

However, the 67% rate of “seroreversion” among the 42 individuals who were identified as new seroconverters at the 12-month timepoint raises questions about whether these individuals represented true anti-HEV sero-naïvety at baseline, or whether they were previously infected and their 12-month “spike” in titer was a response to a reinfection. The baseline titers of these 28 seroreverters ranged from .1 to 19.2 U/ml, with a mean of 12.1 U/ml. The fluctuating trajectory of antibody titer shown in Figures 11 and 12 illustrate this phenomenon, suggesting that reinfections may be misclassified as novel infections, leading to overestimates of incidence rates. Figure 12 follows the anti-HEV titer of the 28 allegedly sero-naïve individuals from baseline to 18months, and
demonstrates what might very well have been a series of immune-boosting re-exposures in the 0-12 month period, rapidly declining back to a “seronegative” status once the exposure subsided.

Evidence of immune fluctuation, rather than consistently waning antibodies over time, may also explain seasonal epidemics in endemic populations like Nepal or parts of India; against a backdrop of continuous, fluctuating levels of environmental HEV exposure, antibody titers may respond according to the level of the insult. As dose increases, so does antibody response. A study of acute HEV patients (identified by viremia, fecal shedding and symptoms) in Nepal was unable to detect antibody in ~20% of cases, leading to a suggestion that some infections may not even trigger an antibody response (143). Similar findings had also been reported earlier (277), although questions were raised about the ability of the assay used to detect that genotype of HEV. This study, fortunately, uses an assay that is recognized as highly sensitive and specific as well as cross-reactive against known genotypes of HEV (94;190).

Some have proposed that hepatitis E illness itself is immunopathologic (17;95). Conceivably, then, only when the environmental dose is sufficiently elevated (compared to “normal” environmental levels of HEV) would we see clinical illness in response to a sharp rise in boosted, anamnestic antibody levels. In animal models of HEV, the production of disease can require challenge doses over a thousand times greater than that required for infectivity (164). In a riverine ecology like much of rural Bangladesh, where annual floods are predictable and sanitation is generally poor, continuous environmental exposure to HEV is quite conceivable.
These observations have important implications both for HEV vaccination and for the estimation of populations at risk of sporadic disease and epidemics. If HEV infection is not sufficient to protect individuals from future infection and illness as suggested by some (85;241), further examination and long-term follow-up of incident infections in endemic populations is warranted.

Finally, the fact that both antibody seroprevalence and infection rates are low in this cohort’s children, theories of infection early in infancy, followed by rapid antibody decline below the threshold of antibody detection / clinical illness are difficult to support (20). It is more likely, at least in this population, that 1) the increase in age-specific incidence is due to a dramatic change in exposures in early adulthood or 2) the dose of HEV to which young children are exposed is insufficient to trigger either a detectable immune response and/or illness. The role of genotypic variation also deserves closer attention and presently genotype analysis of HEV isolated from this cohort is ongoing and may shed additional light on these epidemiologic observations.

Although there is no evidence of long-term sequelae of HEV infections, a continued elucidation of HEV epidemiology and the long term kinetics of antibodies to this virus is important (17;190). A 1999 study of the socioeconomic costs of hepatitis E in the Kathmandu Valley found that this disease costs wage earners about 19% of their yearly income (67). Data on the population burden of HEV in rural Bangladesh is not available and considering the ~146 million population, ~70% of whom are rural inhabitants, it is important to clearly define the burden of this potentially costly infection in this context.
G. Conflicts of Interest

The authors have no known conflicts of interest concerning the work reported in this paper.

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7. Study 3 -
Risk factors for hepatitis E disease in rural Bangladesh
**A. Abstract**

Hepatitis E virus (HEV) is the major cause of epidemic and sporadic hepatitis illness globally. Although studies of outbreaks have clearly demonstrated the association of epidemic HEV with dramatic fecal contamination of drinking water supplies, how HEV is maintained in a community between epidemics is less evident. In contrast to the epidemiology of HEV in Nepal and India, where HEV epidemics and sporadic disease co-exist, communities in rural Bangladesh have been identified as HEV-endemic without any evidence of HEV outbreaks. Still, a substantial burden of sporadic hepatitis E illness has been reported from both urban and rural areas of the country. Aside from the widespread problems of poor sanitation and unclean water, the specific risk factors which lead to individual HEV infection and disease are unknown.

A community surveillance system for hepatitis illness was implemented in a southern Bangladeshi community of 23,500 people to identify incident cases of acute hepatitis E disease over 22 months. A score-based hepatitis illness algorithm was used to identify 279 potential cases, among which 46 were acute HEV infections. An exploratory nested case-control study compared these cases to healthy, age-matched, sero-naïve controls to identify putative risk factors for disease (socioeconomic status, travel, water use, domestic and wild animal exposures, parenteral exposures and hygiene behaviors). Nearly 70% of cases were over 15 years old.

Female gender was protective (OR:0.34) against hepatitis E disease, probably due to social restrictions on movement and outdoor employment. Measures of SES were not significant predictors of hepatitis E disease, although outdoor employment, work outside the home, and travel to a town or city were significant risk factors for hepatitis E illness.
Self-reported contact with a “jaundice” patient in the 3 months before illness was statistically associated with incident hepatitis E disease, a finding not previously shown in epidemic situations. The use of sanitary latrines in the home was highly protective (OR:0.28). No increased HEV risk was found between water use patterns or livestock / poultry ownership and associated exposures. An unexpected significant risk (OR:15.50) of injection exposure in the 3 months prior to hepatitis E disease was detected.

This study revealed a number of putative risk factors for incident hepatitis E disease associated with poor sanitation at the household level. Similar to tourists visiting endemic countries, susceptible individuals from within these communities seem to be at increased risk of hepatitis E disease when exposed to sources of HEV, especially during travel to urban areas or when doing outdoor work, in which cases eating or drinking in public establishments is inevitable. This study also raised the possibility of parenteral and person-to-person transmission of HEV in non-epidemic, sporadic disease settings. Most interesting was a suggested association of hepatitis E disease risk and the daily consumption of large amounts of water, which suggests a possible dose-response relationship may be necessary to cause hepatitis E disease instead of simply subclinical infection.

**B. Keywords**

Hepatitis E, HEV, Epidemiology, Hepatitis Illness, Bangladesh, South Asia, hepatitis E virus, antibody, anti-HEV, risk factors
C. Introduction

Hepatitis E virus (HEV) is an RNA virus, now identified as the viral agent responsible for large epidemics of non-A, non-B hepatitis in South Asia even prior to the first published report in the mid-1950’s (20). Today, HEV is recognized as the leading cause of enterically transmitted hepatitis illness both in epidemic form and as the cause of sporadic disease across much of the developing world, especially in countries where community sanitation is poor (17). Although initially thought to be limited in endemicity to developing countries, the geographic distribution of HEV now includes many countries in Europe as well as the United States, albeit in a primarily zoonotic variant, genotype IV, mainly circulating in swine populations (47).

Studies of HEV infection have revealed that only 20-30% of all infections result in clinical symptoms (58). Even then, hepatitis E disease is primarily self-limiting and does not result in severe long-term sequelae (95). The consequences of HEV infections in pregnancy are severe, and have been associated with high case fatality rates (up to 20%), the pathogenesis of which is not entirely clear (3;17;166). A vaccine for HEV has been developed and tested in clinical trials, but has not yet been released for general use (247).

Over the past two decades a large number of well-documented and careful studies have been conducted of HEV epidemics and many seroepidemiologic and cross-sectional studies have assessed the prevalence of antibodies to HEV in countries from Latin America to Southeast Asia. Both large-scale and local outbreaks of hepatitis E are often clearly preceded by a dramatic water contamination event leading to the transmission of high levels of HEV through drinking water (70;166). This either occurs by a) accidental cross-contamination between sewer and water supply lines, b) during seasonal flooding
(common in South Asia) or c) when water supply infrastructures are dilapidated and permit the influx of raw sewage or fecal contaminants in both wet and dry seasons (24;84;278).

Corwin and colleagues summarized several epidemiologic studies conducted in southeast Asia which attempted to describe risk factors for increased HEV seroprevalence and sporadic disease (279). These included outbreak investigations, cross-sectional prevalence studies and hospital-based case control studies. The disposal of human waste in rivers, which are also used as a source for drinking, washing and cooking were found to be most significantly associated with an increased population prevalence of antibodies to HEV (279). Other possible risk factors range from monsoon flooding of homes, unhygienic personal or household behaviors, to livestock ownership or contact with raw animal products and/or excrement. The role of rats, which have been demonstrated to have antibodies to HEV, is unclear in the risk profile for sporadic hepatitis E disease. (16;184) Studies of HEV in travelers nearly always reveal a history of visiting an endemic country prior to infection (150;151). During the visit the individual probably unknowingly consumed infected food or water, resulting in infection and subsequent clinical illness (280).

It remains unclear, though, how HEV is maintained in a population in non-epidemic settings, or even between outbreaks, and what factors predispose individuals to be infected by HEV and, subsequently, progress to disease. An exploratory nested case control study, with longitudinal community-based surveillance for acute hepatitis illness, was undertaken to identify potential risk factors for sporadic hepatitis E disease in a rural Bangladeshi population. The objective of the study was to compare cases of hepatitis E
disease to healthy sero-naïve controls in an attempt to identify any clear putative risk factors (socioeconomic status, travel, water exposures and use patterns, domestic and wild animal exposures, parenteral exposures and hygiene behaviors).

In animal models of HEV, the manifestation of clinical signs is dose-dependent and may require over a 1000-fold difference in the challenge doses compared to that sufficient for infection (164). This observation is also supported by a disease to infection rate as low as 28% (58). If clinical disease in humans also represents the most severe exposures to sources of HEV, the proposed study hoped to draw out the most dramatic potential sources of “sporadic” HEV and potential risk factors for infection in this unique HEV-endemic community, in which no outbreak of the disease has been documented in over 30 years.

**D. Materials and Methods**

**Study site and Subjects**

The Matlab Demographic and Health Surveillance System (MDHSS) cohort of the International Center for Diarrheal Disease Research, Bangladesh (ICDDR,B) is an ideal setting to conduct such a nested case-control study, with prospective case detection, given the existing infrastructure of a decades-old demographic surveillance system. The research area of Matlab covers approximately 147 villages -- a contiguous, rural population near 210,000 (1). Since the late 1970’s, this carefully enumerated population has been under various forms of demographic and health surveillance by a network of trained community health workers, intimately familiar with the communities under their care (11). Although the Matlab population has been exposed to a number of public health
interventions over the past decades, several statistical and demographic indicators support
the assumption of generalizability to the country at large; these include the age structure,
adult literacy, maternal mortality and total fertility rates (10). The causes of deaths among
adults in Matlab are similar to those outside the study area. However, mortality of
children under five (86 per 1000 live births) is significantly lower than the rates seen at a
national level (116 per 1000 live births), due to aggressive immunization strategies and
active health surveillance (10).

In this Matlab cohort, trained community health research workers (CHRWs) visit
every household in the research area and record vital events (births and deaths) and
migration once per month (5). In the area of Matlab under active demographic
surveillance, there are about 57 CHRWs (about 1 per 1800 people) who make monthly
visits (every 28-30 days) to each household. A CHRW covers between 15 to 20
households per day (1).

A 22-month prospective surveillance system was implemented in one of the
sectors of the Matlab study area to identify incident cases of acute hepatitis E illness. An
entire study block of the MHDSS area was selected as the candidate area for hepatitis
illness surveillance based on ease of access for study teams based in the Matlab Hospital
complex. (A prior HEV seroprevalence study in this population found no difference in
anti-HEV seroprevalence between the four MHDSS blocks.)

The objective of the surveillance was to identify, soon after the onset of clinical
signs and symptoms, the community-based incident cases of sporadic hepatitis E disease
and compare the characteristics of individuals who experienced acute clinical hepatitis
illness to age-matched individuals from the same community without a history of symptoms or anti-HEV seropositivity.

**Developing a Surveillance Instrument**

Although sign and symptom-based acute hepatitis screening has been used in hospital-based settings or by clinically-trained field workers to identify incident hepatitis cases (Fix A., Personal Communication, May 2000), we were challenged to develop a locally appropriate screening tool which minimally trained community health research workers (CHRWs) could use to identify cases of acute hepatitis illness.

Three rounds of month-long field testing were conducted to develop and refine a basic scoring method based on clinical signs and symptoms used to identify acute hepatitis in HEV studies in Egypt and Nepal (187). These included specific signs and symptoms of hepatitis infections such as jaundice, scleral icterus, hepatomegaly, dark urine, clay/ash colored stools, right upper quadrant (RUQ) abdominal pain or liver tenderness, as well as non-specific symptoms such as anorexia, nausea or vomiting, weakness/lassitude, malaise and fever, also commonly associated with acute hepatitis infections. A visual card, depicting a patient with scleral icterus, jaundiced skin, and containing a range of color bands to confirm reports of “tea-colored urine” or “clay-colored stools” was developed to accompany the closed-ended scoring questionnaire used by the CHRWs (Figure 13). As the word “jaundice” was found to be commonly used in this community, the association of this term (as it is understood locally) with the above signs and symptoms of hepatitis illness was investigated, in case it could serve as a means of identifying cases.
From February to March, 2004, in the first two months of field testing, 4 CHRWs covered approximately 360 families each, over a period of 4 days using a broad instrument designed to test and refine the language of the screening tool, explore the frequency of morbidities and explore the sensitivity of the locally used terms “jaundice”, interchangeable with two Bangla terms “Holde Palong” and “Maitha Palong”, as a proxy for hepatitis-like illness. From May to June 2004, the field testing was expanded to 5 CHRWs over a period of 15 days, again covering about 360 families each, to further refine the scoring algorithms, weighting signs and symptoms more specific to clinical hepatitis. During this period, supervisors and study medical personnel visited each of the identified individuals with morbidities to validate the responses recorded by the CHRWs. (The local terms, along with self-reported RUQ pain, were removed from the algorithm as these were found to be insensitive in identifying true hepatitis illness, and were over-reported along with other non-specific symptoms.) Fever and severe weakness were combined to form a single category, to help reduce non-specific reporting. The final pretest involved 10 CHRWs from mid-June to mid-July 2004. This phase tested the functionality of the final screening instrument, visual card and the case alert system, through which the study team was notified of a potential case for investigation.

The final screening algorithm (Table 11) involved a point-based system to triage individuals most likely to be cases of acute hepatitis E illness. In the interval since the CHRW’s prior visit (30 days, on average) individuals reporting nausea or vomiting were given 1 point, fever or lassitude (such that normal work is impeded) – 1 point, anorexia (or repulsion by the smell of food, a symptom which many of our physician-confirmed
acute hepatitis cases reported) – 2 points, yellow eyes or yellow skin – 2 points, and dark (tea-colored) urine or ash/clay colored stools – 2 points.

This algorithm was incorporated into bound surveillance logbooks, preprinted with household numbers, valid for a period of three months. Every three months, a new surveillance logbook was provided to each CHRW participating in the hepatitis illness surveillance.

**Hepatitis Disease Surveillance**

As part of several ongoing demographic and health surveillance activities, CHRWs are required to visit every household in their catchment area once every 30 days. The hepatitis surveillance activities were added on August 22, 2004 to the ongoing CHRW activities. During a monthly household visit the CHRW identified a key adult respondent, normally the wife of the household head, whom she would ask whether any member of his/her household had experienced any of the five sets of signs or symptoms described above in the past 30 days. When asking about yellow eyes or skin and dark urine or clay-colored stools, the CHRW followed up any positive response with a probe using the visual card (Figure 13). Any non-zero score was recorded in the surveillance logbook, and the total score was calculated for each person with at least one morbidity. Individuals under 1 year of age were not eligible for enrollment into the study due to cultural restrictions on blood drawing as well as the limited value of data from that demographic in this exploratory study.
Potential Cases

The objective of the surveillance was merely to identify acutely ill individuals with hepatitis-like signs and symptoms for eventual anti-HEV screening. Over the course of the 22 months, in response to a lower-than-expected “potential case” load, three different criteria were used to increase the number of possible cases. Initially, from round 1 to 7, individuals with an illness score of 6, with symptoms onset within 14 days was selected for antibody screening. From round 8 to 11, the illness score cutoff was lowered to 5 in an attempt to identify more “potential cases”. Finally, from rounds 12 to 22, the window of time since the onset of symptoms was increased to 30 days, keeping the score cutoff at 5.

When a candidate “potential case” was identified by a CHRW, she filled out a notification card, containing the candidates HDSS identifiers, score and duration since onset of symptoms. The notification card was dropped off at a local ICDDR B health subcenter, from where a study bicycle messenger would pick up the card and deliver it to the study supervisors. The potential case was logged, and a two-member investigation team comprising an interviewer and a phlebotomist was dispatched to the household within 48 hours of notification. After reviewing the individual’s symptoms again to confirm the illness score and onset time, the study team pre-enrolled the potential case through an informed consent process.

Case Pre-enrollment and Confirmation

Pre-enrollment involved the drawing, by fingerstick, a small amount of blood as described below. This specimen was processed in the Matlab hospital laboratory, and a
30 ul aliquot of the supernatant serum was transferred on ice to the Molecular and Serodiagnostic Unit, Laboratory Sciences Division, ICDDR,B (Dhaka) for testing by commercial enzyme immunoassay (EIA) for anti-HEV IgM (Molecular Biological Service-M.B.S. S.R.L., Italy) and anti-HAV IgM (DiaSorine, Italy) for individuals aged less than 10. This represented the first-tier case screening (Figure 14).

If the anti-HEV IgM result was positive, the individual was confirmed as a tentative case and the study team scheduled a final visit to collect a detailed exposure and history questionnaire. Definitive cases were those who, after identification by the hepatitis disease surveillance network as acutely ill, had a second tier (see Figure 2) screening confirmed infection by the Virology laboratory of the Armed Forces Research Institute of Medical Sciences (AFRIMS), a regional reference laboratory for viral hepatitis, in Bangkok, Thailand. An AFRIMS anti-HEV IgM titer ≥ 100 WRAIR U/ml, or an AFRIMS anti-HEV total Ig titer ≥ 1000 U/ml was considered confirmation of an acute HEV infection. Any cases identified as acute infections by the AFRIMS assay but missed by the first-tier commercial assay were re-visited by the study team, who attempted to enroll them into the study.

**Control Selection**

For each first-tier confirmed case, four age-matched controls were selected from the Matlab HDSS population database immediately after the case was confirmed (incidence-density sampling). A list of eight possible age-matches was provided to the study supervisors, who were visited in the order selected. The principal reason for the age matching is that we anticipated, based on published literature from Nepal and India, that the age distribution of cases would be different (older) from the age distribution in this
population. (Matching also controlled for age as a potential confounder and as a precise matching criterion (year of birth) was used, the result is similar to that if the groups had been exact-frequency matched.)

Controls were visited and, if consenting to participate, screened using a brief interview. Any control reporting having prior jaundice or hepatitis illness was automatically disqualified from enrollment. Furthermore, any history of illness consistent with acute viral hepatitis (using a similar scoring system to the Illness Score -- Table 11) resulted in disqualification. The purpose of control screening was to limit the control pool to individuals who were ‘at risk’ of infection, by absence of prior hepatitis illness. For every case, age-matched controls were visited and screened until four controls were successfully recruited. After recruitment, each control was interviewed with the same risk and exposure assessment instrument used for cases. A small fingerstick blood specimen was also collected from each potential control, which was tested at AFRIMS, as described below, for anti-HEV total Ig. Potential controls with evidence of prior HEV infection (AFRIMS recommended cutoff of ≥ 20 WRAIR U/ml) were ultimately removed from the control pool. As it is not clear whether circulating anti-HEV Ig protects against infection or illness, previously HEV-exposed controls may have a different risk of illness compared to completely sero-naïve controls.

**Specimen Collection**

A fingerstick blood specimen (~350μl) was collected from consenting and eligible cases and controls using a specialized capillary blood collection and separation microtube system (Safe-T-Fill, RAM Scientific, Needham, MA, USA) following procedures established by the manufacturer. In brief, the technique is as follows: the
fingerstick or heelstick site is warmed by gentle rubbing. The finger or heel site is cleaned using an alcohol pad. A spring-loaded 1.5mm wide Microtainer Safety Flow Lancet (Becton Dickinson, NJ, USA) is used to puncture the skin to a depth of 2.0mm and the first drop of blood is wiped with sterile gauze. Blood is then collected by capillary action into a microtube by holding the capillary perpendicular to the blood drop. The capillary continues to draw blood, until the required volume is reached or the finger/heel stick stops bleeding. Microtubes were immediately labeled with preprinted stickers and placed in portable field coolers containing ice. Within 4 hours of collection, the microtubes were centrifuged at 4000g for 10 minutes and the supernatant serum was aliquoted into two sterile 200μl mini-Eppendorf tubes for testing and archival. For potential cases, a 30ul aliquot was sent in a specimen thermos, on ice, to the Molecular and Serodiagnostic Unit, Laboratory Sciences Division, ICDDR,B, in Dhaka. These arrived in Dhaka within 8 hours of shipment, at which time they were frozen at -20°C or processed immediately. Remaining aliquots of case and control sera were stored in -20°C freezers until shipped on solid CO₂ (dry ice) to AFRIMS (Bangkok, Thailand) for definitive anti-HEV testing.

**Laboratory Analysis**

*First Tier Screening*

Before subjecting potential cases to an extensive interview process and recruiting matched controls, a small aliquot of serum was tested by the Molecular and Serodiagnostic Unit of the Laboratory Sciences Division (ICDDR,B). This Unit has over three decades of experience in microbiology and molecular biology and the laboratory
performance is validated annually against international benchmarks. The Medical Biological Service (M.B.S. S.R.L, Milan, Italy) commercial anti-HEV IgM EIA (test kit ref. 1056) was used as the “first tier screening” to detect IgM antibodies to HEV. This first tier screening was necessary for the expedient enrollment of potential cases, before final status confirmation from AFRIMS was available.

In brief, the MBS kit provides 96-well microplates, coated with HEV-specific immuno-dominant synthetic antigens, from conserved regions of HEV. After incubation and antigen capture of any antibodies to HEV, bound IgM are detected by anti-human IgM antibody, labeled with horseradish peroxidase (HRP). A chromogen substrate solution is added to create a photometric reaction proportional to the amount of anti-HEV IgM antibodies in the sample. The test claims a sensitivity of $\geq 98\%$, when compared to a panel of seropositive samples detected by an unnamed FDA-approved kit, and a specificity of $\geq 98\%$ against a panel of negative samples, also selected by an FDA-approved kit. Known positive and negative controls are built into the test for calibration and validation of the assay. Individuals testing anti-HEV IgM positive by this method are interviewed and have controls matched to them.

Previous studies have shown, antibodies to HAV are generally ubiquitous in this population by the age of 10. As such, only children under 10 were tested for anti-HAV IgM in case adjustment for disease caused by concurrent HAV/HEV infections was necessary. A commercial EIA for anti-HAV IgM (DiaSorine, Italy) was used.

**Second Tier Screening**

For the definitive confirmation of case or control status, specimens were tested using commercial and in-house EIAs for hepatitis A, B, and E antibodies by the Virology
laboratory of AFRIMS (Bangkok, Thailand). Although commercial anti-HEV assays are available, the appropriateness of their use for epidemiologic studies is often questioned (94;95). The Walter Reed Army Institute of Research (WRAIR, Bethesda, MD, USA) has developed both quantitative anti-HEV IgM and anti-HEV total Ig EIAs which are recognized as a highly sensitive and specific to identify HEV infections in populations (264;265). Cases were tested for anti-hepatitis E virus IgM (anti-HEV IgM), anti-hepatitis E virus total Ig (anti-HEV Ig), anti-hepatitis B virus core antigen IgM (anti-HBc IgM). Cases under 10 years old were also tested for anti-hepatitis A IgM (anti-HAV IgM) to identify possible cases of co-infection. As previous studies in this population demonstrated a low prevalence of HCV infections, anti-HCV was not tested. Controls were tested for anti-HEV total Ig.

The anti-HAV assay (Abbott/Murex, Dartford, UK) is 98.9% (95%CI: 96.4-99.68%) sensitive and specific in identifying acute immune response to initial hepatitis A virus infections. The anti-hepatitis B core antigen IgM assay (Abbott/Murex, Dartford, UK) identifies acute infections, even when HBsAg is not detectable, and is indicated for use when multiple hepatitis infections are suspected. This EIA has been evaluated by the manufacturer to have a >99% sensitivity and 100% specificity. Initially reactive specimens were tested in duplicate. Positive and negative controls were included in every EIA run.

The AFRIMS in house anti-HEV assays use a quantitative sandwich approach to capture and label human IgM and Ig antibodies to recombinant HEV ORF2 proteins (rHEV) of the Pakistani strain expressed in the baculovirus system. The photometric result is compared to positive controls from a positive reference pool of Nepali HEV
patients, to report antibody concentrations in WRAIR units / milliliter (WRAIR U/ml). For total Ig, a cutoff of ≥20U/ml is used by WRAIR to classify subjects as ‘definite past infections’, and >1000U/ml as definite acute HEV infection (190)(Myint, KSA. Personal Communication, 2006). For anti-HEV IgM, WRAIR uses ≥ 100U/ml as a diagnostic cutoff for acute HEV infection.

In brief, the assay procedure involves the following steps: coating 96-well plates with rHEV antigen, incubations with blocking solution (0.5% casein and 0.5% bovine serum albumin in phosphate buffered saline, pH 7.4), test serum, goat anti-human IgM conjugated to horseradish peroxidase (HRP) (replaced by goat anti-human Ig-HRP for the total Ig assay) and tetramethylbenzidine. There are washing steps between each incubation. The reaction is stopped with sulfuric acid and the plates are read at 450/650 nm in a SpectraMax 340 EIA reader set for four-parameter analysis. The instrument’s software fit a four-parameter dose-response curve to the OD results of the standards, calculate the correlation ($R^2$) between the diluted antibody standard’s measured and expected results, and calculate the antibody content of unknowns from the standard curve equation. (264;265) (K.S.A. Myint, Personal Communication, October 2006)

**Field Quality Control**

Unannounced, random supervisory field visits were conducted by study supervisors throughout the duration of the study to observe the surveillance by the CHRWs. Logbooks were reviewed at the bi-monthly CHRW meetings and scoring was checked. Study supervisors also conducted monthly debriefs and refresher trainings for the 13 CHRWs throughout the duration of the study. Field teams visiting potential cases re-checked the illness score and duration of morbidity for consistency with the CHRW,
and discrepancies were discussed at the monthly meetings. Logbooks were subject to a complete review prior to data entry at the end of each of the 22 surveillance rounds.

Phlebotomy and interview techniques were spot-checked by study supervisors for adherence to protocol, including appropriate consent procedures, data probing to minimize missing values, and recording of responses. The performance of the study field team was evaluated using a weekly field report shared with investigators, and the time between CHRW identification of a potential case, study team notification and pre-screening was evaluated for each case by the study supervisors to minimize delays.

Each potential case and matched control was visited up to three separate times to maximize recruitment. Refusals were visited by study supervisors to ensure that possible cases or controls were not refusing due to inadequate information about the risks or benefits of participation. All severe acute cases were visited by the study medical officer, who conducted a thorough physical examination, and provided supportive treatment as indicated by the patient’s condition. If necessary, patients were offered outpatient or inpatient care at the Matlab Hospital.

**Statistical Methods**

All data were entered using custom-designed data entry screens, with appropriate field range and data consistency checks, built using Visual FoxPro (Microsoft Corp., Seattle, WA, USA). All statistical analyses were performed using Intercooled Stata Version 9.0 for Windows (Stata Corporation, College Station, TX). A p-value of < 0.05 was considered as statistically significant for all analyses.

The number of cases identified each month was counted, and was compared against the expected number of cases, based on prior prevalence data from this population.
and estimated disease incidence from studies in neighboring Nepal (187). Once the confirmatory second tier results were available, the performance of the MBS anti-HEV IgM EIA was evaluated (sensitivity, specificity, positive and negative predictive values) against the AFRIMS IgM EIA (Table 12).

Two case definitions were used in the analysis of risk factors for acute hepatitis E illness. The first, more conservative analysis, uses only second-tier AFRIMS confirmed anti-HEV IgM seropositive cases. The second, more liberal case definition, uses anti-HEV seropositive cases detected by either MBS or AFRIMS methods. A study recruitment diagram was created to illustrate the enrollment of cases and controls (Figure 14).

Case and control groups were compared descriptively. Demographic variables were explored by measures of central tendency and spread. The mean values of continuous variables were compared between groups by paired t-tests, where appropriate or non-parametric comparison of medians if necessary. After selecting out ineligible controls, the 1:4 case-control ratio was disrupted, leading to varying numbers of controls per case. Therefore, to estimate the significance and strength of association between sporadic hepatitis E disease and putative risk factors, accounting for variable case:control ratios, univariate conditional (fixed-effects) logistic regression was used to extend McNemar’s test to multiple, differing numbers of controls per case. The inverse natural log of the coefficient was taken to derive the estimated odds ratio and their associated 95% confidence intervals.

Demographic variables assessed included age, gender, religion, marital status, household size (divided into tertiles), type of primary and secondary employment, gross
nutritional status (proxied by mid-upper arm circumference – MUAC), education, head of household education, income category (lower than median, median, higher than median), and type of household construction. Household construction was proxied by deriving a household score, summing ordinal values issued to the materials used to build the floor, walls and roof of the primary living quarters, in order of increasing expense and durability (eg. wall quality could range from thatch/sticks, bamboo, wood to tin, to bricks/cement). The analysis of marital status and education as associated with hepatitis E illness excluded individuals who were ineligible for those exposures – persons aged 15 or less for marriage, and persons aged less than 7 years for education. The descriptive analysis of MUAC was only among adults over 15 years old, as cutoffs used as gross indicators of malnutrition vary greatly in growing children.

Exposures and behaviors assessed ranged from ownership of livestock and poultry, land use, social empowerment NGO membership, travel to urban areas, food consumption outside the home to contact with generally sick or specifically hepatitis patients, injection and transfusion exposures and normal treatment seeking behaviors. We also asked about sanitary practices both at the individual and household level (toilet type, distance from living quarters, toilet cleaning frequency, handwashing practices after toilet use and before eating), water use for drinking, cooking and washing, storage and/or filtration of water. Finally, given the recent interest in the potential zoonotic component to HEV, we assessed the range of activity between subjects and domestic livestock (feeding, milking, bathing or slaughtering) and creating “exposure” scores reflecting the number of activities an individual engaged in with a particular type of domestic animal. We also explored the degree to which animal dung was used at the household level by
creating a “dung use” score, reflecting the number of ways in which a given household used animal dung (eg. fuel, construction/repair, cleaning, and fertilizer).

Based on the results of univariate analyses, an explanatory model was developed using multivariate conditional logistic regression, controlling for possible confounders such as gender and indicators of socioeconomic status. Categorical dummy variables were used for non-linear, nominal covariates or where effect modification was suspected. Two modeling exercises were performed, based on the conservative and liberal case definitions. Due to the low numbers in any given cell using the conservative case definition, covariates of potential value, significant at the 25% level (p<0.25) in univariate analyses were added manually, and any variables retaining significance at the 25% level were preserved in the final model. Improved explanatory fit was grossly assessed by the pseudo-$R^2$ estimator provided by the software package, with 1.0 representing best fit. When using the liberal case definition, a more stringent cutoff of 15% was used for inclusion in the final manually-fitted model.

A software-based stepwise modeling approach was also used, including any variable significant at the 25% level during univariate analysis. (In an exploratory study, the use of too stringent pre-selection criteria can be counterproductive, as case cell sizes may be small for a particular exposure.) The remaining variables were added in both a forward and backward stepwise manner, excluding or including variables above or below a 20% (p<0.2) significance level, respectively. This cutoff was increased to 10% (p<0.1) when using the liberal case definition. Variables that cause the greatest increase in the maximized log likelihood were added automatically, until no more change in log likelihood was achieved. The step-by-step addition of variables to the multivariate model
identifies, in order of decreasing importance, the variables most strongly associated with the outcome (incident hepatitis E disease) and at the same time adjusts for confounding among variables. A bootstrap method was used to estimate the robustness of the final automatic model with 50 replications.

The final model provided an exploration of possible associated risk factors with incident, sporadic hepatitis E disease, allowing the calculation of an odds ratio for each significant variable, adjusting for the other covariates in the model. However, as this is an exploratory case-control study and not an inference-based or hypothesis-testing design, the final model serves primarily as a guideline for future investigations of fixed hypotheses, indicating putative directions of association, without necessarily reflecting true magnitudes of association.

Although age was adjusted for by matching, the role of age as an effect modifier could be assessed by analyzing odds ratios for specific risk factors and also for the final multivariate model across age strata to explore any age-related trend in risk.

E. Results

Surveillance

The entire population of Block C, comprising of 20 villages, an estimated population of 23,500 (mid-year 2003) was under hepatitis illness surveillance by 13 CHRWs. Between August 22, 2004 and May 31, 2006, a total 22 consecutive surveillance rounds were carried out, each round lasting approximately one month. This accounted for over 286 person-months of CHRW surveillance time, covering a little over 43,000 person-years of exposure time.
**Numbers of Cases**

Over this period, from the CHRW surveillance network, 279 ‘potential cases’ were identified by qualifying Illness Score and time since onset. The median illness score among this group was 6 (n=143, 51.3%), with only the top 12.5% of scores reaching the maximum possible 8 (n=35). The median time of onset was 14 days (mean 15.1±7.3 days) with only 11.1% being met within a week of the onset of signs and symptoms. Only 9 individuals were not met, despite at least 3 attempts to enroll them, and none of the ‘potential cases’ refused enrollment (See Figure 14).

The final 270 ‘potential cases’ were enrolled and pre-screened for anti-HEV IgM using the MBS commercial assay. This first-tier triage identified 43 anti-HEV positive cases, who were subsequently interviewed. Four age-matched controls were selected and interviewed for each case. As a second-tier verification of seropositivity, aliquots of the 270 specimens were forwarded to AFRIMS and tested for anti-HEV IgM and total Ig titers. Of 270, 25 had an anti-HEV IgM > 100 U/ml and 4 had an anti-HEV total Ig > 1000, for a total of 29 AFRIMS-confirmed positive cases.

Table 12 compares the two assays, and using the AFRIMS assay as the gold standard, suggests a low positive predictive value of the MBS assay, at 43.2%. The kappa score (0.50) between the two assays suggests only a moderate level of agreement. (This motivated the use of two case definitions in the analyses, one more conservative, using only AFRIMS-confirmed cases, and one more liberal, using either MBS or AFRIMS-confirmed anti-HEV IgM seropositive cases.)
Number of Controls

For each of the 46 potential cases identified, at least 8 age-matched counterparts were identified from the HDSS database. Each of the 8 prospective controls was visited and screened in the order listed, and a new list of 8 was generated until four controls were successfully recruited. A total of 268 potential controls were pre-selected for screening to match to ‘potential cases’. Three were not met after 3 visits, 47 had moved temporarily from the study area, and 4 had moved permanently out of the Matlab surveillance area. Of the 214 visited, only 6 (2.8%) refused to participate, primarily due to lack of perceived benefit as they were healthy at the time of the visit. The 208 remaining controls were screened for eligibility (disqualified by a previous history of jaundice, and if no history of jaundice, a lifetime hepatitis illness score less than 4); this process disqualified 20 additional individuals. Four controls were dropped from the final analysis as their case counterpart (n=1) moved out of the study area after initial enrollment. Finally, 184 controls were successfully identified, enrolled, interviewed and tested for antibodies to HEV, at a case:control ratio of 1:4 (Figure 14).

None of the 184 age-matched controls selected for the 46 clinically ill case-patients had an anti-HEV IgM titer greater than the AFRIMS recommended cutoff of 100 WRAIR U/ml for acute infections. The median titer was 7.2 U/ml, ranging from 0.1 to 60.4 U/ml (mean: 10.4±10.9 U/ml). As the control screening was based solely on self-reported clinical signs to attempt to exclude individuals with prior clinical hepatitis illness, it remained possible that individuals with historical, subclinical HEV infections were included as controls. Prior HEV infection might influence these controls’ susceptibility to HEV infection and/or illness, making them less appropriate as
comparisons in this study. This prompted the need to exclude them from the control pool, by investigating the anti-HEV total Ig titers of these 184 individuals. The median anti-HEV Ig was 8.5 U/ml (mean 28.6±62.6U/ml). Using the AFRIMS cutoff of ≥20U/ml as indicative of previous HEV infection, 50 (27.2%) controls were likely to have been previously infected. These 50 were excluded as controls from the final analysis, leading to a case:control ratio of 2.9, with 134 controls for 46 cases.

**Conservative Case Definition**

An alternate analysis was conducted, including only the 24 cases confirmed positive by AFRIMS testing. Only ‘potential’ cases selected by illness score and then found to have anti-HEV IgM ≥ 100 U/ml or anti-HEV total Ig > 1000 U/ml were preserved. Four age-matched controls without any lifetime history of jaundice were initially retained for each of the 24 cases. Subsequent screening eliminated 28 controls with anti-HEV total Ig ≥ 20 U/ml, an AFRIMS-recommended cutoff for previous HEV infection. Finally, this analysis was conducted with 24 cases and 68 age-matched controls, a ratio of 2.83 controls per case.

**Dual infections with HAV or HBV**

Potential cases under 10 were also tested for anti-HAV IgM. Among the nine potential cases under ten years old, three were identified by both the AFRIMS and MBS assays as anti-HEV IgM positive and six by the MBS assay alone. Three of the latter MBS-detected cases showed evidence of infection by HAV as well.

Only two of the cases identified by the MBS assay as anti-HEV IgM positive were also identified as acute HBV infections by the Murex Abbott assay. Only one of
these HBV-HEV co-infections was under 10 years of age. These cases were included in the analysis. By the same method, none of the cases identified by AFRIMS as acute HEV infections were co-infected with HBV.

**Etiology of hepatitis illness**

Of the 270 cases of suspected acute hepatitis illness identified, at most 46 (17.0%) individuals were by at least one EIA to be anti-HEV IgM positive. As specimen volumes allowed, specimens were also tested for anti-HBc and anti-HAV IgM. Among 248 tested for both these antibodies, 18 (7.3%) were found to have acute antibodies to HBc, and 33 (13.3%) to HAV. This leaves 174 (64.4%) of the cases of acute hepatitis-like illness with an unexplained etiology, possibly representing non-specific morbidity, or over-reporting of symptoms due to expectation of treatment or perceived benefit of enrollment.

**Characteristics of cases and controls**

Cases were a median age of 18.8 years (mean 23.1±14.7), using the liberal definition, capturing 46 cases. Within the more conservative case definition (n=24), the age was a median of 17.1 years (mean 20.9±11.5). Up to 30% of the cases were aged 15 or less, with only 2 cases under 10 years old using the AFRIMS assay, and 8 under 10 using either assay. As expected, cases were not significantly different in age from controls, confirming that age-matching of the groups was retained even after the withdrawal of non-eligible controls. As cases were matched to controls on age, the association of age with acute hepatitis E illness was not open to investigation. It was also difficult to examine age as an effect modifier due to the low numbers within each stratum.
The univariate analyses were replicated using both case definitions, and the results are displayed in two tables (Table 13 & 14). The first table covers primarily demographic characteristics, and the second describes exposures related to behaviors and possible environmental exposures. Female gender, irrespective of case definition was significantly protective of hepatitis E illness (OR 0.34 to 0.38). Neither marital status, household size, nor blood group was significantly associated with illness in the univariate analysis. Common proxies of socioeconomic status (SES) such as education, household income, and house construction quality were also not associated with acute hepatitis E illness in this study.

In the analysis of various employment categories compared to none/housework, using the conservative case definition this dataset was unable to identify any increased risk in a particular stratum of employment. With the more inclusive case definition, a significant increased risk was seen for fishing/farming and outdoor labor professions (OR: 8.22, 95%CI:1.67-40.49). A second analysis of employment-related risks excluding the influence of persons aged <15, who due to a lower age may be either protected from exposures and thus have a lower likelihood of infection, the estimated risk for fishing/farming and outdoor labor increased to 13.8 (95%CI: 1.66-115.69). In this analysis, the estimated association between hepatitis E illness and office-based employment also became significant (OR 29.07, 95%CI: 1.09-772.69), but this result should be interpreted with caution due to small numbers in each case-control matched group.

Participants reporting their primary work environment as being “outdoors” were at an estimated 3-fold higher risk of hepatitis E illness than indoor working counterparts
(OR 3.17, 95%CI: 1.35-7.47), using the liberal case definition. The conservative
definition also found a 63% increased risk for this exposure, albeit non-significant. Travel
in the three months prior to illness to an urban area (town or city) for these village-
dwelling cases carried a 2- to 10-fold increased risk of hepatitis E disease. Using the
conservative case definition revealed an OR of 10.23 (95%CI: 2.17-48.15) compared to
the liberal case definition’s estimate of 2.37 (95%CI: 1.15-4.87). Also in line with these
results was a significant increased risk among individuals who ate food prepared outside
the home between 1 and 6 times every week (OR 3.15, 95%CI: 1.16-8.52). In the
univariate analysis, however, those eating food prepared outside the home over 7 times
each week were not significantly at risk for hepatitis E illness (albeit again, low numbers
preclude any definitive conclusion from this dataset).

Contact with any sick person in the 3 months prior to hepatitis E illness was not
found to be a significant risk factor, but interestingly, reported contact with any
individual suffering from yellow eyes or skin, or “jaundice” in the past 3 months carried a
roughly 10-fold increased risk of being a case. Irrespective of case definition, this
association held true, with a conservative analysis presenting an odds ratio of 10.83
(95%CI: 2.18-53.79) to a liberal case definition estimate of 9.63 (95%CI: 3.06-30.37).
Subjects were also asked if they had visited a traditional healer (Kabiraj/Ouja/Fakir) in
the three months prior to illness. This behavior was highly associated with incident
hepatitis E illness, with odds ratios over 35, irrespective of case definition.

Tubewells, which are generally considered pathogen-free in most situations, are
nearly universally used as the source for drinking water in this community (91.1%, not
different by case status). Despite the fact that over 90% of households own their own
The characteristic of drinking water which did seem to be associated with an increased risk of hepatitis E illness was the self-reported average amount consumed daily. Those consuming 8 or more glasses of water per day (about 70% of cases), had an OR of 2.83 (95%CI:1.19-6.74), compared to those consuming 7 or fewer glasses. (The median number of glasses of water reported across the entire sample of cases and controls was 8 per day.) Although this association may be confounded by age (younger children being less likely to consume large amounts of water), the ~3 fold positive association persisted using both case definitions. Stratifying this analysis by age category (≤15, 16-30, >30 - data not shown) resulted in low numbers per cell, but the positive association remained significant in individuals 15 or younger (OR 6.57, 95%CI: 1.28-33.59), in which category ~19% of cases reported consuming over 8 glasses per day. For comparison, in the 16-30 age stratum, 63% of cases and in the >30 age stratum, 86%, of cases reported consuming over 8 glasses of water a day. Associations remained positive in the stratified analysis for age categories over 15, but these were not statistically significant.

Of the various data collected on household sanitation, the use of a sanitary latrine (sealed or slab) was found to be significantly protective (using the conservative case
definition) of incident hepatitis E illness (OR 0.28, 95%CI:0.09-0.90), compared to the use of an open pit or “hanging” latrine in the household. The data also suggested that sanitary toilets were more likely to be located near the living rooms of the house compared to unsanitary toilets. Adjusting for location of the toilet, however, (adjacent to or far from the living rooms of the house) had little effect on the magnitude or significance of the protective association. Toilet cleaning practices (frequency) or the personal involvement of the case-subject in cleaning the toilet had no significant association with hepatitis E illness.

Self-reported hand washing before eating was universal. Most respondents used only water, with only 10.4% of subjects washing with soap and water. Interestingly, a mere 16.3% reported washing both hands before eating, a typical cultural practice across rural Bangladesh; this was, however, not different between cases and controls. Hand washing after defecation was also nearly universal (98.3%), with a variety of methods used, ranging from water only (40.0%), to soap and water (39.1%), and earth/ash with water (20.9%); only 68.5% of respondents reported washing both hands. These did not differ significantly between cases and controls.

Livestock (cows, goats) and fowl (chickens, ducks) ownership was common in this rural community at 37%, 24%, 54% and 52%, respectively, among cases and controls. However, the exposure to these animals in terms of bathing, feeding or milking was minimal, and not associated in this dataset with illness. Up to 30% of this population reported involvement in feeding or caring for domestic fowl although also not different by case status. Contact with animal feces, namely cow dung, is common in rural Bangladesh. Up to 87.0% of cases and controls reported using dung in some form in their
households, primarily for house repairing and maintenance (50.0%), and as fuel for cooking fires (32.5%). Increased use of animal dung in the household was not associated with hepatitis E illness. Recent studies have investigated whether domestic pets or pests may serve as risks for the transmission of HEV (184); in this study over 98% of cases and controls had seen a rat in or near their home in the month prior to illness. Domesticated dogs or cats were extremely rare in this population.

The riverine ecology of this rural area is reflected in the ubiquitous presence of ponds or large water bodies near most households – nearly 85% reported a pond in their homestead. We investigated the ownership and size of household ponds as well as the level of the pond water in the month before illness, but neither was associated with hepatitis E disease, irrespective of case definition used. We looked at whether recent flooding may have played a role in illness, but no association was evident in this study.

Also non-significant were NGO membership (as a proxy for improved individual and household sanitary behaviors), 3-month history of injection exposure, transfusions of transfusions, and the first level of health care sought by the individual during illness. Barber use for shaving or haircutting was frequent (overall 79%), but showed no association with hepatitis E illness.

Interestingly, about 23-29% (depending on case definition) of cases and controls together reported having had an injection in the previous 30 days. As this might be attributed to the widespread use of injectable contraceptives, we examined the exposure carefully by gender and history of contraceptive use. Excluding women who also reported having taken injectable contraceptives in the past year (n=5), we still found high
rates among men (26.1% of male cases and controls) and women (27.2% of female cases and controls).

**Multivariate Analysis – Conservative Case Definition**

Unadjusted variables, significant at the 25% level (p<0.25) were included manually and automatically (forward and backward stepwise) in a multivariate conditional logistic regression model. These included, under the conservative case definition, the demographic variables of gender, employment category, house construction score and NGO membership. The exposure variables selected were recent travel to a town or city, work outside the home, fish pond size, level of water in the pond, unsanitary latrine use, ownership of and exposure to cows, chickens or ducks, recent exposure to hepatitis-like illness, level of treatment provider sought for normal illnesses, source of cooking water, source of drinking water, water storage behavior, quantity of water normally consumed daily and the diversity of dung use in the household. The variables finally included in the final automatic model were recent visit to a town (OR 29.53, 95%CI: 2.58-337.72), unsanitary toilet use (OR 5.28, 95%CI: 0.89-31.57), dung use score (OR 0.25, 95%CI: 0.08-0.84), and 8 or more glasses of water consumed daily (OR 4.37, 95%CI: 0.65-29.14).

**Multivariate Analysis – Liberal Case Definition**

A larger number of cases (n=46) were available for this analysis with more matched controls (n=134). The results of both the forced “manual” model and the stepwise models are shown in Table 15. The variables selected (p<0.25 in the univariate analysis) for the liberal multivariate analyses included among the demographic
characteristics: gender, level of education, employment category, income category, house construction score and size of household. The exposure variables selected were recent travel to a town or city, work outside the home, work outdoors, quantity of water normally consumed daily, source of cooking water, the habit of consuming food prepared outside the home, recent exposure to hepatitis-like illness, recent exposure to injections, level of treatment provider sought for normal illnesses, use of unsanitary latrine, practice of cleaning the latrine, ownership of and exposure to cows, chickens or ducks, and the diversity of dung use in the household.

Interestingly, the manual model was quite similar in composition to the stepwise selected model, except gender was forced into the model. Female gender seemed to be protective (albeit not significantly) against hepatitis E illness (OR 0.30, 95%CI:0.07-1.20, p=0.088). Despite the increase in sample size by the liberal case definition, it was still too limited to perform extensive gender- or age-stratified analyses. We looked at certain key variables to see if there were clear correlations between gender and behavior (eg. work outside the home, travel to town/city). Of the 15 female cases, 40% reported visiting a town or city compared to 64.5% among the 31 male cases, not statistically different by gender (Fisher’s exact test, p>0.2). However, when the gender association with hepatitis E illness was adjusted by covariates such as travel to town, employment outside the home and outdoor work, for which gender is likely a proxy, the significance of gender decreased dramatically (data not shown).

The same final fitted model was selected by both forward (beginning with an empty model) and backward (beginning with a full model) stepwise conditional logistic regression. All exposures finally selected were significant at the 5% level (p<0.05),
adjusted for the other variables. Due to the limited sample sizes, and wide confidence intervals of the final estimates of association, the results will focus on direction of association. The most dramatic positive associations were for reported exposure to a hepatitis or jaundice patient in the three months prior to illness (OR 82.50, 95%CI:8.77-776.39), employment outside the home (OR 19.80, 95%CI:1.89-207.96), and injection exposure in the three months before hepatitis E disease (OR 15.50, 95%CI: 1.97-121.76). Other variables with increased risks of hepatitis E disease were outdoor work (farming/fishing or manual labor) (OR 8.63, 95%CI:1.33-56.09), unsanitary toilet use in the household (OR 5.14, 95%CI 1.20-22.01) and travel to a town / city in the past three months (OR 4.25, 95%CI: 1.06-17.10). Possibly protective against hepatitis E illness were increases in household size category (OR 0.17, 95%CI: 0.05-0.56) and improvements in household construction score (OR 0.35, 95%CI: 0.13-0.98). A diagram of a possible explanatory model for sporadic HEV infections is presented in Figure 15.

The results of the 50 replicate “bootstrap” tests of the final model showed increased standard errors for some covariates (3 month history of visit to “jaundice” patient, outdoor work and history of recent injection), and suggest caution in the interpretation of this small sample of cases and controls.

**F. Discussion**

A recent anti-HEV seroincidence study in the Matlab area demonstrated a high population rate of HEV infections (near 60 infections per 1000 person-years), with a lower rate of accompanying disease. In sharp contrast to neighboring countries of Nepal and India, where predictable annual outbreaks of HEV infection and disease occur, there are few reports of outbreaks in Bangladesh. Hospital-based studies, however, indicate a
substantial burden of hepatitis E morbidity in this population (88;259). This study represents one of the first efforts to identify putative risk factors for sporadic HEV infections by focusing on what are likely to represent the most severe examples of HEV infection – those that manifest clinical signs of hepatitis illness.

The difficulties in conducting studies of sporadic HEV are evident in the need for large, population-based surveillance systems to identify and screen incident hepatitis E illness in communities. In order to rapidly identify and enroll potential cases, the use of a commercial anti-HEV assay was necessary, a decision with unfortunate caveats given the relatively lower performance of commercial anti-HEV assays compared to HEV research laboratory in-house assays (Table 12). As a substantial number (n=22) of potential cases were found to be non-reactive during the more sensitive and specific second tier screening (Figure 14), two case definitions were finally used to analyze this study data. As an exploratory venture to identify potential risk factors for sporadic HEV infection and disease, results under either case definition are presented, with accompanying cautionary notes against the overinterpretation of findings from this small sample study.

**Low burden of hepatitis E disease**

The rural Bangladeshi population in which this study was conducted is, by many measures, one in which enteric infections should flourish. The level of sanitation, although improving, remains low, with just under half (44%) of this sample still using unsanitary latrines. Defecating in such “open” or pit latrines can readily transfer human excrement (and potentially fecal-oral viruses such as HEV) to the environment when periodic flooding occurs. In this riverine community, the use of unsafe pond and river water for cooking, washing and bathing is extremely prevalent and exposes individuals to
a panoply of infectious agents. Animal husbandry is also an integral component of life in Bangladeshi villages; chickens, ducks, cows and goats live and roam freely within the homestead, resulting in potentially increased exposures to animal-borne infections. Physical contact with cow dung is a daily necessity within most households as individuals rely on it as a source of fuel, fertilizer and for construction. These are among the reasons why previously studied enteric hepatitis viruses, such as hepatitis A, have been shown to infect most individuals in rural Bangladesh early, well before the age of 5 (88).

Over 22 months of surveillance, clearly no outbreaks of hepatitis E illness were identified in this population of over 23,000. An underlying canvas of non-specific morbidity led to the identification of 279 potential cases, only 35% of which could be etiologically specified as HEV, HAV or HBV infections. Up to 46 individuals were identified as acutely infected hepatitis E illness cases, by either an AFRIMS in-house or MBS commercial EIA. Of the 188 “healthy” controls finally selected as sero-naïve controls, ~27% were finally excluded due to antibody titer evidence of likely prior infection. This low to moderate prevalence of anti-HEV among ‘healthy’ individuals is consistent with a recent prior study in this same population. Recent cross-sectional and population based studies in Egypt, where HEV outbreaks have not been seen despite high anti-HEV seroprevalence and infection rates, suggested that strain-specific differences may influence whether hepatitis E disease manifests as epidemics or as sporadic cases (184). Equally plausible are hypotheses of regional (or population-specific) or genotype/strain-specific differences in HEV virulence, which may be the case between Bangladesh and India or Nepal.
Alternatively, the absence of large outbreaks or greater numbers of sporadic cases may reflect an insufficient environmental dose of HEV to cause clinical disease. Animal models have clearly demonstrated that the infective dose required to cause hepatitis E illness may be several 1000-fold greater than infection alone (164). Although HEV may be pervasive throughout the Matlab community, the level of exposure necessary to cause disease may be rare. As discussed below, certain risk factors may directly increase the level of individual exposure, which then tips the balance towards overt clinical illness.

It is also possible that individuals maintain low levels of anti-HEV from infections early in life (with present titers falling below the cutoffs used to identify prior infections), which results in an underestimation of actual anti-HEV exposure in this population. If this were true, recent exposures to HEV may result in anamnestic responses to reinfection without clinical illness. This was also suggested as a possible reason for high rates of infection without clinical illness seen in a recent study of Egyptian pregnant women (194).

The phenomenon of HEV infection without an antibody response has also been previously described (187), and may result in the underreporting or failure to capture incident HEV infections / disease. By design, this study compared cases to serologically naïve age-matched individuals, thus ensuring that the control group was at a theoretically equal risk of exposure as the cases. (Including non-naïve controls in a study of a pathogen for which the human protective antibody response is still unclear could cloud the ability to distinguish risk factors for incident HEV infection or disease.)
Age, Multiple Infection and HEV Illness

Between 25-30% of the acute HEV cases identified in this study were under 15 years old. Much has been published on the paucity of pediatric HEV infections in this region, as gleaned from the seroepidemiology of outbreak and hospital-based data from neighboring India and Nepal (56). Among the 9 cases under 10 years of age, three (33%) were co-infected by HAV, and one by HBV. The increase in severity and manifestation of clinical illness in children co-infected by multiple hepatotropic viruses has been previously described (78;210). In an environment where multiple enterically transmitted viruses circulate, the increased likelihood of co-infection in susceptible young children is not surprising. As expected, 70% of the cases identified were in their second or third decade of life, supporting the seroepidemiologic evidence on delayed HEV infection from this region (56;81). In contrast, only a single case over 10 (a 16-year old girl) represented a likely dual acute infection (HEV + HBV).

Many early studies of HEV focused on the landmark characteristic of increased morbidity and mortality among pregnant women, especially in the third trimester (17;20). Initial speculation about gender-related differences in susceptibility to infection has been replaced by the understanding that pregnancy exacerbates HEV pathogenesis, rather than increasing the likelihood of infection itself (16). Studies in conservative communities in South Asia, where social restrictions on women’s movement exist, have suggested a protective effect of female gender on HEV infection and illness (26;72). This study, too, suggested that female gender was protective against hepatitis E illness, likely due to social barriers keeping women from work outside the home or frequent travel to urban areas. The social construct of *purdah* also restricts interactions of married and unmarried
women with non-household members, limiting their exposure to sources of HEV in the environment (1).

**Demographic predictors**

Sample size estimates were based on disease rates similar to those seen in India and Nepal, assuming a similar epidemiologic profile in this population. As a result, few differences in demographic characteristics were detectable in this study, likely due to a lower number of cases than expected. No differences in traditional indicators of SES were evident in this study, except the suggestion of increased risk to individuals employed outside the home, notably in professions with significant outdoor activity. Presumably, these types of activity would predispose individuals to have to resort to unsafe water consumption, whereas in the household, pathogenically “safe” tubewell water is readily available whenever needed.

**Behavioral predictors**

In the same vein, among the most significant univariate predictors of hepatitis E disease were characteristics associated with exposures outside the home. In addition to “outdoor” job activities, consuming food cooked outside the home was associated with a three-fold increased risk of hepatitis E illness in one analysis. Cases were significantly more likely than controls to report travel to a town or city (irrespective of gender) in the three months prior to their hepatitis E illness. Surprisingly, in the univariate analysis there was a consistent nearly 10-fold increase in the likelihood of cases reporting contact with a “jaundice” or “hepatitis-illness” patient in the three months prior to illness. Some of this association may be due to differential recall bias between cases and controls, as cases
may be seeking causes to explain their present illness. Others have not shown evidence of person to person transmission in outbreaks or sporadic cases of HEV, as reflected in the 2% secondary attack rates and low intrafamilial transmission (159;281). However, this significant association deserves careful future assessment as a visit to a jaundice case (possibly an HEV infection), may expose a susceptible subject to contaminated drinking water or food, as fecal shedding of HEV has been well documented.

In the univariate analyses, cases were significantly less likely than controls to use sanitary latrines in their household. The use of unsanitary latrines (under the conservative case definition) increased the risk of hepatitis E disease 3.5-fold (95%CI: 1.11-11.49). Open or “hanging” latrines allow human feces to be washed into nearby ponds and rivers, from which water may be collected for washing, bathing, and even cooking. Studies from Nepal and India have independently shown that HEV infected individuals may continue to be viremic and even shed virus in stool for weeks after infection, even in the absence of antibody evidence of infection (186;187). The use of these “unsafe” water sources was not significantly associated in this study with increased risk of illness, in contrast to findings from Indonesia and Vietnam. However, this study did document, as elsewhere in South Asia, the widespread use of these sources for cooking, bathing and washing (122;129). These behaviors, in combination with the widespread use of unsanitary latrines and the phenomenon of extended fecal shedding could work to maintain HEV endemicity, even if only through a chain of subclinical infections.

Finally, an interesting univariate association worthy of discussion was a consistent increase in risk of disease among individuals (across all age categories) who reported consuming 8 or more glasses of water, on average, daily. Although an age-
stratified analysis reduced the significance of the association in the older age groups, the positive risk remained significant in the lowest age category of 15 or younger (OR 6.57, 95%CI 1.28-33.59). As discussed earlier, this raises the issue of whether substantial doses of HEV are needed to cause clinical disease, and despite the ubiquitous environmental presence of HEV, few individuals consume enough to cause disease, compared to the larger number who are exposed and experience only subclinical infection. As suggested by Clayson and colleagues, this ongoing cycle of continued inapparent HEV infections may maintain HEV in communities, even in the absence of large epidemics (58).

An extremely strong association was found between hepatitis E illness and a visit to a village “Traditional Healer” in the three months prior to illness. Although it is possible that their practices (a mixture of herbalism, shamanism, and spiritual healing) may expose subjects to potentially contaminated food or water, we feel that this association likely represents a reverse causation pathway (see Figure 15). Traditional healers (Kabiraj) are often the first line of treatment for many illnesses in rural Bangladesh, and some qualitative data from this population (Labrique, data unpublished) has suggested that as allopathic treatment for acute viral hepatitis is limited, traditional healers are sought for a wide range of alleged cures for hepatitis, soon after classic prodromal signs and symptoms appear.

The attempt to model a combination of exposures to predict the likelihood of sporadic hepatitis E disease was difficult, primarily due to a lower-than-expected number of cases. As a result, the interpretation of this study’s data should focus on the direction of associations, and not necessarily their magnitude. The characteristics which remained in the final model, irrespective of case definition or method of variable selection,
represent risk factors consistent with those predicted from seroepidemiologic cross-sectional studies and outbreaks in this region. Poor household sanitation remained significantly positively associated with hepatitis E disease, although it is also clear that improvements in household construction (also a proxy for improved SES) may impact on overall cleanliness and subsequently decrease the risk of hepatitis E disease. A complex of exposures “outside the home” remained in the model, ranging from employment outside the home and outdoor work (farming / fishing / labor), to travel to urban areas. Each of these behaviors increases the risk of consuming unsafe food or water when outside the home. In rural areas of Bangladesh, the production of food and drinks in small roadside kitchens is often extremely unhygienic and may represent an ideal environment for the maintenance and spread of sporadic HEV.

The persistence in the model of the highly significant positive association of acute hepatitis E disease and reported contact with a jaundice / hepatitis patient in the months prior to illness is difficult to explain. As discussed above, this may be somewhat due to recall bias, but deserves closer investigation under a sporadic transmission paradigm. Increased household size seemed to be protective against illness, an association which we thought might reflect an increased likelihood of early subclinical HEV exposure and subsequent “family” immunity to illness. However, as the selected controls were sero-naïve, their protection from HEV illness was not likely to be due to early subclinical HEV infection. Family size was not significantly associated with income or education, making it difficult to explain as a proxy for improved socioeconomic status. This association is opposite from the increased risk shown for HAV infection in larger families in India (266).
Finally, a strong positive association emerged in the multivariate model of reported exposure to injections (irrespective of gender) in the months preceding illness. This suggestion is perplexing, given the primary mode of HEV transmission in outbreaks has been fecal-oral through a waterborne route. However, there are suggestions in the literature that HEV can be transmitted parenterally (282;283). This route of possible HEV infection needs further elucidation, as does the highly prevalent practice of injection use in an otherwise “healthy” rural population.

**Zoonotic associations**

A substantial literature has recently developed around zoonotic HEV infections in swine, deer, poultry, and domesticated livestock in both endemic and non-endemic countries (51;55;136). Although these infections have been shown to be of different genotypes (GT 3, 4) than that which is normally found in south Asian human cases (GT 1), cases of animal to human infection have been clearly documented (47;53). Even in India, domestic pigs were found to experience HEV (GT 4) infection early in life (284;285). In addition to pigs, a 2001 survey of different Indian animal species suggested high anti-HEV prevalence in cattle and even rodents (*Bandicota bengalensis*) (285), two ubiquitous exposures in this population under study.

As a predominantly Muslim community, pigs were not reported in any of the households studied in Matlab. We were also unable to demonstrate any significant association between other livestock or poultry raising, or with individual involvement in specific practices of feeding, washing or sacrificing. We also assessed the level of animal dung use in the households, but were unable to find any association with disease. Rodents
were reported by nearly all respondents, making it difficult to assess any role of these household pests in the spread or maintenance of HEV.

There are certain population subgroups in Bangladesh (aboriginal tribes, and other non-Muslim minorities) that do raise and consume pigs. Further studies are warranted to describe HEV antibodies, genotype and possible viremia in domestic livestock and fowl in rural Bangladesh, as well as detailed studies of the aforementioned subgroups that may be at increased risk for hepatitis E disease from swine reservoirs (261).

Summary

Most outbreaks of hepatitis E illness have been clearly linked to sudden failures in drinking water processing, leading to high levels of contamination by HEV and other pathogens. Sporadic disease has been more difficult to explain, although the suggestions in this study that virus, although omnipresent in the environment of endemic areas, may be the source of continuous subclinical infection, causing disease in younger individuals when co-infections with other viruses occur, and in older adults (or also in children) when substantial virus ingestion occurs. These exposures that lead to illness likely take place when individuals leave the relative “safety” of their home environment, and resort to the consumption of unsafe water or food, much like when tourists travel overseas to endemic countries and subsequently get HAV or HEV infections. That said, low levels of household sanitation may increase the risk of infection even at home. Further study is needed to clarify the possible parenteral and intrafamilial risks of HEV transmission, as well as continued investigation of the possible role which household pests, such as rats, may play in maintaining and spreading HEV.
This data provides future directions for the study of sporadic HEV, a better understanding of which will help us target high-risk and vulnerable groups for eventual vaccine intervention. However, it seems equally likely that classic measures of improving community sanitation and lowering individual risk (avoiding potentially contaminated food or water) will be as effective in reducing HEV transmission and disease.

G. Conflicts of Interest

The authors have no known conflicts of interest concerning the work reported in this paper.

H. Acknowledgements

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8. Final Discussion

The evolving picture of an elusive virus

Several recent findings in the field of HEV research have resulted in paradigm shifts in our understanding of HEV, ranging from the characterization of four epidemiologically distinct genotypes to the discovery that HEV genotype III and IV variants are ubiquitous in swine populations around the world. Several decades ago, it was presumed that HEV was an emerging agent, limited in scope and burden to the countries of the developing world where conditions of poor sanitation exist. Today, it is understood that HEV, in various forms, is far more widespread and the potential for autochthonous HEV infection exists even in the most developed countries. Improvements in the assays used to detect HEV and antibodies to HEV have also followed as a result of this new knowledge (286). As pieces of the “HEV puzzle” continue to be uncovered, earlier conflicting and often, confusing, reports on HEV epidemiology become clearer.

Genotypic Variation

In addition to the traditional risk factors of poor hygiene and community sanitation leading to hepatitis E disease (primarily attributable to GT I or II), the role of animal reservoirs or vectors in hepatitis E epidemiology and disease is becoming increasingly clear (primarily for GT III and IV). It is also increasingly likely that variants within a single genotype may have different potential for sporadic or epidemic illness (146). New pathways for infection have even been identified for the spread and maintenance of HEV GT III and IV through the consumption of infected meat, or even through close contact with domestic animals (47;184). Still, the role of animal reservoirs
in maintaining and spreading HEV is unclear in settings where the dominant human HEV epidemiology is one of small seasonal outbreaks or large epidemics (GT I or II). Studies are ongoing to identify whether all HEV genotypes have an equal propensity for zoonotic transmission and/or human disease. As mentioned in the introduction, further research is warranted in Bangladesh, to understand whether multiple genotypes of HEV co-circulate, as seen in India and Indonesia, and to determine the determinants of human infection and illness by HEV for each genotype. In fact, in trying to understand the complex epidemiology of HEV, it is probably useful to view each genotype as a separate entity, with unique genotype-specific epidemiologic characteristics.

**Antibody Kinetics and the Immunity Puzzle**

The large variation in the reported pattern of human antibody kinetics following HEV infection continues to puzzle researchers. Early studies in humans and animal models suggested protective immunity after infection (85;99;241) but given the pattern of repeated, large-scale outbreaks in allegedly “endemic” populations, many have suggested that HEV immunity wanes quickly, allowing for repeated infection and illness. Some early studies even suggested that a certain proportion of infected, viremic patients may not even show an immune response; it is more likely, given more the recent information on genotypic variations, that the assays used (based on GT II or III) were unable to detect the antibody response in individuals infected with GT I. Given what we know about variations in HEV subtypes from season to season, and the co-circulation of different genotypes in populations, it is possible that the human immune response and propensity for hepatitis E illness might also be subject to HEV genotype- or subtype-specific characteristics. Further studies like the one recently done by Myint and colleagues,
looking at antibody trajectories over 14 months in a small cohort of acute Nepali HEV cases, are warranted in different populations, including children, with longer follow-up (190). For example, a follow-up of this Matlab cohort for a few additional years would be worthwhile, even if only the acute cases of HEV infection were included.

In this Matlab population, the definition of susceptibility (or HEV seronegativity) or “new” infection was challenging, as observed in the seroincidence and case control studies. Although some individuals demonstrated undetectable levels of anti-HEV, many had antibody titers which fell just below either cutoff used to separate likely ‘susceptibles’ from previously exposed persons. Furthermore, the discussion of “seroreversion” raised the important question of what benefits may be conferred by prior infection in endemic areas like Bangladesh. In a few cases, we observed individuals’ anti-HEV total Ig titer undulate around the “cutoff” of seropositivity, possibly reflecting ongoing, low-level HEV environmental exposures – enough to cause a small total Ig response, without clinical illness. As mentioned earlier, these observations have important implications both for HEV vaccination and for the estimation of populations at risk of sporadic disease and epidemics. If HEV infection is not sufficient to protect individuals from future infection and illness as suggested by some (85;241), further examination and long-term follow-up of incident infections in endemic populations is warranted. Also worthy of examination are the likely causes for rapid loss of anti-HEV in some and not others; the phenomenon may be dependent on intrinsic host factors such as malnutrition, underlying chronic disease, or even compromised immune function.

*The “Protected” Child Paradox*
Given the hypothesis that clinical hepatitis E illness is immunopathologic (59;95), the link between dose, immune response (acute and anamnestic) and clinical symptoms needs further elucidation. What are the conditions under which an anamnestic response can lead to clinical HEV? It is likely that the “tipping point” between ongoing subclinical infections and occasional “sporadic” illness (as seen in Matlab) or full-blown epidemics (as seen in parts of India and Nepal) is not solely due to genotype- or subtype-level differences; the infective dose and underlying herd immunity may be critical factors in the process. A further study looking at potential environmental sources of HEV in the Matlab community would be necessary to lend support to the dose-response hypothesis – looking at concentrations of HEV in the town drinking water supply near Matlab, in a sample of rivers or ponds near household or community toilets, and in the water supplied by small roadside restaurants in and around Matlab.

In an environment of poor community sanitation where other enteric viruses spread easily early in life, what prevents HEV from infecting children? This is one of the most difficult HEV phenomena to explain, unless one of four situations is true: either a) young children are not exposed to environmental sources of HEV, b) children are not exposed to sufficiently large doses of HEV to cause infection and/or subsequent illness, c) children are infected (mostly subclinically) very early in life, and retain some protective immunity to re-infection and illness until their teenage years, or d) there are, again, genotype-level characteristics which increase the likelihood of pediatric infections.

In Egypt, pediatric hepatitis E illness is common and a high seroprevalence (~60%) is reported in children under 10; the prevailing genotype is also GT I (47;193), albeit clustering into a distinct “African” subtype. Although GT III has been identified in
Egyptian pigs, it remains to be seen whether GT III contributes to the pediatric illness in this largely Muslim population (194). In India, most studies of HEV in children have not been able to demonstrate as high a population prevalence of anti-HEV in those under 14 years of age, peaking at ~24% in an rural cohort and ~29% in an urban cohort (287). Even in our Matlab studies, 95% of the 244 children under 10 years old had baseline anti-HEV antibody titers under 6 WRAIR U/ml (the cutoff for possible prior exposure being 20 U/ml). Unless this reflects a much faster drop-off in antibody titers in Indian and Bangladeshi children, compared to Egyptian children after early infection, there are either significant differences in HEV exposures or virus-specific characteristics between these two Muslim, epidemic-free, HEV GT-I endemic populations.

This is not to say children in India or Bangladesh are never infected by HEV; there have been well-documented outbreaks of hepatitis E illness in schoolchildren (288;289), although most sporadic cases of children presenting with hepatitis E illness are cases of multiple hepatotropic infections, as discussed earlier. One must also consider the possibility that other non-hepatitis co-infections (eg. schistosomiasis) may also increase susceptibility to HEV infection and illness in some populations and not others.

**Classical Risk Factors**

The case control study of putative risk factors for hepatitis E illness once again identified the apparently lower risk of disease in younger members of the population under surveillance. Over 75% of the cases identified were adults, and half of the children identified as cases were later shown to be dual hepatitis infections, possibly contributing to the severity of the infection (and subsequent clinical signs). Again, as has been seen in countries where social norms severely restrict female movement and interpersonal
interactions beyond the family, female gender seemed to protect against hepatitis E disease. As discussed earlier, rather than reflecting a biological gender difference, this phenomenon likely reflects decreased HEV exposure linked to the latter social restrictions. A set of behaviors likely to increase the risk of consuming unsafe food or water outside the home were significantly positively associated with acute HEV infection, ranging from work outside the home, notably in agrarian or fishing professions, to travel to an urban area. As mentioned earlier, this is similar in some ways to when a tourist travels to an endemic country and is exposed to a new pathogen or, in our case, higher levels of HEV than they normally experience in their home environment. As might have been expected, classical risk factors of poor sanitary practices at home and the consumption of potentially unsafe food or water when traveling emerged as risk factors for this enteric pathogen, even in HEV-endemic populations.

“Novel” Risk Factors

Another “novel” finding from the Matlab data supports a small number of reports which present evidence that parenteral transmission of HEV may be important in certain populations. A few instances of nosocomial infections have been documented, and a parenteral route suggested as the means of HEV spread, primarily in settings known to be endemic for GT I (171;282). A study among acute HBV-infected patients in Sweden, where GT III is most likely to be prevalent, also found a significantly higher rate of anti-HEV in individuals engaged in high risk sexual behavior and IV drug use, compared to healthy counterparts. Given a considerably long HEV viremic phase during acute infection, it is possible that virus could be transmitted through unsafe needle-sharing (290). The further exploration of this pathway is important, especially in developing
countries where “professional” blood donation is frequent and banked blood is not extensively screened for pathogens such as HEV. As mentioned in the discussion of the case-control study, an unusually high proportion of “healthy” individuals in this rural Bangladesh population reported injection exposures; a large number of untrained quacks and medicine shops have ready access to a range of injectable drugs and vitamins, and it is unclear whether single-use syringes are always used by these treatment providers.

The extremely significant association between acute hepatitis E cases and recent reported contact with a person with overt jaundice is one that, although subject to numerous recall biases, is worthy of exploration. In outbreak settings, secondary infection rates are low, and infections are thought to share a common source (20;38). It is difficult to explain this as a genotype or subtype specific viral characteristic which results in differing degrees of transmissibility between “outbreak” strains and “sporadic” strains. It is more likely that poor standards of hygiene result in the sharing of a contaminated water or food source. There is no evidence of direct transmission of HEV (except perhaps parenterally) on which to propose any other explanation for this observed association.

A Gold Standard is Needed

The case control study also raised, as other have before, the importance of understanding potential limitations of commercial anti-HEV assays for epidemiologic studies (94). Although both the NIH and WRAIR (AFRIMS) have developed extremely sensitive and specific assays for HEV research purposes, the newest commercial assays from Abbott (Abbott Diagnostika, Wiesbaden-Delkenheim, Germany) and Genelabs Diagnostics (GLD Pty, Singapore) purportedly perform similarly well, at least in outbreak situations (286). However, even these assays, although extremely specific, were
not very sensitive in identifying subclinical HEV infections independently confirmed by RT-PCR. It is not clear whether this is due to an antibody response in subclinical cases lower than that needed to meet the “cutoff” value set for acute infection. If we hold fast the hypothesis that hepatitis E illness is due to the immunopathologic consequences of infection, this explanation may be valid. Given the scope for confusion from inaccurate estimations of HEV prevalence in a population, or the poor positive predictive value of an assay in a clinical setting, it is important that anti-HEV assays be able to detect infections by any of the four HEV genotypes. Assays should also be evaluated against a gold standard such as RT-PCR, and researchers should be careful to appreciate the limitations of commercial assays used in studies of HEV, especially in non-endemic settings.

**HEV in Bangladesh**

Among the identified gaps in the field of HEV research enumerated in the introductory pages was a lack of data from countries such as Bangladesh, which although thought to be HEV endemic has not reported any large outbreaks as seen in Nepal, India, and other developing countries. These Matlab studies have clearly demonstrated a previously undescribed burden of HEV infections in rural Bangladesh, in a context where other hepatitis viruses (HAV and HBV) are also common. Others have shown that HEV is etiologically responsible for most acute hepatitis illness in this country (88;259), but not in a large, otherwise healthy population.

The overall anti-HEV prevalence in Matlab of 22.5%, when described by age, reflects the epidemiologic patterns seen in most other HEV GT-I endemic countries – low seroprevalence of antibodies in the youngest tier of the population, dramatically
increasing in the second and third decade of life. Our data does not support the hypothesis that young children are infected early in life, and maintain low antibody titers which disappear in adolescence. The Matlab evidence clearly suggests that infections, as proposed in points (a) and (b) above (The Protected Child Paradox), are likely not occurring in the pediatric segments of this population. The lower seroprevalence observed in women lends further strength to the argument that this age/gender seroprevalence pattern has to do with exposures to sources of HEV in the environment outside the homestead. In early adolescence, when male children traditionally begin to contribute to the agricultural labor of the household, or take on apprenticeships outside the home, we see a corresponding increase in anti-HEV seroprevalence.

The anti-HEV seroconversion rate, estimated in our study at 60.3 per 1000P-Y, remains comparable to the estimated incidence rate in an epidemic-prone Nepalese population. The absence of outbreaks, then, in our study setting remains perplexing. A possible explanation for this phenomenon would be that the environmental “availability” of HEV is sufficient to cause infection, but not illness. An alternative explanation is possible: waning antibody titers from subclinical infections in early adolescence results in the misclassification of individuals as “susceptible”, whereas their low titer is actually protective from hepatitis E illness. Basically, after their initial “first infection”, adolescents’ anti-HEV titers drop below present cutoffs for seropositivity, and upon re-exposure to the virus, they experience a slight anamnestic response, without clinical symptoms. Only 7 of our 49 seroconverters were under 20 years of age, and 2 of them had baseline titers over 15 U/ml (where the cutoff for seropositivity is 20 U/ml). Although this number is extremely low, it seems unlikely that this latter explanation
holds true for most adolescents, and that the most plausible explanation remains that the HEV dose in the infections causing seroconversion is insufficient in our setting to cause hepatitis E illness.

Stoszek et al. proposed earlier this year that in their Egyptian studies, the likely reason for high HEV seroconversion without overt illness is either a) the low-level protective antibody hypothesis presented above or b) the circulation of an avirulent, zoonotic genotype of HEV (eg. GT-III), which continually infects humans and stimulates an anti-HEV response (194). In Matlab, early evidence suggests that the predominant circulating HEV is GT-I, which has not been shown to be zoonotic in nature. Also, current phylogenetic analysis HEV GT-I strains do not support the existence of a low-virulence subtype, as most sequenced strains have been linked to clinical disease, either from sporadic cases or large outbreaks (47).

**Limitations**

One of the major difficulties in designing these studies was the absence of reliable prior data on HEV in Bangladesh. Several calculations, including sample size estimates, were made with the assumption of an underlying similarity between the Matlab population and rural communities in neighboring countries. Data on the burden of clinical HEV infection in urban centers of Bangladesh were likely to be inaccurate due to an important referral bias; treatment seeking and good health care in Bangladesh are closely linked with socio-economic factors, so any association between low SES and infection risk for a virus would likely result in the under-representation of that virus in the etiologic breakdown reported from urban, clinic-based studies. Although the prevalence and incidence studies were adequately powered to estimate those population characteristics of
HEV, the case-control studies were likely underpowered to accurately identify small, but potentially important, differences between groups -- especially for ubiquitous exposures such as animal husbandry or the use of animal dung.

Although assay reliability and laboratory errors are always a concern, the extensive experience of the AFRIMS Virology Department and several layers of laboratory quality control (QC) and redundancy measures served to minimize the likelihood of laboratory or assay-related problems. Positive specimens were repeated on the same plate, and ultimately showed low inter-assay variability. Commercial assays used by AFRIMS were extensively tested prior to use, and represented the latest-generation assays commercially available for anti-HAV, anti-HBc, and anti-HCV, with high sensitivity and specificity. AFRIMS, a recognized leader in the field of HEV research, maintains close collaborations with other HEV research groups, including Robert Purcell's hepatitis lab at NIH/NIAID and the active HEV study groups at Genelabs and GlaxoSmithKline. The assays and algorithms used in this study have been extensively tested in both laboratory and field settings in South Asia, with high sensitivity and specificity (264;265;286).

Although several months of extensive field testing went into the development of a locally-appropriate visual algorithm for acute hepatitis illness, it may not have been sensitive enough to capture low-grade or mild illness attributable to HEV. This would have led to an underestimation of the actual hepatitis E disease rate, and consequently, the disease to infection ratio. Also, it is difficult to ascertain whether this algorithm approach was equally sensitive across age groups in this cohort. Algorithm specificity is less of an issue as each potential case was anti-HEV confirmed by EIA.
In terms of the comparability of findings from the Matlab area, many have begun to question whether, after over three decades of research and intervention, this population is less representative of rural Bangladesh as a whole. Several health education-based interventions may have created a high level of community awareness that surface water is unsafe for consumption, especially following the recent discovery of extensive arsenic contamination of shallow aquifers. This knowledge may have led to some amount of reporting bias, as participants may have provided inaccurate information on actual exposure to unsafe water, attempting to give 'desirable' responses to interviewers. However, the data does reflect the ubiquitous use of surface water for cooking, which suggests that if this bias existed, it was likely minimal. Proxy interviews for young children or disabled persons may also incorrectly estimate exposures, especially if recall bias was significant in parents of sick children (cases).

As the sampling frame for the first two specific aims and the control group is based on a large, heterogeneous population, there was some potential for participation or response bias. The refusals and losses to follow-up were examined closely for any important differing characteristics, which were discussed in previous sections. The HEV study surveillance system relied on regular CHRW visits to identify incident cases early in their clinical course, reducing the potential for prevalence-incidence bias. The average duration of clinical illness normally ranges from two to four weeks, depending on severity, which allowed sufficient time to be captured by the CHRW surveillance network.

Finally, membership bias (to a cohort under surveillance) may limit the generalizability of study findings to the country at large. Recent investigations of the
spectrum of infections and the rates of other enteric infectious diseases (i.e. cholera, diarrheal diseases) have shown very small differences between the Matlab population and other rural Bangladesh sites (10). The continued occurrence of annual diarrheal epidemics in Matlab indicates that the environmental conditions there are still favorable for the maintenance and spread of enteric pathogens (K. Zaman, Personal Communication, 2006). However, the high rate of infant measles vaccinations (with subsequent reduced infant mortality) and high contraceptive use may imply local health behaviors different from most of rural Bangladesh; this would result in an underestimation of both the burden of hepatitis E disease and the risk of HEV infection in the country. On the other hand, this improvement in the Matlab population's health-seeking behavior might also have led to improved reporting of hepatitis-like illness.

**Strengths**

These studies were meticulously organized to maximize structural efficiency and accurate data collection. Study organizational tools such as Daily Activity Diaries and Weekly Performance Reports were used to maximize supportive supervision and oversight of field team activities, as well as to actively report progress to study investigators. Refusal Logs were maintained for each study, to document and analyze reasons for non-participation, and to improve the field teams’ efforts in improving compliance and minimizing attrition. All study contacts and assay outcomes were centrally maintained in manual and electronic “tracking logs” (eg. Case Tracking Logbook, Control Tracking Logbook) which allowed supervisory staff to instantly assess performance and study progress. Examples of these tools are supplied as appendices.
All field procedures were documented prior to training, and Field Operations Manuals were written for study supervisors and field workers for each aspect of the study. These were translated into the Bangla language and made available to all the HEV Study team members. Standardized operational definitions were used to ensure homogeneity in the way interviewers explained questions in the field.

Questionnaires were designed using either extensively pre-tested modules from demographic and health studies in Matlab and elsewhere in Bangladesh, but were again pilot-tested for local comprehension and to incorporate specific differences in local dialect. Field testing also ensured that the maximum number of possible options were included as structured response codes for each question. Questionnaires were finally back-translated into English to ensure that the intent of the questions was preserved and the codes were correctly sequenced. Codebooks were also designed to facilitate data entry programming and data analysis. All data was recorded on custom-designed, press-printed, color-coded forms and questionnaires to prevent the loss of any data. Questionnaires were designed using layouts that maximize both interview and data entry accuracy.

Although all study interviewers were experienced in interviewing and data collection, extensive refresher training sessions were held to first standardize character and digit writing to reduce the possibility of data entry errors. Redundant measures were also enforced (circling response codes) to ensure the correct code was data entered for every field. Interviewers were also re-trained on interview technique, appropriate probing techniques, and methods of minimizing inconvenience to the participant while
maximizing the accuracy of responses (eg. waiting until the subject completed household chores, provided care to young children, etc., before interviewing).

To maximize the consent of selected healthy individuals to provide a small fingerstick sample (in the baseline and follow-up studies, and the controls in the third study), extensive initial field testing was performed to assess social restrictions in this community to providing blood. Many local beliefs about the scarcity of blood in the human body make blood drawing from “healthy” individuals extremely challenging. It is believed that the loss of a single drop blood may take 40 days to regenerate; in this context, where many perceive themselves to be generally “ill” or “poorly-nourished”, a cut or finger prick leading to blood loss is taken quite seriously. Information sheets were prepared with standard responses to help field teams address common questions about blood specimens and the potential consequences (or lack thereof) to a participant’s health.

In terms of field QC, random, unscheduled supervisory visits were regularly performed every week to observe field teams and ensure the strict adherence to study protocols. Interviewers were given periodic feedback, as a group, to avoid introducing any individual-level biases. Unfortunately, we were unable to mask the interviewers to the case status of the interviewee, to reduce potential interviewer bias, given the acute illness of the cases. HEV Study supervisors also attended the monthly CHRW group meetings, during which time any issues regarding the ongoing surveillance for acute hepatitis illness were discussed. During the initial phases of the surveillance system, the CHRWs were shadowed by HEV Study team members, to ensure adherence to the protocol, and correct calculation of hepatitis illness scores from the algorithm.
After the completion of interviews, questionnaires were entered into custom-designed FoxPro data entry programs formatted with skip and jump patterns and range/error checking. A random sample of questionnaires was double-entered for quality assurance purposes and error-rate estimation. During analysis, any data inconsistencies were investigated manually against the responses provided in the physical questionnaire, or in the laboratory reporting forms.

Summary

These data illustrate, as accurately as possible, the current epidemiologic profile of HEV in a representative community of rural Bangladesh. The meticulous organization of the studies themselves, nested within the well-defined infrastructure of the Matlab HDSS, provided the necessary elements for high-quality epidemiologic investigation. The intersection of expertise from Johns Hopkins, ICDDR,B, and AFRIMS all strengthened the scientific caliber of these studies.

These studies estimated prevalence and incidence rates of HEV in a non-epidemic population, under sporadic transmission conditions. Some insights into possible environmental sources of HEV were provided, and other, more predictable associations between improved hygiene and protection from illness were confirmed. Some epidemiologic questions have been addressed by these studies, whereas other important questions have been raised. As HEV continues to gain recognition as an important contributor to the global burden of infection and illness, not only in the developing world, continued research in this field is warranted and necessary to prevent death and morbidity. We must seek to understand the role of this emerging pathogen in pregnancy and subsequently, maternal mortality in developing countries. The cohorts established in
these studies offer unique opportunities for long-term longitudinal follow-up to elucidate the persistent questions surrounding the human antibody response to HEV.

Finally, as the prospects for an HEV vaccine become more tangible, appropriate targeting strategies must be developed to optimize the cost:benefit ratio of such an intervention. Pre-conceptional vaccination may be an appropriate strategy to limit maternal deaths subsequent to HEV infection (or possibly, illness due to re-infection) in pregnancy. Vaccine effectiveness in a population context to prevent infections or hepatitis E illness could also be assessed in this cohort, given the demonstrated high rate of infections in a short period of time. As HEV researchers, we must continue to pursue the main ‘unanswered’ questions of HEV epidemiology, the answers to which will bring us closer to eventually controlling and reducing the global costs of this elusive emerging pathogen.
References Part I: Tables
### Table 1. Prevalence of antibodies to hepatitis E virus (HEV) in healthy and clinical populations (Indian Subcontinent). Studies listed by country, ranked by sample size.*

<table>
<thead>
<tr>
<th>Population Sampled</th>
<th>Total</th>
<th>Age (Mean)</th>
<th>Calendar Period</th>
<th>% HEV Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nepal (81)</td>
<td>4486</td>
<td>7 - 30</td>
<td>1992-1993</td>
<td>10</td>
</tr>
<tr>
<td>India (287)</td>
<td>2070</td>
<td>&lt;10**</td>
<td>1996</td>
<td>26</td>
</tr>
<tr>
<td>Nepal (25)</td>
<td>692</td>
<td>Adult</td>
<td>1995</td>
<td>30</td>
</tr>
<tr>
<td>India (56)</td>
<td>664</td>
<td>1 - 45+</td>
<td>1992</td>
<td>26</td>
</tr>
<tr>
<td>India (57)</td>
<td>600</td>
<td>1 - 41+</td>
<td>2004</td>
<td>4</td>
</tr>
<tr>
<td>India (291)</td>
<td>500</td>
<td>All Ages</td>
<td>2000</td>
<td>36</td>
</tr>
<tr>
<td>India (56)</td>
<td>474</td>
<td>1 - 45+</td>
<td>1982</td>
<td>18</td>
</tr>
<tr>
<td>Tibet (292)</td>
<td>426</td>
<td>8 - 49</td>
<td>2006</td>
<td>31</td>
</tr>
<tr>
<td>Myanmar (293)</td>
<td>371</td>
<td>7 - 80</td>
<td>2001</td>
<td>32</td>
</tr>
<tr>
<td>Bangladesh (88)</td>
<td>273</td>
<td>15 - 70</td>
<td>1996</td>
<td>60</td>
</tr>
<tr>
<td>India (162)</td>
<td>250</td>
<td>0 - 50</td>
<td>1994</td>
<td>4</td>
</tr>
<tr>
<td>India (196)</td>
<td>185</td>
<td>0 - 12</td>
<td>2003</td>
<td>17</td>
</tr>
<tr>
<td>Nepal (254)</td>
<td>114</td>
<td>Adult (31)</td>
<td>1995</td>
<td>37</td>
</tr>
<tr>
<td>Pakistan (254)</td>
<td>109</td>
<td>Adult (32)</td>
<td>1995</td>
<td>62</td>
</tr>
<tr>
<td>India (254)</td>
<td>107</td>
<td>Adult (30)</td>
<td>1995</td>
<td>37</td>
</tr>
<tr>
<td>Bangladesh (254)</td>
<td>105</td>
<td>Adult (31)</td>
<td>1995</td>
<td>27</td>
</tr>
<tr>
<td>Vietnam (129)</td>
<td>100</td>
<td>Adult</td>
<td>1994</td>
<td>38</td>
</tr>
<tr>
<td>Nepal (82)</td>
<td>94</td>
<td>7 - 73 (32)</td>
<td>1993</td>
<td>12</td>
</tr>
<tr>
<td>India (116)</td>
<td>75</td>
<td>0 - 18</td>
<td>1997</td>
<td>64</td>
</tr>
<tr>
<td>India (162)</td>
<td>40</td>
<td>20 - 50 (33)</td>
<td>1988-1991</td>
<td>5</td>
</tr>
<tr>
<td>India (116)</td>
<td>20</td>
<td>&gt; 18</td>
<td>1997</td>
<td>50</td>
</tr>
<tr>
<td>Hepatitis Patients†</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>India (294)</td>
<td>381</td>
<td>All Ages</td>
<td>2000</td>
<td>17</td>
</tr>
<tr>
<td>India (162)</td>
<td>190</td>
<td>2 - 76 (35)</td>
<td>1988-1991</td>
<td>18</td>
</tr>
<tr>
<td>Bangladesh (259)</td>
<td>180</td>
<td>&lt;6 – 30+</td>
<td>2000</td>
<td>53</td>
</tr>
<tr>
<td>India (197)</td>
<td>172</td>
<td>≤ 14 (5.6)</td>
<td>1997-2000</td>
<td>24</td>
</tr>
<tr>
<td>Nepal (145)</td>
<td>154</td>
<td>All Ages</td>
<td>1997</td>
<td>56</td>
</tr>
<tr>
<td>Nepal (219)</td>
<td>95</td>
<td>Adult</td>
<td>1987-1988</td>
<td>95</td>
</tr>
<tr>
<td>Nepal (187)</td>
<td>76</td>
<td>13 - 72</td>
<td>1993</td>
<td>88</td>
</tr>
<tr>
<td>India (291)</td>
<td>75</td>
<td>All Ages</td>
<td>2000</td>
<td>53</td>
</tr>
<tr>
<td>India (77)</td>
<td>69</td>
<td>18 - 42</td>
<td>1996</td>
<td>33</td>
</tr>
<tr>
<td>Pakistan (295)</td>
<td>65</td>
<td>Adult (Pregnant)</td>
<td>2001</td>
<td>57</td>
</tr>
<tr>
<td>India (76)</td>
<td>57</td>
<td>Adult</td>
<td>1992-1994</td>
<td>25</td>
</tr>
<tr>
<td>India (24)</td>
<td>57</td>
<td>0 - 44</td>
<td>1993-1994</td>
<td>11</td>
</tr>
<tr>
<td>India (42)</td>
<td>25</td>
<td>All Ages</td>
<td>2006</td>
<td>56</td>
</tr>
<tr>
<td>Bangladesh (68)</td>
<td>19</td>
<td>Adult</td>
<td>1987</td>
<td>90</td>
</tr>
<tr>
<td>Patients with fulminant hepatitis (FH) or acute liver failure (ALF)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>India (296)</td>
<td>67</td>
<td>≤ 12 (5.8)</td>
<td>1997-2000</td>
<td>36</td>
</tr>
<tr>
<td>India (181)‡</td>
<td>50</td>
<td>Adult</td>
<td>1987-1993</td>
<td>39</td>
</tr>
<tr>
<td>India (174)‡</td>
<td>50</td>
<td>15 - 60</td>
<td>1988-1992</td>
<td>62</td>
</tr>
<tr>
<td>India (210)</td>
<td>40</td>
<td>0 - 13</td>
<td>1993-1994</td>
<td>45</td>
</tr>
<tr>
<td>Bangladesh (88)</td>
<td>22</td>
<td>18-60</td>
<td>1995-1996</td>
<td>64</td>
</tr>
</tbody>
</table>

* Prevalence in healthy populations was determined by anti-HEV IgG, IgM or IgG/IgM detected in participants and thus represents both historical and acute infections. Clinical prevalence (percent clinical illness attributable to HEV) was determined by anti-HEV IgM, serum HEV RNA or a positive IgM Western blot, indicating acute infection. Mean ages are listed when available.

† Study conducted among patients presenting with acute / clinical hepatitis or liver disease.

‡ Percent HEV prevalence determined by serum RNA from a subset of the total sample.

‡‡ Study was conducted in a pediatric hospitalized population not presenting with hepatic complaints.
Table 2. Seroprevalence of viral hepatitis infections in rural Bangladesh (Matlab).

<table>
<thead>
<tr>
<th>EIA Assay</th>
<th>Manufacturer</th>
<th>Tested (n)</th>
<th>Positive (n)</th>
<th>% Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-HEV (Total Ig)*</td>
<td>WRAIR</td>
<td>1134</td>
<td>146</td>
<td>12.9</td>
</tr>
<tr>
<td>Anti-HBc (Total Ig)</td>
<td>Abbott-MUREX</td>
<td>1080</td>
<td>380</td>
<td>35.2</td>
</tr>
<tr>
<td>Anti-HCV (IgG)</td>
<td>Abbott-MUREX</td>
<td>917</td>
<td>14</td>
<td>1.5</td>
</tr>
<tr>
<td>Anti-HAV (Total Ig)</td>
<td>Abbott-MUREX</td>
<td>124</td>
<td>116</td>
<td>93.5</td>
</tr>
</tbody>
</table>

* (≥ 40 WRAIR U/ml), using a cutoff of ≥ 20 WRAIR U/ml yields a % prevalence of 22.5%
<table>
<thead>
<tr>
<th>Age Group (years)</th>
<th>Antibody Test Result</th>
<th>Seroprevalence Estimates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>Anti-HEV Ig</td>
</tr>
<tr>
<td>1 - 5</td>
<td>149</td>
<td>1</td>
</tr>
<tr>
<td>6 - 10</td>
<td>116</td>
<td>6</td>
</tr>
<tr>
<td>11 - 15</td>
<td>137</td>
<td>11</td>
</tr>
<tr>
<td>16 - 20</td>
<td>110</td>
<td>19</td>
</tr>
<tr>
<td>21 - 25</td>
<td>89</td>
<td>15</td>
</tr>
<tr>
<td>31 - 35</td>
<td>72</td>
<td>17</td>
</tr>
<tr>
<td>36 - 40</td>
<td>87</td>
<td>18</td>
</tr>
<tr>
<td>41 - 45</td>
<td>64</td>
<td>10</td>
</tr>
<tr>
<td>46 - 50</td>
<td>54</td>
<td>5</td>
</tr>
<tr>
<td>51 - 55</td>
<td>40</td>
<td>7</td>
</tr>
<tr>
<td>56 - 60</td>
<td>34</td>
<td>5</td>
</tr>
<tr>
<td>61 - 65</td>
<td>38</td>
<td>7</td>
</tr>
<tr>
<td>66 - 70</td>
<td>21</td>
<td>2</td>
</tr>
<tr>
<td>71 - 75</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>76 - 80</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>81 - 85</td>
<td>6</td>
<td>2</td>
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<tr>
<td>86 - 90</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>1134</td>
<td>146</td>
</tr>
</tbody>
</table>

* (≥ 40 WRAIR U/ml)
# Table 4. Characteristics of enrolled subjects, in aggregate and by anti-HEV status.

<table>
<thead>
<tr>
<th>Demographic Characteristics</th>
<th>Total Population (n=1134)</th>
<th>Anti-HEV Ig Reactive (≥40 U/ml) (n=146)</th>
<th>Anti-HEV Ig Negative (&lt;40 U/ml) (n=988)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median Range</td>
<td>Median Range</td>
<td>Median Range</td>
</tr>
<tr>
<td>Age</td>
<td>24 (1 – 88)</td>
<td>32 (1 – 85)</td>
<td>22 (1 – 88)</td>
</tr>
<tr>
<td>Male MUAC (cm, n=307)</td>
<td>24.6 (17.6 – 34.6)</td>
<td>24.4 (20.8 – 29.6)</td>
<td>24.6 (17.6 – 34.6)</td>
</tr>
<tr>
<td>Female MUAC (cm, n=425)</td>
<td>24.4 (16.8 – 33.6)</td>
<td>24.6 (18.2 – 31.6)</td>
<td>24.4 (16.8 – 33.6)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>520 (45.9)</td>
<td>78 (53.4)</td>
<td>442 (44.7)</td>
</tr>
<tr>
<td>Female</td>
<td>614 (54.1)</td>
<td>68 (46.6)</td>
<td>546 (55.3)</td>
</tr>
<tr>
<td>Religion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muslim</td>
<td>956 (84.3)</td>
<td>125 (85.6)</td>
<td>831 (84.1)</td>
</tr>
<tr>
<td>Hindu</td>
<td>178 (15.7)</td>
<td>21 (14.4)</td>
<td>157 (15.9)</td>
</tr>
<tr>
<td>Marital Status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single (≤15y)</td>
<td>402 (35.5)</td>
<td>18 (12.3)</td>
<td>384 (38.9)</td>
</tr>
<tr>
<td>Single</td>
<td>147 (13.0)</td>
<td>29 (19.9)</td>
<td>118 (11.9)</td>
</tr>
<tr>
<td>Married</td>
<td>514 (45.3)</td>
<td>91 (62.3)</td>
<td>423 (42.8)</td>
</tr>
<tr>
<td>Divorced</td>
<td>7 (0.5)</td>
<td>1 (0.7)</td>
<td>6 (0.6)</td>
</tr>
<tr>
<td>Widowed</td>
<td>64 (5.6)</td>
<td>7 (4.8)</td>
<td>57 (5.8)</td>
</tr>
<tr>
<td>Employment Location</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indoors</td>
<td>532 (47.1)</td>
<td>81 (55.9)</td>
<td>451 (45.8)</td>
</tr>
<tr>
<td>Outdoors</td>
<td>598 (52.9)</td>
<td>64 (44.1)</td>
<td>534 (54.2)</td>
</tr>
<tr>
<td>1º Employment Category</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Housework</td>
<td>363 (32.0)</td>
<td>53 (36.3)</td>
<td>310 (31.4)</td>
</tr>
<tr>
<td>Farming / Fishing / Man. Labor</td>
<td>147 (13.0)</td>
<td>32 (21.9)</td>
<td>115 (11.6)</td>
</tr>
<tr>
<td>Own business / Rickshaw</td>
<td>75 (6.7)</td>
<td>17 (11.6)</td>
<td>59 (6.0)</td>
</tr>
<tr>
<td>Office-based Service</td>
<td>42 (3.7)</td>
<td>10 (6.9)</td>
<td>32 (3.2)</td>
</tr>
<tr>
<td>Student</td>
<td>281 (24.8)</td>
<td>24 (16.4)</td>
<td>257 (26.0)</td>
</tr>
<tr>
<td>Child</td>
<td>172 (15.2)</td>
<td>2 (1.4)</td>
<td>170 (17.2)</td>
</tr>
<tr>
<td>Other</td>
<td>53 (4.7)</td>
<td>8 (5.5)</td>
<td>45 (4.6)</td>
</tr>
<tr>
<td>2º Employment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1018 (89.8)</td>
<td>120 (82.2)</td>
<td>898 (90.9)</td>
</tr>
<tr>
<td>Any</td>
<td>116 (10.2)</td>
<td>26 (17.8)</td>
<td>90 (9.1)</td>
</tr>
<tr>
<td>Education</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None (Age ≤7)</td>
<td>172 (15.2)</td>
<td>1 (0.7)</td>
<td>171 (17.3)</td>
</tr>
<tr>
<td>None</td>
<td>263 (23.2)</td>
<td>40 (27.4)</td>
<td>223 (22.6)</td>
</tr>
<tr>
<td>Class 1-5</td>
<td>341 (30.1)</td>
<td>55 (37.7)</td>
<td>286 (29.0)</td>
</tr>
<tr>
<td>Class 6-11</td>
<td>304 (26.8)</td>
<td>42 (28.8)</td>
<td>262 (26.5)</td>
</tr>
<tr>
<td>Class 12+</td>
<td>54 (4.8)</td>
<td>8 (5.5)</td>
<td>46 (4.7)</td>
</tr>
<tr>
<td>Monthly Household Income</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ Taka 3000 (~US$ 45)</td>
<td>293 (25.8)</td>
<td>43 (29.5)</td>
<td>250 (25.3)</td>
</tr>
<tr>
<td>Taka 3000 – 4999 (US$ 45-75)</td>
<td>414 (36.5)</td>
<td>58 (39.7)</td>
<td>356 (36.0)</td>
</tr>
<tr>
<td>&gt; Taka 5000 (~US$ 75)</td>
<td>427 (37.7)</td>
<td>45 (30.8)</td>
<td>382 (38.7)</td>
</tr>
</tbody>
</table>

† $p < 0.001$, Nonparametric equality-of-medians k-sample test comparing reactive and negative groups

†† Comparison of MUAC was restricted to participants > 15 y.o., due to difficulty of comparing MUAC among rapidly growing infants. No significant differences.

* For this comparison, students and children reported their primary location of activity / study.

‡ $p < 0.05$, $\chi^2$ Test comparing reactive and negative groups.

** Comparison of groups excluded participants < 15 y.o., and therefore ineligible to be married. No significant difference.

*** Comparison of groups excluded employment categories of “Child” and “Student”. No significant difference.

‡‡ $p < 0.01$, $\chi^2$ Test comparing reactive and negative groups. Comparison excluded participants < 15 y.o.

¶ $p < 0.01$, $\chi^2$ Test comparing reactive and negative groups. Comparison excluded participants < 7 y.o., and therefore ineligible for school. No significant difference.
Table 5. Characteristics associated with anti-HEV seroprevalence.

**Model 1: Anti-HEV Ig ≥ 40 WRAIR U/ml**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>(n=1130)</th>
<th>Unadjusted</th>
<th>Adjusted Model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>OR</td>
<td>95% C.I.</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 15</td>
<td></td>
<td>0.22</td>
<td>0.13 – 0.37</td>
</tr>
<tr>
<td>16 – 30</td>
<td></td>
<td>1.71</td>
<td>1.18 – 2.48</td>
</tr>
<tr>
<td>30 – 44</td>
<td></td>
<td>1.89</td>
<td>1.27 – 2.81</td>
</tr>
<tr>
<td>≥ 45</td>
<td></td>
<td>1.29</td>
<td>0.86 – 1.95</td>
</tr>
<tr>
<td><strong>Gender (0=Male, 1=Female)</strong></td>
<td></td>
<td>0.71</td>
<td>0.50 – 1.00</td>
</tr>
<tr>
<td><strong>Work Location (0=In, 1=Out)</strong></td>
<td></td>
<td>0.67</td>
<td>0.47 – 0.95</td>
</tr>
</tbody>
</table>

**Model 2: Anti-HEV Ig ≥ 20 WRAIR U/ml**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>(n=1130)</th>
<th>Unadjusted</th>
<th>Adjusted Model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>OR</td>
<td>95% C.I.</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 15</td>
<td></td>
<td>0.15</td>
<td>0.10 – 0.23</td>
</tr>
<tr>
<td>16 – 30</td>
<td></td>
<td>1.55</td>
<td>1.15 – 2.11</td>
</tr>
<tr>
<td>30 – 44</td>
<td></td>
<td>2.49</td>
<td>1.80 – 3.44</td>
</tr>
<tr>
<td>≥ 45</td>
<td></td>
<td>1.59</td>
<td>1.15 – 2.20</td>
</tr>
<tr>
<td><strong>Gender (0=Male, 1=Female)</strong></td>
<td></td>
<td>0.71</td>
<td>0.53 – 0.94</td>
</tr>
<tr>
<td><strong>Work Location (0=In, 1=Out)</strong></td>
<td></td>
<td>0.76</td>
<td>0.57 – 1.01</td>
</tr>
</tbody>
</table>
Table 6. Frequency of Dual-Antibody Seroprevalence to HBV Core, HCV, HEV.

<table>
<thead>
<tr>
<th>Antibodies to</th>
<th>HEV + HBc</th>
<th>HEV + HCV</th>
<th>HBc + HCV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number Positive (%)</td>
<td>60 (5.6%)</td>
<td>5 (0.6%)</td>
<td>6 (0.7%)</td>
</tr>
<tr>
<td>Number Tested for both</td>
<td>1080</td>
<td>917</td>
<td>866</td>
</tr>
<tr>
<td>(X^2) p-value</td>
<td>0.024</td>
<td>0.008</td>
<td>0.559</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antibodies to</th>
<th>HEV** + HBc</th>
<th>HEV** + HCV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number Positive (%)</td>
<td>117 (10.8%)</td>
<td>9 (1.0%)</td>
</tr>
<tr>
<td>Number Tested for both</td>
<td>1080</td>
<td>917</td>
</tr>
<tr>
<td>(X^2) p-value</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* Anti-HEV total Ig > 40, anti-HBc total Ig, anti-HCV IgG
** Anti-HEV total Ig > 20, anti-HBc total Ig, anti-HCV IgG
Table 7. Seroprevalence of viral hepatitis infections in rural Bangladesh at three timepoints.

<table>
<thead>
<tr>
<th>EIA Assay</th>
<th>12/’03 to 04/’04 Baseline Seroprevalence % (n/N)</th>
<th>12/’04 to 04/’05 12-Month Follow-Up Seroprevalence % (n/N)</th>
<th>06/’05 to 11/’05 18-Month Follow-Up Seroprevalence % (n/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-HEV (Total Ig)*</td>
<td>22.5 (255/1134)</td>
<td>26.3 (270/1025)</td>
<td>24.4 (247/1011)</td>
</tr>
<tr>
<td>Anti-HBc (Total Ig)**</td>
<td>35.2 (380/1080)</td>
<td>38.4 (369/960)</td>
<td>35.3 (357/1011)</td>
</tr>
<tr>
<td>Anti-HCV (IgG)**</td>
<td>1.5 (14/917)</td>
<td>Not Tested</td>
<td>Not Tested</td>
</tr>
<tr>
<td>Anti-HAV (Total Ig)**</td>
<td>93.5 (116/124)</td>
<td>Only &lt;10y Tested</td>
<td>Only &lt;10y Tested</td>
</tr>
</tbody>
</table>

* (≥ 20 WRAIR U/ml)  
** Abbott-MUREX Commercial Assay
Table 8. Age-specific anti-HEV seroincidence in rural Bangladesh. *

<table>
<thead>
<tr>
<th>Age Group (years)</th>
<th>N (%)</th>
<th>0 – 12 Months Seroincidence Estimates **</th>
<th>12 – 18 Months Seroincidence Estimates</th>
<th>Overall Rates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. Susceptible</td>
<td>Losses</td>
<td>Infections</td>
</tr>
<tr>
<td>1 – 10</td>
<td>265 (23.4)</td>
<td>255</td>
<td>30</td>
<td>3</td>
</tr>
<tr>
<td>11 – 20</td>
<td>247 (21.8)</td>
<td>200</td>
<td>22</td>
<td>4</td>
</tr>
<tr>
<td>21 – 30</td>
<td>177 (15.6)</td>
<td>126</td>
<td>14</td>
<td>6</td>
</tr>
<tr>
<td>31 – 40</td>
<td>159 (14.0)</td>
<td>93</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>41 – 50</td>
<td>118 (10.4)</td>
<td>86</td>
<td>6</td>
<td>16</td>
</tr>
<tr>
<td>51 – 60</td>
<td>74 (6.5)</td>
<td>52</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>61+</td>
<td>94 (8.3)</td>
<td>67</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>1134</td>
<td>879</td>
<td>84</td>
<td>49</td>
</tr>
</tbody>
</table>

* Seronegative individual experiencing an increase in total anti-HEV Ig to > 20 WRAIR U/ml
** p < 0.001, log-rank test comparing infection rates between age categories
† PY: Person-Years.
The Incidence Density is calculated as events per person-years: \((\# \text{ of Infections}) / (\# \text{ of Non-events} + 0.5(\# \text{ Infections} + \# \text{ Losses})) \times 1000\)
Non-events: \((\# \text{ Susceptible} - (\# \text{ Losses} + \# \text{ Infections}))\)
Table 9. Characteristics of susceptible participants at baseline and seroconverters at 12 months / 18 months follow-up.

<table>
<thead>
<tr>
<th>Demographic Characteristics</th>
<th>Baseline Susceptibles (n=879)</th>
<th>Seroconverters (0-12M) (n=49)</th>
<th>Seroconverters (12-18M) (n=26)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-10</td>
<td>255/879 (29.0)</td>
<td>3/49 (6.1)</td>
<td>7/26 (26.9)</td>
</tr>
<tr>
<td>11-20</td>
<td>200/879 (22.8)</td>
<td>4/49 (8.2)</td>
<td>7/26 (26.9)</td>
</tr>
<tr>
<td>21-30</td>
<td>126/879 (14.3)</td>
<td>6/49 (12.2)</td>
<td>5/26 (19.2)</td>
</tr>
<tr>
<td>31-40</td>
<td>93/879 (10.6)</td>
<td>9/49 (18.4)</td>
<td>3/26 (11.5)</td>
</tr>
<tr>
<td>41-50</td>
<td>86/879 (9.8)</td>
<td>16/49 (32.7)</td>
<td>0/26 (0)</td>
</tr>
<tr>
<td>51-60</td>
<td>52/879 (5.9)</td>
<td>5/49 (10.2)</td>
<td>4/26 (15.4)</td>
</tr>
<tr>
<td>60+</td>
<td>67/879 (7.6)</td>
<td>6/49 (12.2)</td>
<td>0/26 (0)</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>386/879 (43.9)</td>
<td>21/49 (42.9)</td>
<td>9/26 (34.6)</td>
</tr>
<tr>
<td>Female</td>
<td>493/879 (56.1)</td>
<td>28/49 (57.1)</td>
<td>17/26 (65.4)</td>
</tr>
<tr>
<td><strong>Nutritional Status</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>22/188 (11.7)</td>
<td>1/16 (6.3)</td>
<td>0/4 (0)</td>
</tr>
<tr>
<td>Female</td>
<td>77/315 (24.4)</td>
<td>7/27 (25.9)</td>
<td>1/11 (9.1)</td>
</tr>
<tr>
<td><strong>Religion</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muslim</td>
<td>739/879 (84.1)</td>
<td>41/49 (83.7)</td>
<td>21/26 (80.8)</td>
</tr>
<tr>
<td>Hindu</td>
<td>140/879 (15.9)</td>
<td>8/49 (16.3)</td>
<td>5/26 (19.2)</td>
</tr>
<tr>
<td><strong>Marital Status</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single (≤15y)</td>
<td>376/879 (42.8)</td>
<td>6/49 (12.2)</td>
<td>11/26 (42.3)</td>
</tr>
<tr>
<td>Single</td>
<td>97/879 (11.0)</td>
<td>3/49 (6.1)</td>
<td>1/26 (3.9)</td>
</tr>
<tr>
<td>Married</td>
<td>351/879 (39.9)</td>
<td>36/49 (73.5)</td>
<td>14/26 (53.9)</td>
</tr>
<tr>
<td>Divorced</td>
<td>6/879 (0.7)</td>
<td>0/49 (0)</td>
<td>0/26 (0)</td>
</tr>
<tr>
<td>Widowed</td>
<td>49/879 (5.6)</td>
<td>4/49 (8.2)</td>
<td>0/26 (0)</td>
</tr>
<tr>
<td><strong>Education</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None (&lt;7y)</td>
<td>170/879 (19.3)</td>
<td>2/49 (4.1)</td>
<td>7/26 (26.9)</td>
</tr>
<tr>
<td>None</td>
<td>185/879 (21.1)</td>
<td>17/49 (34.7)</td>
<td>6/26 (23.1)</td>
</tr>
<tr>
<td>Class 1-5</td>
<td>255/879 (29.0)</td>
<td>12/49 (24.5)</td>
<td>5/26 (19.2)</td>
</tr>
<tr>
<td>Class 6-11</td>
<td>232/879 (26.4)</td>
<td>17/49 (34.7)</td>
<td>6/26 (23.1)</td>
</tr>
<tr>
<td>Class 12+</td>
<td>37/879 (4.2)</td>
<td>1/49 (2.0)</td>
<td>2/26 (7.7)</td>
</tr>
<tr>
<td><strong>Monthly Household</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; Taka 3000 (~US$45)</td>
<td>222/879 (25.3)</td>
<td>12/49 (24.5)</td>
<td>7/26 (26.9)</td>
</tr>
<tr>
<td>Taka 3000 – 4999 (~US$45-75)</td>
<td>311/879 (35.4)</td>
<td>20/49 (40.8)</td>
<td>11/26 (42.3)</td>
</tr>
<tr>
<td>&gt; Taka 5000 (~US$75)</td>
<td>346/879 (39.4)</td>
<td>17/49 (34.7)</td>
<td>8/26 (30.8)</td>
</tr>
<tr>
<td><strong>Primary Employment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indoors</td>
<td>399/879 (45.6)</td>
<td>28/49 (57.1)</td>
<td>13/26 (50.0)</td>
</tr>
<tr>
<td>Outdoors</td>
<td>477/879 (54.5)</td>
<td>21/49 (42.9)</td>
<td>13/26 (50.0)</td>
</tr>
<tr>
<td><strong>Primary Employment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None/Housework</td>
<td>263/455 (57.8)</td>
<td>26/42 (61.9)</td>
<td>11/16 (68.8)</td>
</tr>
<tr>
<td>Farming/Fishing/Labor</td>
<td>91/455 (20.0)</td>
<td>6/42 (14.3)</td>
<td>1/16 (6.3)</td>
</tr>
<tr>
<td>Own business/Rickshaw</td>
<td>40/455 (8.8)</td>
<td>8/42 (19.1)</td>
<td>2/16 (12.5)</td>
</tr>
<tr>
<td>Office-based service</td>
<td>27/455 (5.9)</td>
<td>0/42 (0)</td>
<td>2/16 (12.5)</td>
</tr>
<tr>
<td>Other</td>
<td>34/455 (7.5)</td>
<td>2/42 (4.8)</td>
<td>0/16 (0)</td>
</tr>
</tbody>
</table>

*p<0.001, \( \chi^2 \) between incident cases and non-seroconverters, significant only between 0-12 months. Logrank test of incidence between age categories also significant (p<0.001)

**Percent malnourished defined by MUAC<22.5. MUAC was restricted to participants > 15 y.o. No significant difference between incident cases and non-seroconverters.

†Comparison of groups excluded participants ≤15 y.o., and therefore ineligible to be married. No significant difference.

‡Comparison of groups excluded participants < 7 y.o., and therefore ineligible for school. No significant difference.

§Comparison excluded participants <15 y.o. due to non-significant employment prior to this age. No significant difference.
Table 10. Self-reported morbidities among seroconverters and non-seroconverters between baseline and 12-months of follow-up, by seroconversion cutoff.

<table>
<thead>
<tr>
<th>Symptom</th>
<th>BL to 12M , &gt; 20 U/ml Cutoff</th>
<th>BL to 12M , &gt; 40 U/ml Cutoff</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-Seroconverters (N = 746)</td>
<td>Seroconverters (N = 49)</td>
</tr>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>Anorexia</td>
<td>307 (41.2)</td>
<td>21 (42.9)</td>
</tr>
<tr>
<td>Nausea / Vomiting</td>
<td>209 (28.0)</td>
<td>15 (30.6)</td>
</tr>
<tr>
<td>Severe Weakness</td>
<td>208 (27.9)</td>
<td>18 (36.7)</td>
</tr>
<tr>
<td>Yellow Eyes / Skin *</td>
<td>38 (5.1)</td>
<td>6 (12.2)</td>
</tr>
<tr>
<td>Fever</td>
<td>274 (36.7)</td>
<td>13 (26.5)</td>
</tr>
<tr>
<td>Clay Colored-Stools</td>
<td>49 (6.6)</td>
<td>1 (2.0)</td>
</tr>
<tr>
<td>Dark Urine</td>
<td>57 (7.6)</td>
<td>4 (8.2)</td>
</tr>
<tr>
<td>Upper Right Quadrant /</td>
<td>206 (27.6)</td>
<td>18 (36.7)</td>
</tr>
<tr>
<td>Liver Pain</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* p<0.05, $\chi^2$ test for differences between groups at ≥ 20 U/ml cutoff
Table 11. Sign and symptom-based visual algorithm for identifying potential cases.

<table>
<thead>
<tr>
<th>Components of Illness Score</th>
<th>Points</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nausea or Vomiting</td>
<td>1</td>
</tr>
<tr>
<td>Fever or Lassitude (such that normal work is impeded)</td>
<td>1</td>
</tr>
<tr>
<td>Anorexia (or repulsion by smell of food)</td>
<td>2</td>
</tr>
<tr>
<td>Yellow Eyes or Yellow Skin</td>
<td>2</td>
</tr>
<tr>
<td>Dark Urine or Ash/Clay-colored stools</td>
<td>2</td>
</tr>
</tbody>
</table>
Table 12. Correlation between AFRIMS and commercial anti-HEV IgM Assay.

<table>
<thead>
<tr>
<th></th>
<th>AFRIMS</th>
<th></th>
<th>Total</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MBS**</td>
<td>19</td>
<td>24</td>
<td>43</td>
<td>PPV: 44.2%</td>
</tr>
<tr>
<td>-</td>
<td>6</td>
<td>220</td>
<td>226</td>
<td>NPV: 97.3%</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
<td>244</td>
<td>269</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sensitivity</td>
<td>Specificity</td>
<td>88.5% Agreement</td>
<td>Kappa: 0.30</td>
</tr>
<tr>
<td></td>
<td>76.0%</td>
<td>90.2%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* AFRIMS in-house anti-HEV IgM Immunoassay as described in Myint 2006 (190)
** Anti-HEV IgM Enzyme Immunoassay 1056, Medical Biological Service (MBS), Milano, Italy
### Table 13. Demographic characteristics of acute hepatitis E disease patients and age-matched controls.

<table>
<thead>
<tr>
<th>Demographic Characteristics</th>
<th>Conservative Case Definition</th>
<th></th>
<th>Liberal Case Definition</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cases (N=24)</td>
<td>Controls (N=68)</td>
<td>Odds Ratio (95% CI)</td>
<td>Cases (N=46)</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;15 years</td>
<td>6 (25.0)</td>
<td>23 (33.8)</td>
<td>-</td>
<td>14 (30.4)</td>
</tr>
<tr>
<td>16-30 years</td>
<td>14 (58.3)</td>
<td>40 (58.8)</td>
<td>-</td>
<td>19 (41.3)</td>
</tr>
<tr>
<td>&gt;30 years</td>
<td>4 (17.4)</td>
<td>5 (7.4)</td>
<td>-</td>
<td>13 (28.3)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>17 (70.8)</td>
<td>27 (39.7)</td>
<td>1.0</td>
<td>31 (67.4)</td>
</tr>
<tr>
<td>Female</td>
<td>7 (29.2)</td>
<td>41 (60.3)</td>
<td>0.34 (0.13-0.93)</td>
<td>15 (32.6)</td>
</tr>
<tr>
<td>Religion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muslim</td>
<td>14 (58.3)</td>
<td>37 (54.4)</td>
<td>1.0</td>
<td>33 (71.7)</td>
</tr>
<tr>
<td>Hindu</td>
<td>10 (41.6)</td>
<td>31 (45.6)</td>
<td>0.76 (0.28-2.03)</td>
<td>13 (28.3)</td>
</tr>
<tr>
<td>Marital Status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single (&lt;15y)</td>
<td>6 (25.0)</td>
<td>23 (33.8)</td>
<td>-</td>
<td>14 (30.4)</td>
</tr>
<tr>
<td>Married</td>
<td>11 (45.8)</td>
<td>30 (44.1)</td>
<td>1.0</td>
<td>15 (32.6)</td>
</tr>
<tr>
<td>Household Size</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤4</td>
<td>7 (29.2)</td>
<td>15 (22.1)</td>
<td>0.37 (0.04-3.70)</td>
<td>17 (37.0)</td>
</tr>
<tr>
<td>5-6</td>
<td>12 (50.0)</td>
<td>32 (47.1)</td>
<td>1.04 (0.25-4.41)</td>
<td>19 (41.3)</td>
</tr>
<tr>
<td>≥7</td>
<td>5 (20.8)</td>
<td>21 (30.9)</td>
<td>0.51 (0.12-2.15)</td>
<td>11 (23.9)</td>
</tr>
<tr>
<td>Primary Employment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None / Housework</td>
<td>2 (9.5)</td>
<td>14 (20.9)</td>
<td>1.0</td>
<td>4 (8.9)</td>
</tr>
<tr>
<td>Farming / Fishing / Labor</td>
<td>4 (19.1)</td>
<td>5 (7.5)</td>
<td>3.69 (0.53-25.5)</td>
<td>9 (22.0)</td>
</tr>
<tr>
<td>Own business / Rickshaw</td>
<td>3 (14.3)</td>
<td>4 (6.0)</td>
<td>0.99 (0.06-16.99)</td>
<td>6 (14.6)</td>
</tr>
<tr>
<td>Office-based service / Teaching</td>
<td>2 (9.5)</td>
<td>1 (1.5)</td>
<td>8.53 (0.48-151.78)</td>
<td>2 (9.1)</td>
</tr>
<tr>
<td>Student</td>
<td>9 (42.9)</td>
<td>38 (56.7)</td>
<td>1.88 (0.28-12.43)</td>
<td>17 (37.0)</td>
</tr>
<tr>
<td>Minor Child</td>
<td>1 (4.8)</td>
<td>5 (7.5)</td>
<td>1.15 (0.03-41.76)</td>
<td>3 (7.3)</td>
</tr>
<tr>
<td>Gross Nutritional Status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MUAC ≥ 22.5 cm</td>
<td>12 (66.7)</td>
<td>23 (31.1)</td>
<td>1.0</td>
<td>21 (65.6)</td>
</tr>
<tr>
<td>MUAC &lt; 22.5 cm</td>
<td>6 (33.3)</td>
<td>22 (31.8)</td>
<td>0.64 (0.16-2.51)</td>
<td>11 (34.4)</td>
</tr>
<tr>
<td>Education</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>2 (8.3)</td>
<td>8 (11.8)</td>
<td>1.0</td>
<td>6 (13.0)</td>
</tr>
<tr>
<td>Class 1-5</td>
<td>12 (50.0)</td>
<td>26 (38.2)</td>
<td>2.17 (0.35-13.34)</td>
<td>21 (45.7)</td>
</tr>
<tr>
<td>Class 6+</td>
<td>10 (41.7)</td>
<td>34 (50.0)</td>
<td>1.16 (0.20-6.93)</td>
<td>19 (41.3)</td>
</tr>
<tr>
<td>Monthly Household Income</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; Taka 3000 (&lt;US$45)</td>
<td>2 (8.3)</td>
<td>7 (10.3)</td>
<td>1.0</td>
<td>7 (15.2)</td>
</tr>
<tr>
<td>Taka 3000–4999 (&lt;US$45-75)</td>
<td>16 (66.7)</td>
<td>33 (48.5)</td>
<td>1.39 (0.26-7.37)</td>
<td>25 (54.4)</td>
</tr>
<tr>
<td>&gt; Taka 5000 (&gt;US$75)</td>
<td>6 (25.0)</td>
<td>28 (41.2)</td>
<td>0.64 (0.11-3.75)</td>
<td>14 (30.4)</td>
</tr>
<tr>
<td>Household Construction</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Impermanent (Thatch, mud, reeds)</td>
<td>4 (16.7)</td>
<td>9 (13.2)</td>
<td>-</td>
<td>7 (15.2)</td>
</tr>
<tr>
<td>Semi-permanent (Tin walls)</td>
<td>20 (83.3)</td>
<td>56 (82.4)</td>
<td>-</td>
<td>39 (84.8)</td>
</tr>
<tr>
<td>Permanent (Cement walls)</td>
<td>0 (0.0)</td>
<td>3 (4.4)</td>
<td>0.58 (0.28-1.22)</td>
<td>0 (0.0)</td>
</tr>
</tbody>
</table>

NB. Conservative case definition includes only individuals found to be anti-HEV IgM seropositive by the AFRIMS assay. The liberal case definition includes individuals found positive by either AFRIMS or the MBS assay.

* p<0.05, Univariate conditional (fixed effects) logistic regression comparing cases and controls
** Comparison of marital status excluded individuals ineligible for marriage as an exposure as age <15
Divorced/Widowed not shown (n=2)
† “Don’t Know” responses excluded, each category tested against “None/Housework”, adjusting for other categories of employment in a multivariate conditional logistic regression model.
¶ Poor nutritional status defined by Mid-upper Arm Circumference (MUAC) <22.5. MUAC comparison was restricted to participants > 15 y.o.
§ Comparison of education excluded individuals ineligible for schooling as an exposure as age < 7
† A household score was created based on the sum of ordinal values issued for the type of roof, floor and wall construction. The OR was calculated for every one unit increase in household score (p>0.05, NS)
Table 14. Exposure characteristics of acute hepatitis E patients and age-matched controls.

<table>
<thead>
<tr>
<th>Exposure Characteristics</th>
<th>Conservative Case Definition</th>
<th>Liberal Case Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cases (N=24)</td>
<td>Controls (N=68)</td>
</tr>
<tr>
<td><strong>Primary Employment</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indoors</td>
<td>11 (45.8)</td>
<td>40 (58.8)</td>
</tr>
<tr>
<td>Outdoors</td>
<td>13 (54.2)</td>
<td>28 (41.2)</td>
</tr>
<tr>
<td><strong>Livestock Ownership</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cows (Any)</td>
<td>5 (20.8)</td>
<td>27 (39.7)</td>
</tr>
<tr>
<td>Goats (Any)</td>
<td>5 (20.8)</td>
<td>19 (27.9)</td>
</tr>
<tr>
<td>Chickens (Any)</td>
<td>11 (45.8)</td>
<td>38 (55.9)</td>
</tr>
<tr>
<td>Ducks (Any)</td>
<td>14 (58.3)</td>
<td>34 (50.0)</td>
</tr>
<tr>
<td><strong>Recent</strong> travel to town/city</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>6 (25.0)</td>
<td>45 (66.2)</td>
</tr>
<tr>
<td>Any</td>
<td>18 (75.0)</td>
<td>23 (33.8)</td>
</tr>
<tr>
<td><strong>“Eating Out” frequency</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>17 (70.8)</td>
<td>57 (83.8)</td>
</tr>
<tr>
<td>&lt; 7 times / wk</td>
<td>5 (20.8)</td>
<td>6 (8.8)</td>
</tr>
<tr>
<td>7+ times / wk</td>
<td>2 (8.3)</td>
<td>5 (7.4)</td>
</tr>
<tr>
<td><strong>Recent “Jaundice” contact</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>17 (73.9)</td>
<td>64 (97.0)</td>
</tr>
<tr>
<td>Any</td>
<td>6 (26.1)</td>
<td>2 (3.0)</td>
</tr>
<tr>
<td><strong>Recent Traditional Healer Visit</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>5 (20.8)</td>
<td>59 (86.8)</td>
</tr>
<tr>
<td>Any</td>
<td>19 (79.2)</td>
<td>9 (13.2)</td>
</tr>
<tr>
<td><strong>Recent Injection</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>16 (66.7)</td>
<td>55 (80.9)</td>
</tr>
<tr>
<td>Any</td>
<td>8 (33.3)</td>
<td>13 (19.1)</td>
</tr>
<tr>
<td><strong>Type of Household Latrine</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Open/Hanging</td>
<td>16 (66.7)</td>
<td>30 (44.1)</td>
</tr>
<tr>
<td>Sealed / Slab (Sanitary)</td>
<td>8 (33.3)</td>
<td>38 (55.9)</td>
</tr>
<tr>
<td><strong>Drinking Water Source</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubewell</td>
<td>23 (100.0)</td>
<td>58 (85.3)</td>
</tr>
<tr>
<td>Pond</td>
<td>0</td>
<td>2 (2.9)</td>
</tr>
<tr>
<td>River</td>
<td>0</td>
<td>8 (11.8)</td>
</tr>
<tr>
<td><strong>Cooking Water Source</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubewell</td>
<td>2 (8.7)</td>
<td>3 (4.4)</td>
</tr>
<tr>
<td>Pond</td>
<td>17 (73.9)</td>
<td>43 (63.2)</td>
</tr>
<tr>
<td>River</td>
<td>4 (17.4)</td>
<td>22 (32.4)</td>
</tr>
<tr>
<td><strong>Average Water Consumption</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 7 glasses per day</td>
<td>7 (29.2)</td>
<td>37 (54.4)</td>
</tr>
<tr>
<td>≥ 8 glasses per day</td>
<td>17 (70.8)</td>
<td>31 (45.6)</td>
</tr>
</tbody>
</table>

NB. Conservative case definition includes only individuals found to be anti-HEV IgM seropositive by the AFRIMS assay. The liberal case definition includes individuals found positive by either AFRIMS or the MBS assay.

* “None” is not shown, comparison of livestock ownership uses “None” as the reference category (OR=1.0)
** Recent is defined as in the three months prior to the onset of illness / interview
† Defined as the number of times eating food prepared outside the home in an average week.
‡ p<0.005, Univariate conditional (fixed effects) logistic regression comparing cases and controls
§ p<0.05, Univariate conditional (fixed effects) logistic regression comparing cases and controls
†† In an age category stratified analysis (<15, 16-30, >30) only the ≤15 group remained significantly at risk, likely due to low n’s in each age category.
‡‡ This association likely reflects a local practice of seeking initial care from traditional “faith” healers early during hepatitis-like illnesses.
### Table 15. Multivariate conditional logistic regression model† of risk factors for HEV disease.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Manual (forced) Model*</th>
<th></th>
<th></th>
<th>Stepwise Selected Model**</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR</td>
<td>95% C.I.</td>
<td>P Value</td>
<td>OR</td>
<td>95% C.I.</td>
<td>P Value</td>
</tr>
<tr>
<td>Gender (0=Male, 1=Female) §, ‡</td>
<td>0.30</td>
<td>0.07 - 1.20</td>
<td>0.088</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Recent Exposure to “Jaundice” patient‡</td>
<td>63.50</td>
<td>8.07 - 499.50</td>
<td>&lt;0.000</td>
<td>82.50</td>
<td>8.77 - 776.39</td>
<td>&lt;0.000</td>
</tr>
<tr>
<td>Travel to town / city in past 3 months ‡</td>
<td>2.80</td>
<td>0.78 - 10.08</td>
<td>0.114</td>
<td>4.25</td>
<td>1.06 - 17.10</td>
<td>0.041</td>
</tr>
<tr>
<td>Unsanitary toilet use ‡</td>
<td>4.39</td>
<td>1.02 - 18.98</td>
<td>0.048</td>
<td>5.14</td>
<td>1.20 - 22.01</td>
<td>0.027</td>
</tr>
<tr>
<td>Work outside the home</td>
<td>19.36</td>
<td>1.39 - 269.75</td>
<td>0.027</td>
<td>19.80</td>
<td>1.89 - 207.96</td>
<td>0.013</td>
</tr>
<tr>
<td>Outdoor Work (Farming/Fishing/Labor )</td>
<td>4.17</td>
<td>0.73 - 23.78</td>
<td>0.108</td>
<td>8.63</td>
<td>1.33 - 56.09</td>
<td>0.024</td>
</tr>
<tr>
<td>Injection in the last 3 months</td>
<td>18.44</td>
<td>2.10 - 162.05</td>
<td>0.009</td>
<td>15.50</td>
<td>1.97 - 121.76</td>
<td>0.009</td>
</tr>
<tr>
<td>Household size (≤4, 5-6, ≥7 members)</td>
<td>0.49</td>
<td>0.29 - 0.84</td>
<td>0.009</td>
<td>0.17</td>
<td>0.05 - 0.56</td>
<td>0.004</td>
</tr>
<tr>
<td>Household construction score (1-6)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.35</td>
<td>0.13 - 0.98</td>
<td>0.045</td>
</tr>
</tbody>
</table>

† Only the models constructed using the liberal case definition are shown as the conservative case definition was extremely restricted by a low sample size. Variables also significant using the conservative model are those indicated above by an (‡).

* Model reflects explanatory variables of interest significant at the 25% level (p<0.25) in univariate conditional logistic regression, and retained at least 15% (p<0.15) significance in the multiple, adjusted model.

** Model selected by forward and reverse stepwise conditional logistic regression, including variables significant at the 25% level (p<0.25) in univariate analysis and retained in the final model if significant at the 10% (p<0.10) level.

§ Gender was left out of the stepwise model as female gender was strongly inversely correlated with work outside the home or with performing outdoor work. Stratified analysis was not possible or appropriate due to extremely small numbers in each cell.
References Part II: Figures
Figure 1. Geographic location and layout of the ICDDR,B Matlab Health Research Center.

Figure 2. Gender-specific population pyramid of Matlab, based on 2004 mid-year census data. *

Figure 3. Global distribution of hepatitis E virus. *

* Dark shaded areas represent areas where documented outbreaks of HEV infections have occurred or where clinical/serological evidence suggests that most sporadic non-A, non-B, non-C viral hepatitis is attributable to HEV. Striped areas indicate where HEV is endemic in swine and other animals, but where few human cases are documented, or where a small number of autochthonous cases of HEV have been reported (likely linked to zoonotic HEV). The absence of shading either represents non-endemicity or a lack of adequate data (eg. Russia).
Figure 4. Geographic distribution by year of the important enterically transmitted non-A, non-B hepatitis and confirmed HEV outbreaks in the Indian Subcontinent. *

* The two larger circles indicate the first documented epidemic in Delhi (1955-1956) and the largest South Asian epidemic in Kanpur, India (1991). A “?” indicates a suspected high seroprevalence area without any large reported outbreaks; the year beside these refers to the date of an HEV survey.
Figure 5. Immune electron micrograph of hepatitis E virus (HEV) particles and a suggested structural model *

Figure 6. An overview of the course of hepatitis E virus (HEV) infection based on studies of nonhuman primates, human volunteers and human outbreak studies *

* The dotted line represents the trajectory of anti-HEV Immunoglobulin M (IgM) after infection at 0 months. The dashed line follows the course of anti-HEV Immunoglobulin G (IgG) up to 5 months post infection. The top arrows define the acute and convalescent phases of the clinical illness. The gray bar shows the time during which HEV particles may be detectable in an infected patient's stool; the black bar represents the period when HEV antigen may be detectable in the liver; and the hashed bar represents the period of serum viremia, as detectable by polymerase chain reaction (PCR). The earliest sign of infection is viral replication in the liver, detectable by immunofluorescence of HEV antigen, immune electron microscopy of HEV particles or by PCR of HEV RNA (95). ALT, alanine aminotransferase.
**Figure 7.** Age-specific seroprevalence of anti-HEV *

* Anti-HEV total Ig ≥ 40 WRAIR U/mL, n=146
Figure 8. Age-specific anti-HEV seroprevalence estimates by gender (bars represent 95% confidence intervals)

* Anti-HEV total Ig ≥ 40 WRAIR U/mL, n=146
Figure 9. Enrollment and follow-up of the baseline cohort to estimate seroprevalence and incidence of antibodies to HEV.
Figure 10. Follow-up of baseline susceptibles by anti-HEV status over 18 months follow-up.**

* Seroconversion events used in incidence density calculations
** Gray-shading represents seroreversion; Black represents return to seropositivity after reversion
Figure 11. “Sero-reversion” among baseline anti-HEV seropositive cases (n=230) with 18 months of complete follow-up.

* By 12M, 9/230 (3.9%) revert to anti-HEV seronegativity (one not shown due to 18M loss to follow-up)

** Between 12 and 18M, 11/209 (5.3%) revert to anti-HEV seronegativity (2, shown as ---, return to seropositive status)
Figure 12. “Sero-reversion” among 12M anti-HEV seroconverters (n=42) after 6 months of additional follow-up.

* At 18M, 28/42 followed for 6 months (66.7%) reverted to anti-HEV seronegativity
Figure 13. Sample Visual Card used by CHRW to perform hepatitis illness surveillance.

(Front) 

(Back) 

(Transliteration)
Figure 14. Case and control screening and selection diagram

Potential Case Identification

First Tier Case Screening (MBS Assay)

Second Tier Case Screening (AFRIMS Assays *)

Control Screening (History of jaundice, Lifetime Illness Score > 4)

Control Screening (AFRIMS Assay *)

* Anti-HEV IgM ≥ 100 WRAIR U/ml OR Anti-HEV total Ig > 1000 U/ml

** Only 3 of these 8 interviews were successful, resulting in total 46 Case Interviews

† Anti-HEV Ig > 20 WRAIR U/ml withdrawn from control pool

---

22 Months Surveillance n=23,500

Illness Score > 5 n=279

Not Met 3 Times n=9

Specimen Obtained n=270

Not Met 3 Times n=1

Anti-HEV IgM- n=226

Anti-HEV IgM+ n=43

Anti-HEV - n=218

Anti-HEV + n=8 **

Anti-HEV - n=22

Anti-HEV + n=21

Potential Controls Visited n=268

Ineligible by history n=20

Not met > 3 times n=3

Moved out of study area n=51

Refused to participate n=6

Controls Recruited (4:1) n=188

Controls Dropped n=50

Final Controls n=134

Controls dropped b/c Case Not Met n=4
Figure 15. Conceptual framework of sporadic HEV infection and illness in rural Bangladesh.
References Part III : Appendices
Appendix A. HEV Study Field Operations Manual (for Field Team Use)

A random list will be generated by the HDSS of 1300 individuals from all over Matlab Intervention Area aged > 1 years. These individuals will be listed in the Baseline Survey Contact Logbook (BSCL), ordered by Block# and CHRW#. Each individual will be pre-assigned a Study Number, comprising of the letters BL and four digits. (The same study number will be used for the 12 and 18 month follow-ups, except the prefix BL will be changed to 12M or 18M.)

**Daily Activities**

**Morning Work Assignments**

Each morning of the surveys, the team will meet in the HEV Project Office, where the MO/SFRO will assign the day’s contact lists to the field teams (1 FRA, 1 Lab Tech), and review the plan of movement in the field.

**MO/SFRO**

The END of each workday, the MO/SFRO will help each team to complete the Daily Activity Diary (DAD) for the following day, using the BSCL, by completing the Study Number, CID, and Visit # of the individuals who should be visited the next day. This must be done the previous day, so that the appropriate transportation requisitions may be placed for the field teams.

The MO/SFRO will obtain pre-printed Identifiers sections for the appropriate questionnaire (Baseline, 12 months or 18 months Follow-Up Survey) and attach them to the form. This is done to avoid excess paper use for individuals who refuse consent.

The MO/SFRO will also attach using a paper clip, the appropriate consent form(s) for each questionnaire.
**Field Teams**

At the END of each workday, each field team will use the Daily Checklist (See Appendix A) to ensure that their equipments and supplies have been replenished for the following day’s activities. This will reduce the amount of time spent the next morning checking their supplies.

Before going to the field, the Field Teams must ensure that they have one questionnaire and one consent form per subject listed on their DAD, with the Identifiers section preprinted by the MO/SFRO. Each team must also take their Witness Logbook (WL) to the field to document the identity of the witness to the consent process.

**Field Activities**

**Finding the subjects**

When ready, the teams will go to the field, according to their action plan, and try to find the subject whose CID has been listed on their DAD. Transportation to Blocks A&B is available by vehicle, whereas Blocks C&D are normally accessed by speedboat. The appropriate requisitions will have been made the previous day by the MO/SFRO.

The Field Teams should use the identifiers section of their BLQ/FUQ forms to find the individuals.

**Not Met Scenario**

If the individual is not found in the field, the Field Team should write this in the Comments section of the BLQ/FUQ and also place a “0” in the Visit Complete column of the DAD, with accompanying comments. It is important that the Field Team tries to
determine when the individual will be available for a visit, and note this information in the Comments Section, and also in their notebooks, if additional space is needed.

Upon returning to the HEV office, they should inform this to the MO/SFRO, who will copy this information onto the BSCL, and re-schedule a second or third visit, as per the recommendations of the Field Team. The MO/SFRO should already pre-fill the Visit Date 2 on the BLQ, BSCL and also list the name on the appropriate page of the DAD, as well as the Visit # (First, Second, Third).

If the subject is not met three times, the field team will place a ‘2’ in the Form Status box of the Survey Questionnaire. The MO/SFRO will place a check mark in the Never Met column of the BSCL.

Obtaining Consent

Once a subject is identified, the Field Team should introduce themselves. They should then, depending on the age of the subject, administer the appropriate consent form. The Field Team should refer to the laminated “Consent Form Guideline” sheet to determine which consent form is appropriate.

The consent form should be read, and the consent or assent must be documented by signature or by thumbprint, as per ICDDR,B regulations. The Subject ID should be placed on the consent form. One witness must observe the procedure and the witness’s identity must be recorded in the WL.

Refusals

If any subject refuses to participate in the survey, the Field Team should place a ‘6’ in the form status box of the Survey Questionnaire. They should place a “1” in the
Visit Completed box of their DAD. The field team should also try to understand why the subject refuses to participate in the study and note this in their DAD.

At the end of the day, the MO/SFRO will then place a check mark in the “Visit completed” column of the BSCL Visit Record, and also complete the refusal logbook. The MO/SFRO may choose to make a follow-up visit to the subject if he feels that the subject may be motivated to participate.

Upon obtaining consent, the Field Team should first collect the fingerstick specimen. Fingerstick specimen collection procedures are described in Appendix B.

The BLQ, and FUQ should be completed according to the Guidelines for Completing the BLQ, FUQ, available in Bangla. Upon completion of the questionnaire, the Field Team should place a “1” in the form status box of the BLQ/FUQ. The field team should also place a “1” in the Visit Complete box of the DAD.

**Field Procedures Complete – Returning to the HEV Office**

After the collection of specimens and completion of the questionnaires the team will return to the HEV Office and explain their performance to SFRO and MO, according to their DAD. If any rescheduling is needed, the MO/SFRO will do this, as described above.

The MO/SFRO will also update the BSCL, and complete the Refusal Logbook, if needed. Any necessary advocacy or field visits can be planned at this time.

**Digital copies**

A digital copy of the BSCL / 12MCL / 18MCL should also be maintained in Excel format.

**Transmitting Samples to the Lab**
When the field teams return from the field, the Lab Technician on Laboratory Duty should be ready to receive the specimens from the field. All BL/12M/18M specimens should be kept in the cool box, with fresh ice packs until ready to be processed. The Lab Technician should have the Lab Processing Logbook ready.

The Lab Technician should check the sample IDs carefully, and ensure that a corresponding form is available for each sample ID. The Lab Technician should complete the Receiving section of the logbook, and then process the specimens as per protocol. After processing all specimens, the Lab Technician should copy the logbook into the digital copy of the Lab Logbook.

Two aliquots should be separated into separate Archive (AR) and Shipping (SH) boxes. Do not mix different specimen types (stool [Stool was ultimately not collected during the baseline / follow-up study, as it was not logistically feasible, nor necessary in the presence of a serologic anti-HEV assay.] / RBC / serum) in one freezer box. Do not mix Baseline and 12 Month follow-up specimens in one box. Box numbers should be labeled starting from 001 to 100 etc... Specimen type and study section should also be clearly indicated on the box cover and bottom (in case these get mixed up).

**Weekly Field Report**

At the end of each workweek, the MO/SFRO should submit a Weekly Field Report, with the appropriate performance statistics and cumulative totals. This report should be saved as a Word document: HEV Weekly Field Report_Sept XX_03.doc

The document should be printed and kept in the Weekly Field Report Binder file and one copy should be emailed to the Investigator Team: K. Zaman, A. Labrique, K. Nelson, J. Ticehurst.
During their monthly community visits, CHRWs in block C will visit every household along with the HEV Visual Card with the objective of identifying potential Hepatitis E clinical cases over a period of 45 months. (This period was expanded from 15 to 22 months to maximize the number of potential cases enrolled from this population surveillance.)

Our case definition for potential cases is $\leq 14$ days onset, a score of 6-8, and residence in Block C of the HDSS Area. From Round 1-7, these criteria were used. However, as the potential caseload was low, from Rounds 8-11, the recruitment criteria was loosened to include individuals with a score of 5-8. From Round 12 to 22, the onset time was increased to 30 days.

**HEV Visual Card**

This is a two-sided, color-printed card, on hard paper, which is laminated. The visual card also contains the guidelines for completion of the Hepatitis Surveillance Logbook (HSL). The pictures on the Card are used to guide the respondent through the questions of the HSL. After the respondent has given an answer to any question of the HSL (either Yes or No), the appropriate image should be shown for confirmation to the respondent. The images correspond to questions 6 and 7.

**Hepatitis Surveillance Logbook (HSL)**

Every CHRW in the intervention area is issued an HSL book, in two books. Each HSL is labelled Volume 1 (Surveillance Units 1-8) and 2 (Surveillance Units 9-15), splitting the total workload for one CHRW into...
two parts, for ease of carrying. Each page of this book refers to a different family in the CHRW’s area. The first book was valid for a period of three months, or three surveillance rounds. Each subsequent book was valid for a period of four months.

At each household visit, the CHRW will use the text side of the HSL to ask the question “Has anyone of your family members had __________”, where the blank is filled in with the options 1 through 8. The CHRW should try to meet with any member of the family who is capable of answering questions on the health status of the members of that family. If any response to the questions is Yes, then the appropriate score value will be given to that family member. Up to six separate family members can be tracked by the CHRW. The CHRW carries two colors of notification cards- one red, the other blue. Red cards inform the HEV team that a recent-onset, severe potential case has been identified, who may still be shedding virus for the virology aim. Blue cards inform the team that a recent potential case has been identified, but it is unlikely they are still shedding virus, and therefore will not be eligible for venous blood sampling, requiring the MO’s attention.

If the respondent claims that one of the family members has had “jaundice” (q1), then the CHRW should skip to q9, and ask the question 10, listed on the text side of the visual card. (THIS WAS CUT AS PRETESTING FOUND JAUNDICE TO BE VERY NON-PREDICTIVE FOR SYMPTOMS ASSOCIATED WITH HEPATITIS ILLNESS)

a) If the onset of the illness symptoms is within 7 days, the CHRW will fill up a Red Card. Once each day, a team of 2 project messengers visit each CHRW to collect any completed cards. The paramedics (SHRA)
of the sub-center will receive the card from the messenger and put it in the HEV mailbox. **A sum of 50 TK will be paid to the messenger.**

When a card is received, the SHRA phones the HEV Office and informs them that a card has been submitted. **On a daily basis, the HEV Messenger comes to the Matlab HEV office with this card and contacts the Medical Officer of the project.** The MO will ask the SHRA the age of the potential case. As per the CHR approved protocol, any person <15 years old will be excluded from the virology aim. As this is a red card, the MO must complete the Case Tracking Logbook, from which he obtains a Study ID number for the case. In the column labeled “Assigned to Team #” the MO should write “VIR”. The Medical Officer will visit the case immediately with his medical instrument box and a cool box with ice packs, in case venous blood specimens are obtained.

After confirming the identity of the case, the MO will repeat the CHW’s algorithm to verify whether in fact a score of 6, 7, or 8 is obtained. Only then will he administer consent (both for virology and case control) and motivate the patient to accompany him to a nearby sub-center for a physical examination and sample collection (venous blood and stool, if possible).

*See Virology Procedures for more details.*

The Medical officer then returns to the office and assures that the samples are processed and preserved at -80°C according to the procedure described in the Appendix E: Virology. A small aliquot (30ul) of the serum will be kept in (-20°C) until it can be sent to Dhaka for pre-enrollment procedures of Case Control Study in the same manner as which other non-virology specimens are handled.
If the onset of the illness symptoms is **between 8 and 14 days** (this was extended to 30 days from Rounds 12 to 22) the CHRW will fill up the Blue Card (Hepatitis Case Report) and wait for the arrival of the HEV Messenger. The HEV Messenger visits every CHRW of Block C once every day, according to a roster prepared and maintained by the SFRO. The Messenger will pick up any cards that the CHRW has filled out and carry it to the SubCenter.

**Case Tracking Logbook (CTL)**

Upon receipt of a Blue Card, the MO/SFRO will complete the Identifiers section of the digital Case Tracking Logbook (CTL). This process results in a Study ID (Case ID) being issued to that subject. The blue cards are then hole-punched and stored in a ring binder, by Study ID.

The case tracking logbook serves as the means of monitoring every interaction with each potential case, and as a guide for the MO/SFRO to ensure that the pre-enrollment processing takes place, enrollment interviews are completed and that control data is requested in a timely fashion. The CTL remains in the HEV Project Office, and is to be managed only by the MO/SFRO.

**Case Pre-Enrollment Form (CPE)**

Once the CTL is filled out, then a Case Pre-Enrollment form is filled out (Section A and B) for each blue card (or <15 red card) received. The MO/SFRO must staple the FORM and specimen labels to the HEV-CPE form to be used by the Field Teams. The MO/SFRO must complete the identifiers of the CPE.

**Daily Activity Diary (DAD)**
Once a CPE has been filled out and is ready for the Field Team, then the MO/SFRO should schedule the visit on the appropriate team’s DAD. The Field Team will conduct the visit. The Team will visit the potential Case and verify the CHRW’s scoring of the patient. If the score by the team is 6, 7 or 8, then ONLY will the team proceed to obtain consent for the Case Control study.

**Specimen Collection**

Fingerstick blood should be collected as described in Appendix B. Each specimen will be placed in the cool box as soon as possible. The Laboratory Technician will receive the samples from the Field Teams and process the specimen as described in Appendix D. As the specimen is a CA-prefixed sample, the Lab Technician must complete the Serum Transmittal Form (STF), which is used to send a small aliquot (30ul) of the specimen to Dhaka (ICDDRB – Virology Lab) for pre-testing. The STF is a carbon-copy pad, and one copy of each STF is preserved in the HEV Office by the MO/SFRO in the STF folder, by Study ID. Each pair of STF’s has an identical Serial Number (SN) on the top right hand corner of the form.

Each Dhaka aliquot must have an accompanying STF. The aliquots are placed in a sealed thermos, with an accompanying Ice Pack. The thermos is then placed in a hard-case cool box, which is clip-locked. The transmittal to Dhaka is then arranged by the MO/SFRO, who must ensure that the Office Attendant in Dhaka is ready to receive the box and transmit this to the Virology lab. The MO/SFRO should call ICDDRB, Dhaka (CHU) in advance to notify them that a specimen is on the way.
The lab in Dhaka will process the specimen, complete the STF copy which accompanied the specimen. These STFs will be filed at the lab, until they are picked up by the Office Attendant in Dhaka, when delivering the next specimen. These completed forms should then be transmitted to the Matlab HEV office as soon as possible through interoffice Mail.

The MO/SFRO should now update the Case Tracking Logbook (CTL), completing the Pre-Enrollment Processing columns. If the Hepatitis E test result is positive, then the MO/SFRO should proceed with the Case Control Questionnaire completion (CCQ) by scheduling a Field Team visit through the DAD.

**Completion of the Case Control Questionnaire (CCQ)**

The MO/SFRO should complete Identifiers section (A) of the CCQ. The Field Teams should then take this CCQ to the field, to visit the case, for whom consent has already been obtained. The Field Teams should read the brief statement to the individual regarding their positive test result before administering the CCQ.

As for the BLQ and FUQ, any not met visits should be recorded on the DAD, the top of the CCQ, after which, the MO/SFRO will reschedule another visit and complete the DAD and Case Tracking Logbook accordingly.

If a Case refuses to consent, then only the CCQ Cover Sheet is completed and the Continuation pages are NOT attached to the CCQ Cover Sheet. A code “6” is placed in the Form Status Box on the Cover Sheet.

**Control Selection**
After the Case interview is complete, the Control Selection process begins. A Control Data Request Form is completed and submitted to the DSS office, Matlab. The DSS provides 8 age-matched candidates as possible controls from the HDSS database.

Before adding any Name or CID to the Control Tracking Log, the MO/SFRO should check the Control Tracking Log to see if this individual has been previously selected as a Control. Placing the mouse cursor on the “C” Column head, and selecting the whole C column, then press “CTRL-F”, which brings up the FIND menu. Type in the CIDs of the new Controls (one at a time), to see if there is any overlap. If the control was previously selected, was eligible AND was visited for a previous Case, then this control is no longer eligible to be a control. A control may serve ONE TIME ONLY. HOWEVER, if the control was NOT MET in the previous selection, he is still eligible to serve as a control.

If the control is not eligible, his/her name and CID is NOT to be transferred from the DRF to the Control Tracking Log. **This person does NOT receive a Study ID twice.** If a control was not met a previous time and IS eligible, then he should be entered AGAIN in the Control Tracking Log, and should be issued a NEW Study Control ID.

The MO/SFRO should now transfer the eligible Identifiers (Name, CID, RID, Bari Name, Head Name, Sex, DOB) from the Control Data Request Form to the Control Tracking Log. The Control Study ID’s obtained from the tracking log should be noted on the Data Request Form for future reference in the last gray column of the DRF.

A total of eight controls are selected per case, with the objective of meeting and interviewing exactly four age-matched, block-matched, HEV seronegative controls per case. If one control is not met, the Team should move on to the next control on their list.
Of six potential controls, at least 4 should be enrolled, whenever possible. (Although the sampling frame requires only 2 controls, it is possible that some controls will turn out to be ineligible, then two “reserves” will be available to choose from.)

The MO/SFRO should keep track of each case’s Control set, and “close” the control search once they are satisfied that the 4 controls have been identified. If four controls are not recruited, then the MO/SFRO may choose to reschedule a visit to a previously not met control, OR resubmit a new Control Data Request Form.

Meeting and Enrolling a Control

When a Control subject is met, he is first put through the Screening Process for Controls using the Control Screening Card. The screening process aims to remove as candidates any person who may previously have had hepatitis E. As the issue of immunity is unclear, controls may not be exposed to the same “risk of illness” as a case due to pre-existing immunity. We reduce the likelihood of enrolling and interviewing “false” controls by this screening to see if they have ever had Jaundice, or any combination of symptoms which would indicate likely hepatitis illness. If the score is < 4, then the team should proceed with the CCQ. If the score is \( \geq 4 \), then the subject is not eligible to serve as a control. The Team must place a “4” in the form status box of the CCQ Cover Page. If the Control is eligible, then the Team should administer the appropriate consent form(s) and then code the forms accordingly.

Processing Control Specimens

Note that for Controls, there is NO pre-enrollment process (as there is for Cases). This means that the Field Teams MUST remember to collect a blood specimen (fingerstick) from controls as per the procedures in Appendix A. There is a reminder to
do so in Section H of the CCQ. Also remember to place the FORM sticker corresponding to the specimen onto the CCQ. The lab processing of specimens will follow the instructions as described in Appendix D.

Weekly Field Report

At the end of each workweek, the MO/SFRO should submit a Weekly Field Report – Case Control / Virology, with the appropriate performance statistics and cumulative totals. This report should be saved as a Word document: HEV Weekly Field Report CCV_Sept XX_03.doc

The document should be printed and kept in the Weekly Field Report CCV Binder file and one copy should be emailed to the Investigator Team: K. Zaman, A. Labrique, K. Nelson, J. Ticehurst.

Quality Control

The SFRO/MO will make unscheduled surprise visits to the field teams to check work progress and performance. Scheduled visits to specific villages or areas where problems are being faced in motivating or recruiting participants will also be planned as needed. The MO/SFRO will check each questionnaire as they are submitted by the Field Teams for consistency, correct codes, missing values, illegible handwriting, etc. The MO/SFRO are then responsible for the correction of these values by sending the teams back to visit the respondent if necessary.

Periodically, the MO/SFRO will observe interviews of randomly selected respondents to ensure that the quality of the interviews is maintained. The MO/SFRO will also attend the CHRW meetings to respond to any questions or issues faced by the CHRWs.
Intended to be blank.
Field Operations Manual Part C: Field Team Checklists

Logistics for FRA’s bag
1. Daily Activities Diary (1)
2. Questionnaires (Based on DAD)
3. Consent Form (Based on DAD)
4. Umbrella (1)
5. Pen – Black (2)
6. Clip Board (1)
7. Towel (1)
8. MUAC Tape (1)
9. Stamp Pad (1)
10. Gems Clip (1 box)
11. Stapler (Full) (1)

Logistics For Lab Technician:
1. Tool Box with all Fingerstick Supplies
   a. Lancet (20)
   b. Alcohol Swab (30)
   c. Bandage (30)
   d. Safe-T-Fill Capillary Tube (20)
   e. Gloves (20 Pairs)
   f. Cotton (1 Ziploc bag full)
2. Cool Box
   a. 3 Frozen Ice Packs
3. Biohazard Box
4. Label (Based on DAD for Serosurvey, As per MO/SFRO for CC)
5. Soap and Soap Case
6. Umbrella

Logistics For MO:
1. Virology Consent Forms and Questionnaire
2. Ice Box with Frozen Ice Packs (2)
   a. Vacutainer Holder Rack
3. Biohazard Box
4. Tool Box with Fingerstick and IV Blood Drawing Supplies
   a. Lancet (5)
   b. Safe-T-Fill Capillary Tube (5)
   c. Alcohol Swab (20)
   d. Bandage (20)
   e. Tourniquet (5)
   f. Butterfly Kits (5)
   g. Vacutainer – Red Camouflage Top (5)
   h. Vacutainer Holder (2)
   i. Cotton (1 Ziploc Bag full)
   j. Stethoscope
   k. BP Cuff
   l. Thermometer
   m. Pencil flashlight (1) – working order
5. Soap and Soap Case
6. Towel
7. Umbrella
The Training Video from Safe-T-Fill Inc. should be watched by all HEV Project Field Staff before attempting to collect fingerstick specimens.

The following items must be ready and available for each subject’s fingerstick, placed on a piece of laminated paper towel.

1. Alcohol Swab
2. Lancet
3. Safe-T-Fill Capillary Tube
4. Cotton

Beside the paper towel, the sharps / biohazard container must be placed, ready for the used cotton, lancet, etc.

The Lab Technician must wear gloves at all times when performing the fingerstick procedures. First the Lab Technician must open the alcohol swab and rub vigorously the left hand ring fingertip, if possible, of the subject. This must then be allowed to air dry for 30 seconds or so. The cover of the lancet must be twisted off, and then carefully pierce the lateral edge of the fingertip. Using the cotton, quickly wipe off the first drop of blood and then collect the formed drop of blood as per the video/MO/SFRO instructions. The objective is to collect between 300 and 500ul of blood.

Once the Safe-T-Fill tube is full, or the blood flow has stopped, wipe the patient’s fingers with the cotton, and a new alcohol swab if needed to clean dried blood. Place a small bandage on the place of the fingerstick. If necessary, the Lab Technician may wash his/her hands with soap and water from any available tubewell. The appropriate Label
must be placed on the tube, before placing the tube into the Cool Box, in the foam rack.

Be sure that the label number matches the questionnaire exactly.
Field Operations Manual Part E. Lab processing for Survey Specimens

1. Each specimen should be placed in the microcentrifuge, with an appropriate balancing tube at the opposite position.

2. The specimens should be centrifuged at 4,000 rpm for 10 minutes.

3. After removing the specimen tubes, carefully aliquot the supernatant, being careful not to aspirate any buffy coat or RBCs.

4. If for any reason the specimen is disrupted (falls down, hand shaking, or pierced buffy coat) the specimen can be centrifuged again at the same speed for 5 to 10 minutes depending on the degree of disruption.

5. The Eppendorf with the remaining RBCs and buffy coat layer should also be placed in -20°C, in a separate box.

Before processing specimens:

1. The microeppendorf tubes should be labeled with the appropriate labels and placed in the Eppendorf rack.

2. Two aliquots of equal volume should be preserved for each specimen.

3. These aliquots should be placed in sequential order in the freezer boxes (A-1, A-2, A-3, etc...).
Field Operations Manual Part F. Lab Processing for Case Control Specimens and Other Lab Responsibilities

1. Case Control Specimen Processing

When a specimen for the Case Control study (ID Prefix CO or CA) is received, after completing the appropriate columns of the Lab Processing Logbook, the specimen(s) should be placed in the microcentrifuge, with an appropriate balancing tube at the opposite position.

1. The specimens should be centrifuged at 4,000 rpm for 10 minutes.

2. After removing the specimen tubes, carefully aliquot the supernatant, being careful not to aspirate any buffy coat or RBCs, into one 200ul micro-Eppendorf tube.

3. If this is a CA-prefix specimen, 30 microliters of the specimen should be aliquoted into a separate micro-Eppendorf and labelled with the DAC label, for transmittal to Dhaka.

4. If for any reason the specimen is disrupted (falls down, hand shaking, or pierced buffy coat) the specimen can be centrifuged again at the same speed for 5 to 10 minutes depending on the degree of disruption.

Before processing specimens:

1. The microeppendorf tubes should be labelled with the appropriate labels and placed in the Eppendorf rack.

2. One aliquot of maximum volume should be preserved for each specimen.

3. These aliquots should be placed in sequential order in the freezer boxes (A-1, A-2, A-3, etc...)

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2. Freezer Temperature Log

Each morning and evening, the Lab Technician should check the freezer temperature and record this in the Temperature Logbook, which is attached to the Freezer. The Lab Technician should place his worker ID and initials beside each time he has checked the temperature. If for any reason the Lab Technician cannot check the freezer temperature, the MO/SFRO may do this in his place.
Field Operations Manual Part G. MO Procedures at Subcenter for Virology Aims

The MO, depending on the physical condition of the patient, should first ensure that the patient has a place to sit or lie down. The MO should conduct a general patient examination and then move on to completing the HEV-VA form. The form is self-explanatory. The identifiers should be copied from the Hepatitis Case Report Card (RED). If possible, a stool sample may be obtained from either the home or when the patient is at the clinic. The stool sample should be placed in the Cool Box, and the filled vacutainer should be carefully and very gently inverted a total of 5 times to allow the anticoagulant activity to take place. The test tube should be placed in the foam vacutainer holder in the Cool Box for transportation back to the lab.

The patient, after the completion of the exam, will then be released by the MO, with any drugs the MO feels would be helpful to managing this case, which include but are not limited to: vitamin supplements, ORS.

The MO will also pay the return journey cost for the patient and any accompanying relatives / support persons. The MO must also complete the CPE Form so that the serum can be processed as for other potential cases.
Field Operations Manual Part H: Lab processing for Virology Specimens

The vacutainer of blood should be spun at 10,000 rpm for 10 minutes to separate the blood from the serum. Once this is complete, the lab technician should use the large volume pipettor to extract three aliquots of serum, of 1.0mL each, into cryovials for storage at -80°C.

Fecal specimens, if available, will be used to create roughly 10% w/v suspensions (1.5 mL final volume). Stool will be homogenized in phosphate-buffered saline (PBS), pH 7.4, and centrifuged at 2000 x g for 30 minutes. Three aliquots 1000 microliters will be made of the stool suspension.

These specimens will then be placed in the –80°C freezer.

The Lab Technician should complete the Lab Processing Logbook, both physical and digital copies. Any hemolysis or other problems should be noted in the comments section of the logbook.

If it is found that the patient does not have IgM antibodies to HEV through the CPE process, then these specimens should be removed and placed in a separate box, where all non-HEV acute hepatitis sera are kept.
Appendix B. Hepatitis Illness Surveillance – Hepatitis Case Report (≤ 7d onset)

<table>
<thead>
<tr>
<th>Column</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPATITIS CASE REPORT</td>
<td></td>
</tr>
<tr>
<td>(≤7 Days Onset)</td>
<td></td>
</tr>
<tr>
<td>CTD</td>
<td></td>
</tr>
<tr>
<td>d/d</td>
<td></td>
</tr>
<tr>
<td>m/m</td>
<td></td>
</tr>
<tr>
<td>YYYY</td>
<td></td>
</tr>
<tr>
<td>Name</td>
<td></td>
</tr>
<tr>
<td>Block</td>
<td></td>
</tr>
<tr>
<td>YYYY</td>
<td></td>
</tr>
<tr>
<td>d/d</td>
<td></td>
</tr>
<tr>
<td>mm</td>
<td></td>
</tr>
<tr>
<td>Village Code</td>
<td></td>
</tr>
<tr>
<td>Year</td>
<td></td>
</tr>
<tr>
<td>Family Number</td>
<td></td>
</tr>
<tr>
<td>HET Score</td>
<td></td>
</tr>
<tr>
<td>Days since onset</td>
<td></td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td></td>
</tr>
<tr>
<td>Date of Birth</td>
<td></td>
</tr>
<tr>
<td>d/d</td>
<td></td>
</tr>
<tr>
<td>m/m</td>
<td></td>
</tr>
<tr>
<td>Initials</td>
<td></td>
</tr>
<tr>
<td>MO</td>
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</tbody>
</table>
### HEPATITIS CASE REPORT

**Hepatitis Illness Surveillance – Hepatitis Case Report (8+ days onset)**

<table>
<thead>
<tr>
<th>Field</th>
<th>Description</th>
<th>Format</th>
</tr>
</thead>
<tbody>
<tr>
<td>CID</td>
<td>Case Identification Number</td>
<td>D d m y</td>
</tr>
<tr>
<td>RII</td>
<td>Reporting Institution Identification Number</td>
<td>D d m y</td>
</tr>
<tr>
<td>Name</td>
<td>Patient's Name</td>
<td></td>
</tr>
<tr>
<td>Bari Name</td>
<td>Village or District Name</td>
<td></td>
</tr>
<tr>
<td>Head of Household Name</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Block</td>
<td>Administrative Block Number</td>
<td></td>
</tr>
<tr>
<td>Village Code</td>
<td>Village Code Number</td>
<td></td>
</tr>
<tr>
<td>Family Number</td>
<td>Family Number</td>
<td></td>
</tr>
<tr>
<td>Date of Birth</td>
<td>Date of Onset</td>
<td>D d m y</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>Patient's Sex</td>
<td></td>
</tr>
<tr>
<td>HEV Score</td>
<td>HEV Score Indicator</td>
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</tr>
<tr>
<td>Days since onset</td>
<td>Days since onset of symptoms</td>
<td></td>
</tr>
<tr>
<td>CHR/W ID</td>
<td>Case Reference or Work Identification Number</td>
<td></td>
</tr>
<tr>
<td>Initials</td>
<td>Initials</td>
<td></td>
</tr>
</tbody>
</table>

**HEV Messenger**
| Date of Visit | 1) Nausea OR Vomiting | 2) Fever and weakness disabling normal daily work | 3) Anemia OR Food smells foul like rotten fish | 4) Yellow Eyes OR Skin | 5) Tea-colored urine or Ash/Clay stools | 6) Do you believe you had jaundice or holdo palang? | 7) Total Score \((1\times2\times3\times4\times5)\) | 8) Number of Days since Onset (\(\geq 5\), CONTINUE; \(< 5\), STOP) | WHO? | (9) Name | (10) CID | (11) RID |
|---------------|------------------------|-----------------------------------------------|---------------------------------------------|-----------------------|----------------------------------------|---------------------------------------------|---------------------------------|---------------------------------|-----------------|--------------|-------------|
| 1             |                        |                                               |                                             |                       |                                        |                                             |                                 |                                 |                 |              |             |
| 2             |                        |                                               |                                             |                       |                                        |                                             |                                 |                                 |                 |              |             |
| 3             |                        |                                               |                                             |                       |                                        |                                             |                                 |                                 |                 |              |             |
| 4             |                        |                                               |                                             |                       |                                        |                                             |                                 |                                 |                 |              |             |
### Appendix D2. Hepatitis Illness Surveillance – Hepatitis Surveillance Logbook (Bangla)

<table>
<thead>
<tr>
<th>WHO?</th>
<th>RD</th>
<th>CID</th>
<th>Name</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

**HEPATITIS E SURVEILLANCE LOG BOOK**

**Round 1+2+3+4**

**CHR # 142**

**Village Code K00**

**Family # 0048**

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
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</tbody>
</table>

**STOP**

- If yes 1
  - If no 2
    - If yes 3
      - If no 4

Appendix E. Hepatitis Illness Surveillance – Visual Algorithm Card

I will now ask you some questions about the health of the members of your household. In the last 30 days, or since my last visit until today…. Has any member of your family had ………………?

1. Nausea OR vomiting ?
2. Fever AND weakness such that you could not perform your daily work ?
3. Loss of appetite OR feel that your food has a foul, raw or rotting fish-liko smell ?
4. Yellow eyes OR skin became yellow  
   (* CHRW- use the visual card to check the answer.)
5. Urine the color of tea liquor OR ash/clay-colored stool ? 
   (* CHRW- use the visual card to check the answer.)
6. Do you think that this person / you had “jaundice” or “holde palong” ?

7. How many days ago did these problems begin ?
Appendix F1. Data Collection Instrument – Baseline Questionnaire (BLQ)

HEV Baseline Questionnaire (HEV - BLQ)

Worker ID: _______ _______ _______  Worker Initials: ________________________________
Attempt: 1 - 2 - 3 -

Section A: Identifiers
Field Team Note: Complete Identifiers before going to field
Name _______ _______ _______ _______ _______ _______ _______ Study ID: _______
CID _______ _______ _______ _______ RID _______ _______ _______ _______ _______ _______
Bari Name _______ Head Name _______
Sex 1= Male 2= Female 9= Don’t Know
Date of Birth dd/mm/yy
Form Status 1= Form Complete 2= Not Met 3 Times 6= Refused 7= Permanently Moved from Study Area 8= Died
STOP INTERVIEW

Section B. Demographic and Socioeconomic Assessment

1. Marital Status: _______ 1= Single 2= Married 3= Divorced 4= Widowed 6= Refused 9= Don’t Know

2. Religion _______ 1= Muslim 2= Hindu 3= Tribal 6= Refused 8= Other 9= Don’t Know

3. Khana Size _______ 01-30= Size 99= Don’t Know

4. What kind of work do you spend most of your time doing?
   _______ 00= Housework
   01= Work on own farm or as sharecropper
   02= Day, unskilled laborer (agricultural & migrant)
   03= Fisherman/woman
   04= Contracted laborer (long-term domestic, agricultural)
   05= Own business (shopkeeper, vendor, merchant, seller, artisan)
   06= Private service (maidservant, skilled factory and office worker, salesperson, skilled laborer)
   07= Government service (all GOB-paid employees)
   10= Child
   11= Student
   88= Other
   99= Don’t know
5. Is the work you do mainly indoors or outside?  
   1= Inside  
   2= Outside  
   9= Don’t Know

6. Do you have a secondary occupation for which you are paid in cash or kind?  
   If yes, what is it?  
   0 = None (Go to 7)  
   1 = Work on own farm or as sharecropper  
   2 = Day, unskilled laborer (agricultural & migrant)  
   3 = Fisherman/woman  
   4 = Contracted labor (long term domestic, agricultural)  
   5 = Own business (shopkeeper, vendor, retailer/odf, artisan)  
   6 = Private service (sathpata, skilled factory and office workers, salesman, skilled laborer)  
   7 = Government service (all GOR-paid employees)  
   8 = Other  
   9 = Don’t know

6a. Is this work you do mainly indoors or outside?  
   1= Inside  
   2= Outside  
   9= Don’t Know

7. What was the highest class you completed in school?  
   00 = No schooling  
   01-09= From class 1 to class 9  
   10= SSC passed  
   11= 11 years completed  
   12= HSC passed  
   13= 13 years completed  
   14= Degree or higher  
   99= Don’t know

8. What is the highest class completed by the head of your household?  
   00 = No schooling  
   01-09= From class 1 to class 9  
   10= SSC passed  
   11= 11 years completed  
   12= HSC passed  
   13= 13 years completed  
   14= Degree or higher  
   66= Not applicable (same as 7)  
   99= Don’t know

9. What is your family’s average monthly income?  
   0= No income  
   1= <100 taka  
   2= 100-199 taka  
   3= 200-299 taka  
   4= 300-499 taka  
   5= 500-699 taka  
   6= 700-899 taka  
   7= 900-1199 taka  
   8= >1200  
   9= Don’t know/Refused
Section C. Health and Exposure Evaluation

1. Over the past three months how many times have you traveled to a town or city?
   
   00-99 Number of Times
   99= Don’t know

2. Are you currently suffering from any illness which has lasted for more than 30 days?
   
   0= No (Go to 3)
   1= Yes
   9= Don’t Know

2a. What illness have you had?

3. Over the past 3 months have you had any contact with any person with jaundice or yellow eyes or yellow skin?
   
   0= No
   1= Yes
   9= Don’t Know

4. Over the past 3 months have you had any contact with any sick person?
   
   0= No
   1= Yes
   9= Don’t Know

5. Over the past 3 months has any doctor or health worker told you that you had jaundice or hepatitis?
   
   0= No
   1= Yes
   9= Don’t Know

6. Over the past 3 months have you had _______________?

   a. Extreme weakness
   b. Nausea or vomiting
   c. Fever
   d. Loss of appetite
   e. Yellow eyes or yellow skin
   f. Clay colored stools
   g. Dark colored urine
   h. Upper right quadrant pain

6b. If YES, How many days did this _______________ last?
   
   00= Less than 1 day
   01-98= Number of days
   99= Don’t know
7. Over the past 3 months have you had any injections?
   0= No
   1= Yes
   9= Don’t Know

8. Over the past 3 months have you had any blood transfusions?
   0= No
   1= Yes
   9= Don’t Know

9. Over the past 3 months have you had visited any ouja / fakir / kabiraj?
   0= No
   1= Yes
   9= Don’t Know

10. MUAC Measurement: ______ mm

Section D: For married woman (If Age < 12, GO TO SECTION E)

1. Are you currently pregnant?
   0= No
   1= Yes (Go to 1a)
   9= Don’t Know

1a. How many running months pregnant are you?
   00= < 1 month
   01-09= Number of Months
   99= Don’t Know

2. Have you used Injection or ‘Kati’ contraceptive in the past one year?
   0= No
   1= Yes
   9= Don’t Know
Section E: For Participants < 12 yrs Only (If Age > 12, GO TO SECTION F)

1. Has this child been in contact with any sick children over the past 3 months?
   - 0 = No
   - 1 = Yes
   - 9 = Don’t Know

2. Is this child currently breastfeeding?
   - 0 = No
   - 1 = Yes
   - 9 = Don’t Know

Section F: Fingerstick Specimen

I will now collect a few drops of blood from the tip of your finger, as I did last time.

1. Specimen collected:
   - 0 = No
   - 1 = Yes
   - 6 = Refused Specimen

FIELD TEAM NOTE: Remember to place ID LABEL on the TUBE (☑): □
Place Second ID Label Here: □
Appendix F2. Data Collection Instrument – Baseline Questionnaire (BLQ) - Bangla

HEV Baseline Questionnaire (HEV - BLQ)

Worker ID: [Blank] - [Blank] Worker Initials: [Blank]

Attempt: 1 - [Blank], 2 - [Blank], 3 - [Blank]

Section A: Identifiers

Field Team Note: Complete Identifiers before going to field

Name

CID

Bari Name

Head Name

Sex

1 = Male

2 = Female

9 = Don’t Know

Date of Birth

dd / mm / yy

Form Status

1 = Full Complete

2 = Not Met 3 Times

6 = Refused

7 = Permanently Moved from Study Area

8 = Died

STOP INTERVIEW

Section B. Demographic and Socioeconomic Assessment

1. জনস্বাস্থ্য অবস্থা:

   1 = অসুস্থ

   2 = তরানামাত্র

   3 = লালস্থ

   4 = বিকল্প

   5 = অস্থিত

   6 = অকল্পনা

   7 = অন্যায়

   9 = ধারণা না

2. ধর্ম:

   1 = হিন্দু

   2 = বুদ্ধ

   3 = শাস্ত্রীয়

   4 = বিদ্যালয়

   5 = মুসলমান

   6 = আন্তর্জাতিক

   7 = অন্যায়

   9 = ধারণা না

3. পরিবারের সদস্য সংখ্যা

   (যারা একই পাকে খান)

   01-30 = ভাবে

   99 = ধারণা না

   00 = ব্যাপক অর্থ

4. আপনি এখানে অন্য কোন রাশের কাজ দিয়াছেন কেন?

   02 = নিজের রাশ, কিছু শ্রমিক (নিজের গাড়ি বা কারণ একেবারে কাজ দেওয়া)

   03 = কঙ্কাল

   04 = রাজনৈতিক প্রথম (সীমাবদ্ধতার পুরুষ বা মহিলা প্রথম)

   05 = শ্রমিক দানী (বেশ উচ্চ, কিছু শ্রমিক, কিছু শ্রমিক চাঁদ, রাজনৈতিক)

   06 = সন্তান চাঁদী (রান্না শ্রমিক নয় কিছু শ্রমিক নয় কিছু শ্রমিক নয় কিছু শ্রমিক)

   07 = সন্তান চাঁদী

   10 = কর্ম

   11 = কষ্ট

   12 = তৃষ্ণ

   88 = অন্যরা

   99 = ধারণা না

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5. অপনি নে কাজে নিয়ন্ত্রিত সেটি কি ঘরের তিতর না বাহিতে করেছে?

1 = ঘরের তিতর
2 = বাহিতে
9 = জানি না / অপরাধ না

6. অপনি নে কাজে নিয়ন্ত্রিত সেটি ছাড়াও অন্য কোন পেশায় নিয়ন্ত্রিত আছেন কি?

0 = অন্য কোন পেশায় নাই (Go to 7)
1 = নিজের কম্পিউটারের ব্যবহারের জন্য
2 = প্রশ্নের মূল্য, অফিস পরিকল্পনা (নিজের এলাকায় বা অন্য এলাকায় সবচেয়ে নেওয়া)
3 = উপায়
4 = মূল্যায়ন শিক্ষা (রাজনীতি, মাল্টিইলি প্রক্রিয়া বা পৃথিবী প্রক্রিয়া)
5 = নিজস্ব ব্যবসা (রাজনীতি, বর্তিতির নেওয়া, মালামাল চাই, পৃথিবী প্রক্রিয়া)
6 = তোদরের চাই (তোদরের নিজের শিক্ষা বা মালামাল চাই, নেওয়া অথবা আর্থিক শিক্ষা শিক্ষা)
7 = সকলকেই চাই
8 = আসাদ
9 = জানি না

6a. এই কাজটি কি ঘরের তিতরে না বাহিতে করেছে?

1 = ঘরের তিতর
2 = বাহিতে
9 = জানি না / অপরাধ না

7. অপনি একাডেমিক ক্লাস পর্যটন পড়ালেখা করেছেন?

00 = এখানে কারা নাই
01-09 = এখানে একাডেমিক পর্যটন পড়ালেখা
10 = একাডেমিক পর্যটন
11 = একাডেমিক পর্যটন পড়ালেখা
12 = একাডেমিক পর্যটন
13 = ১১ পর্যটন পদ্ধতি
14 = তিনি পড়ালেখা বা তাঁর উপরের
99 = জানি না

8. অপনার পরিবারের একাডেমিক ক্লাস পর্যটন পড়ালেখা করেছেন?

00 = এখানে কারা নাই
01-09 = এখানে একাডেমিক পর্যটন পড়ালেখা
10 = একাডেমিক পর্যটন
11 = একাডেমিক পর্যটন
12 = একাডেমিক পর্যটন
13 = ১১ পর্যটন পদ্ধতি
14 = তিনি পড়ালেখা বা তাঁর উপরের
99 = জানি না

9. অপনার পরিবারের পর্যটন মাসিক আয় কত?

0 = এখানে কারা নাই
1 = ১০০০ টাকা
2 = ২০০০-৩০০০ টাকা
3 = ৩০০০-৪০০০ টাকা
4 = ৪০০০-৫০০০ টাকা
5 = ৫০০০-৬০০০ টাকা
6 = ৬০০০-৭০০০ টাকা
7 = ৭০০০-৮০০০ টাকা
8 = > ১২০০০ টাকা
9 = জানি না / উপর পর্যন্ত পর্যটন মাসিক আয় না
Section C. Health and Exposure Evaluation

1. গত তিন মাসে আপনি কট বা ছোট বা বড় শহরে আসা গেয়েছেন?
   
   0-9 = কট বাস
   99 = জানি না

2. গত ৩০ দিনেরও বেশী ছুটি কেন রোগে কি আপনি বর্তমানে আছেন?
   
   0 = না (0-3)
   1 = জানি
   9 = জানি না

2a. আপনার কি অসুখ ছিল?

3. গত তিন মাসে আপনি কি কোন জরুরি/হালনা পালনের সাধারণে এসেছিলেন?
   
   0 = না
   1 = জানি
   9 = জানি না

4. গত তিন মাসে আপনি কি কোন অসুস্থ যাত্রা সাধারণে এসেছিলেন?
   
   0 = না
   1 = জা
   9 = জানি না

5. গত তিন মাসে আপনাকে কোন ভাবায় বা মাথা কমলি কি দেখেছে যে আপনার জরুরি হচ্ছে?
   
   0 = না
   1 = জানি
   9 = জানি না

6. গত তিন মাসে আপনার **************** ছিল?
   
   6a. যদি হই হয়, তাহলে তা কতদিন ছাড়াই হয়েছিলো?

   a. দুইটি দূরন্ত যাত্রে কাছ করা সহজ হয়েছে
   b. আহর বা আহর গ্রহণ
   c. জ্বালা
   d. আলি/ধামায় রক্ত মানুষের
e. হৃদগত রক্ত হৃদগত হৃদাক
f. ছাঁই তার আনুপাতিতা
g. সুদৃশ বাদায় সার পর্যায়
h. উপর প্যেল্টের জনসিকে বাড়া
7. প্রতিনিয়তায় আপনি কি কেন ইংরেজিতে নিয়েছেন?

- 0 = না
- 1 = হাঃ
- 9 = জ্ঞানিনা

8. প্রতিশোধ মাঝে আপনি কি কেন রাখ নিয়েছেন?

- 0 = না
- 1 = হাঃ
- 9 = জ্ঞানিনা

9. প্রতিনিয়তায় আপনি কি আপনার অসুস্থতার জন্য কেন ওয়াল/ফোকল/ফিকারজের সিক্টর নিয়েছেন?

- 0 = না
- 1 = হাঃ
- 9 = জ্ঞানিনা

10. MUAC Measurement: □□□ mm

Section D: কোনও বিশেষ অভিজ্ঞার জন্য (যদি বর্তমান ১২ বছরের সময় হয় তাহলে Section E এক্ল হয়)

1. আপনি কি গর্ভধারণে তাব্লভতি?

- 0 = না
- 1 = হাঃ (১৪ শেষ তথ্য)
- 9 = জ্ঞানিনা

1a. আপনি গর্ভধারণে তাব্লভতি?

- 00 = < ১ মাসের অন্ত
- 01-09 = মাসের সংখ্যা
- 99 = জ্ঞানিনা

2. আপনি প্রতি ১ বছরে কেন নিরোধক হিসেবে কেন বাবুল বা ইংরেজিতে ব্যবহার করেছেন?

- 0 = না
- 1 = হাঃ
- 9 = জ্ঞানিনা
Section F: Fingerstick Specimen

আমি এখন আপনার আমলের মাথা হতে করেক ব্যক্তি রক দিয়েছি।

Specimen collected:

0 = না  
1 = হ্যাঁ  
6 = অপর্যাপ্ত

FIELD TEAM NOTE: টিউটোর ID LABEL সূচনা করা হয় করা ।  
ফ্যাক্ট্রোম প্রেসার এর অধিকারী:

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Appendix G1. Data Collection Instrument – Follow-Up Questionnaire (FUQ)

HEV 12/18 Month Follow-up Questionnaire (HEV - FUQ)

Follow Up ☐ 12 Month ☐ 18 Month

Worker ID: ______________________ Worker Initials: ______________________

Attempt: 1 - ______________________
2 - ______________________
3 - ______________________

Section A: Identifiers
Field Team Note: Complete Identifiers before going to field
Name ______________________ Study ID: ______________________
CID ______________________ RID ______________________
Bari Name ______________________ Family Name ______________________
Sex 1= Male 2= Female 9= Don’t Know
Date of Birth dd mm yy

Form Status 1= Form Complete 2= Not Met 3 Times 6= Refused
7= Permanently Moved from Study Area 8= Died

STOP INTERVIEW

Section B. Demographic and Socioeconomic Assessment

1. Marital Status:
☐ 1= Single
☐ 2= Married
☐ 3= Divorced
☐ 4= Widowed
☐ 6= Refused
☐ 9= Don’t Know

2. Khana Size
☐ 01-30= Size
☐ 99= Don’t Know

3. In the past three months, what kind of work did you spend most of your time doing?
☐ Housework
☐ Work on own farm or as share cropper
☐ Day, unskilled laborer (agricultural & migrant)
☐ Fisherman/woman
☐ Contracted laborer (long term domestic, agricultural)
☐ Own business (shopkeeper, vendor, nickel-smoker/paper, artisan)
☐ Private service (pastry, skilled factory and office workers, nannies, skilled laborer)
☐ Government service (
☐ 10= Children
☐ 11= Student
☐ 12= Other
☐ 99= Don’t know

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4. Has this work been mainly indoors or outside?
   1 = Inside
   2 = Outside
   9 = Don’t Know

5. Did you have a secondary occupation?
   9 = None (Go to Section C)
   1 = Work on own farm or as sharecropper
   2 = Day, unskilled laborer (agricultural & migrant)
   3 = Fisherman/woman
   4 = Contracted laborer (long term domestic, agricultural)
   5 = Own business (shopkeeper, vendor, rfahia/van-puller, artisan)
   6 = Private service (salaried, skilled factory and office workers, stelemen, skilled laborer)
   7 = Government service (all GOB-paid employees)
   8 = Other
   9 = Don’t know

5a. Is this work you do mainly indoors or outside?
   1 = Inside
   2 = Outside
   9 = Don’t Know

Section C. Health and Exposure Evaluation

1. Over the past three months how many times have you traveled to a town or city?
   00-98 Number of Times
   99 = Don’t know

2. Are you currently suffering from any illness which has lasted for more than 30 days?
   0 = No (Go to 3)
   1 = Yes
   9 = Don’t Know

2a. What illness have you had?

3. Over the past 3 months have you had any contact with any person with jaundice or yellow eyes or yellow skin?
   0 = No
   1 = Yes
   9 = Don’t know

4. Over the past 3 months have you had any contact with any sick person?
   0 = No
   1 = Yes
   9 = Don’t know
5. Over the past 3 months has any doctor or health worker told you that you had “jaundice” or “hepatitis”?  
   □ 0= No  
   □ 1= Yes  
   □ 9= Don’t Know

6. Over the past 3 months have you had ________________?  

   a. Extreme weakness  
   b. Nausea or vomiting  
   c. Fever  
   d. Loss of appetite  
   e. Yellow eyes or yellow skin  
   f. Clay colored stools  
   g. Dark colored urine  
   h. Liver pain

6b. If YES, How many days did this last?  

   □ 0= No  
   □ 1= Yes  
   □ 9= Don’t Know  

   □ 00= Less than 1 day  
   □ 01-99= Number of days  
   □ 99= Don’t know

7. Over the past 3 months have you had any injections?  
   □ 0= No  
   □ 1= Yes  
   □ 9= Don’t Know

8. Over the past 3 months have you had any blood transfusions?  
   □ 0= No  
   □ 1= Yes  
   □ 9= Don’t Know

9. Over the past 3 months have you visited any ouja / fakir / kahiraj?  
   □ 0= No  
   □ 1= Yes  
   □ 9= Don’t Know

10. MUAC Measurement: □ □ mm
Section D: For married woman (If Age < 12, GO TO SECTION E)

1. Are you currently pregnant?
   - 0 = No
   - 1 = Yes (Go to 1a)
   - 9 = Don’t Know

   1a. How many running months pregnant are you?
   - 0 = < 1 month
   - 1 = 1 - 9 months
   - 9 = Don’t Know

2. Have you used Injection or ‘Kati’ contraceptive in the past one year?
   - 0 = No
   - 1 = Yes
   - 9 = Don’t Know

Section E: For Participants < 12 yrs Only (If > 12, GO TO SECTION F)

1. Has this child been in contact with any sick children over the past 3 months?
   - 0 = No
   - 1 = Yes
   - 9 = Don’t Know

2. Is this child currently breastfeeding?
   - 0 = No
   - 1 = Yes
   - 9 = Don’t Know

Section F: Fingerstick Specimen

I will now collect a few drops of blood from the tip of your finger, as I did last time.

1. Specimen collected:
   - 0 = No
   - 1 = Yes
   - 9 = Rejected Specimen

FIELD TEAM NOTE: Remember to place ID LABEL on the TUBE (☑):

Place Second ID Label Here:
Appendix G2. Data Collection Instrument – Follow-Up Questionnaire (FUQ) - Bangla

HEV 12/18 Month Follow-up Questionnaire (HEV - FUQ)

Follow Up: [ ] 12 Month [ ] 18 Month

Worker ID: ___________________________ Worker Initials: ___________________________

Attempt: 1- [ ] [ ] [ ] [ ] 2- [ ] [ ] [ ] [ ] 3- [ ] [ ] [ ] [ ]

Section A: Identifiers
Field Team Note: Complete Identifiers before going to field
Name: ___________________________
CID: ___________________________ RID: ___________________________

Bari Name: ___________________________
Head Name: ___________________________

Sex: [ ] 1=Male [ ] 2=Female [ ] 9=Don’t Know
Date of Birth: [ ] [ ] [ ]

Form Status: [ ] 1=Form Complete [ ] 2=Not Met 3 Times [ ] 3=Refused
[ ] 4=Permanently Moved from Study Area [ ] 5=Died

STOP INTERVIEW

Section B. Demographic and Socioeconomic Assessment

1. নৈবেদিত্ব অবস্থা: [ ]
   1=নিয়মিত
   2=নিয়মিত
   3=তালাক প্রাপ্ত
   4=বিবাহ
   6=উচ্চ স্তর যৌবন
   9=অন্য না

2. পরিবারের সদস্য সংখ্যা
   (খালী একই পক্ষে বাছ
   01-30=সন্তান
   99=অন্য না

3. আপনি প্রথমে কি ধরনের কাজে নিয়োগ করেছেন?
   [ ]
   00 = গৃহপত্নী
   01 = নিয়মে যথাযথ কর চাঁদ সময়বাদ
   02 = সমস্ত হস্তবাদ, অন্য যৌবন (নিয়ম অধিকার বা অন্য নিয়ম বলা কেন্দ্র)
   03 = সমাজ
   04 = পালিয়ে গৃহপত্নী (সুরক্ষায় বা পুলিশ প্রতি)
   05 = গৃহপত্নী হয় (সুরক্ষায় বা পুলিশ প্রতি)
   06 = লোকযোগী পুলিশ (সুরক্ষায় বা পুলিশ প্রতি)
   07 = সমাজ
   10 = বিদেশ
   11 = স্কুল
   12 = স্কুল
   88 = অন্য না
   99 = অন্য না

1
4. আপনি যে কাজে নিয়োজিত সেটি কি ধরনের ভিত্তিতে না বাহিতে করেন?
   1- ধরনের ভিত্তি
   2- বাহিত
   9- জানি না

5. আপনি যে কাজে নিয়োজিত সেটি ছাড়াও অন্য কোন পেশায় নিয়োজিত আছেন কিনা?

5a. এই কাজটি কি ধরনের ভিত্তিতে না বাহিতে করেন?
   1- ধরনের ভিত্তি
   2- বাহিত
   9- জানি না/চেয়ারম্যান না

Section C. Health and Exposure Evaluation

1. গত দিন মাসে আপনি কত দৈনের কষ্ট বা ব্যাধি সহ্য করার সময় মাস করেছেন?
   0= অন্য কোন পেশায় না (Section C তে যেন)
   1= নিজের কষ্ট অন্য কোন পেশায় সহ্য করার সময়
   2= নিজের কষ্ট, অন্য পেশায় না ছিল এমনকী কোন সময় (যেন অন্য পেশায় সহ্য করে)
   3= পেশায়
   4= অন্য পেশায় সহ্য (যেকোনো কষ্ট অন্য পেশায় সহ্য করে)
   5= নিজের কষ্ট, অন্য পেশায় একেবারে জিনিস পত্তন তুলা না করা যাবে
   6= একেবারে জিনিস পত্তন তুলা না করা যাবে (যেকোনো কষ্ট নিজের পেশায় তুলা না করা)
   7= অন্য কোন পেশায়
   8= জানি না

2a. আপনার কি অপরু ছিল?

3. গত দিন মাসে আপনি কি কোন অভিজ্ঞতাকে পাইলেন বা পাইলেন যে সংস্পর্শ সংক্রামক ছিল?
   1= না
   2= হ্যাঁ
   9= জানি না

4. গত দিন মাসে আপনি কি কোন অপরু বাচির সংস্পর্শ পাইলেন?
   1= না
   2= হ্যাঁ
   9= জানি না
5. পত্র তুলে মাঝে আপনাকে কেন ঢাকাটা বা ভাঙ্গা কর্নী কি বলেছে যে আপনারা কমিল হয়েছে?
   
   0= না
   1= হ্যা
   9= জ্ঞানিত

6. পত্র তুলে মাঝে আপনার ——————————— ছিল ?
   6a. যদি হ্যা হয়, তাহলে তা কতদিন জ্ঞানী হয়েছিলো?

| a. পৃথিবী পৃষ্ঠায় কাজ কর্নী সচ্চ হয়নি | 0 = না |
| b. বিন্য বা বিন্য বিন্য আবে | 1 = হ্যা |
| c. ঝুর | 9 = জ্ঞানিত |
| d. অফিস/পাইলটারা কোথা নাই এমন | 00 = এককের কম |
| e. পৃথিবী চোখের বা চাপা হয়নি | 01-98 = নিজের সহায়তা |
| f. ছুটি রং এর সাধনা | 99 = জ্ঞানিত |
| g. পৃথিবী চোখের ভক্ত পেশাধিরাি | |
| h. উপর পেটের ভক্তি দিনিকে ব্যাপার | |

7. পত্র নিম্নমুখে আপনি কি কোন ইসনামক নিয়েছেন?
   
   0= না
   1= হ্যা
   9= জ্ঞানিত

8. পত্র তুলে মাঝে আপনি কি কোন রক নিয়েছেন?
   
   0= না
   1= হ্যা
   9= জ্ঞানিত

9. পত্র নিম্নমুখে আপনি কি কোন ওকা/ফকিকা/কর্মভাবের লিখ নিয়েছেন?
   
   0= না
   1= হ্যা
   9= জ্ঞানিত

10. MUAC Measurement: ______ mm
Section D: কেবলমাত্র সৌন্দর্য মহিলাদের জন্য (যদি বাস্তু ২২ বছরের কম হয় তাহলে Section E তে যান)

1. আপনি কিছু কর্মজীবনে গল্পরচনা?
   
   [☐] 0- না (২২ বছরের মধ্যে)
   [☐] 1- হা
   [☐] ৯= জ্ঞানিনা

1a. আপনি কর্মজীবনে গল্পরচনা?
   
   [☐] 0- না (<২ মাসের মধ্যে)
   [☐] 1- হা
   [☐] ৯= জ্ঞানিনা

2. আপনি পর ১ বছরের জন্য নিরোধক হিসাবে কোন কাজ বা ইন্টারেসশন অবহার করেছেন?
   
   [☐] 0- না
   [☐] 1- হা
   [☐] ৯= জ্ঞানিনা

Section E: কেবলমাত্র ২২ বছরের কম বয়সী শিশুদের জন্য (যদি বাস্তু ২২ বছরের বেশী হয় তাহলে SECTION F তে যান)

1. এই শিশুর স্বাস্থ্য কর্মজীবনে কি বিশেষ বিশেষ সংস্থার সাথে সমাপ্ত করেছিলেন?
   
   [☐] 0- না
   [☐] 1- হা
   [☐] ৯= জ্ঞানিনা

2. শিশুর কিছু কর্মজীবন স্থানে যুক্ত হয় হয়েছে?
   
   [☐] 0- না
   [☐] 1- হা
   [☐] ৯= জ্ঞানিনা

Section F: Fingerstick Specimen

আপনি এখন আপনার আধ্যাত্মিক মাধ্যম হতে করকের ফিংগার স্পীক দিন

Specimen collected:

[☐] 0= না
[☐] 1= হা
[☐] ৬= আপ্রমাণ

FIELD TEAM NOTE: চিত্রে ID LABEL লিপ্তার জন্য যান করুন (২৭)

কর্মকর্তা'র চেয়েন্টা বাবু নাম: [☐]

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Appendix H. Data Collection Instrument – Serum Transmittal / Result Reporting Form (STF)

Serum Transmittal / Result Reporting Form (STF)

Transmittal Date: 

Study Number: 

Age: (From Case Tracking Logbook)

HEV anti-IgM

HAV anti-IgM

(If < 10 years of age)

Tests Requested (☐): _____________

Requested by: ______________________ ID: ____________

Signature: _______________________

DHAKA LAB USE ONLY

Processed by: ________________ Processing Date: ___/___/___

Hepatitis E anti-IgM Opinion (+/-) ☐ (Reagent Lot #____________)

Sample Rate: __________ Cutoff Rate: __________

Hepatitis A anti-IgM Opinion (+/-) ☐ (Reagent Lot #____________)

Sample Rate: __________ Cutoff Rate: __________

PLEASE COMMUNICATE RESULTS AS SOON AS POSSIBLE TO:

DR. ZAHID HOSSAIN ☐

MR. PARIMAL SAHA ☐

HEPATITIS E STUDY-MATLAB

Mobile: 0172257619

WHEN COMPLETE, FILE SERIALLY BY SN (Serial Number).

Call Received by: ______________________ ID: ____________

Signature: _______________________

Case Tracking Logbook Updated (☐): ☐
Control Data Request Form

**Date:**

**Requested by:**

**Designation:**

**ID No:**

**Number of Persons:**

**Birth Year:**

**Study ID:**

**Block (circle one):** A B C D

**HDSS Please Note:** Please select eight individuals randomly from the set of eligible persons of this birth year in this block. Do not sort by any criteria.

<table>
<thead>
<tr>
<th>SN</th>
<th>Name</th>
<th>CID</th>
<th>RID</th>
<th>Bari Name</th>
<th>Head Name</th>
<th>Sex</th>
<th>Date of Birth</th>
<th>HEV Study Staff Only: Control ID # (IF Subject not met in Previous Selection)</th>
</tr>
</thead>
<tbody>
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</table>
Appendix J1. Data Collection Instrument – Control Screening Card

Control Screening Card_v1.0_August 14, 2004

SCREENING PROCESS FOR CONTROLS

FIELD INTERVIEWER NOTE: ASK THESE QUESTIONS TO ALL CONTROLS BEFORE CONSENT PROCESS. IF THE CONTROL IS AGED < 15 THEN YOU MUST ASK A PARENT OR GUARDIAN.

1. In your lifetime, have you ever had jaundice or holde palong?

   IF YES, THANK THE PARTICIPANT AND STOP THE INTERVIEW. PROCEED TO NEXT CONTROL CANDIDATE.

   IF NO, GO TO #2.

2. Have you ever have the following symptoms at the same time...

   a. weakness, such that you could not work?  YES NO
      +1  0
   b. nausea or vomiting?  YES NO
      +1  0
   c. fever?  YES NO
      +1  0
   d. loss of appetite?  YES NO
      +1  0
   e. yellow eyes or yellow skin?  YES NO
      +2  0
   f. ash-colored stools or dark urine?  YES NO
      +2  0
   g. liver pain (upper right quadrant)?  YES NO
      +2  0

ADD THE SCORE, AND IF < 4, THEN PROCEED WITH THE CONSENT PROCESS FOR THE HEV CASE CONTROL STUDY.

IF THE SCORE IS ≥ 4, THEN STOP. PLACE A ‘4’ IN THE CCQ FORM STATUS BOX, AND WRITE IN COMMENTS SECTION OF DAD “NOT ELIGIBLE”
Appendix J2. Data Collection Instrument – Control Screening Card (Bangla)

SCRENNING PROCESS FOR CONTROLS

FIELD INTERVIEWER NOTE: Consent নেবার পূর্বে এই সকল প্রশ্নগুলো control list এর ধরে তুলে করা দিন। যদি বয়স <১৫ বছরের কম হয় তাহলে তার বাবা, মা বা অভিজ্ঞকে জিজ্ঞাসা করন।

1. আপনার জীবনে কি কিছু বিতর্ক বা হালনা পালন হয়েছিলো?
   
   যদি হয়, তাহলে ধর্মবাদ নিয়ে সন্তানতন্ত্র বদন করন এবং পরবর্তী ব্যক্তির সন্তানতন্ত্র নিয়ে অন্তর্ভুক্ত করন।  
   
   যদি না হয়, তাহলে ২ নং প্রশ্নটি জিজ্ঞাসা করন।

2. আপনার কি কিছু হিসেবে নির্দেশ করার সাধারণ একসাথে ছিলো?

   a. ...শুধুমাত্র জন্ম করতে পারেন নি ?
   b. ...ফুথি বং ডাক বা বামি...
   c. ...ফুথি...
   d. ...সর্চ খাওয়ার মর্ম নাই এমন অবস্থা...
   e. ...চাই হলদ হয়ে গেছে বা চাই হলদ ...
   f. ...ফুথি রং এর পারস্পরিতা বা বুঝি হয় ডাকের সত্ত্ব রয়ে গেলাদ ...  
   g. ...ক্রিকেট খাদ্য (উপর পেটের জন নিকে)...

   YES NO
   +1  0
   +1  0
   +1  0
   +1  0
   +2  0
   +2  0

সন্তানতন্ত্রীনন্দনস্তের নিয়ে হুঁসঃ score যেতে হবে এবং যদি score < ৪ এর কম হয় তাহলে উপর ব্যাখ্য সম্পর্কে প্রকৃতি করা হবে করন।

যদি score ≥ ৪ হয় তাহলে তাহলে সন্তানতন্ত্রীনাদের থেকে বদন করন এবং তার COMMENTS SECTION OF DAD “NOT ELIGIBLE” কে লিখুন।
Appendix K1. Data Collection Instrument – Case Pre-Enrollment Form (CPE)

HEV_CPE_v1.0_August 14, 2004

HEV Case Pre-Enrollment (HEV - CPE)
Worker ID: [ ] - [ ] Worker Initials: _______________________

Attempt: 1 - [ ] [ ] [ ] [ ] Comments: _______________________

2 - [ ] [ ] [ ] [ ]

3 - [ ] [ ] [ ] [ ]

Section A: Identifiers
Interviewer Note: Please complete Identifiers before going to field.

<table>
<thead>
<tr>
<th>Name</th>
<th>HEV Study ID [ ] - [ ] [ ]</th>
</tr>
</thead>
<tbody>
<tr>
<td>CID</td>
<td>RID</td>
</tr>
<tr>
<td>Bari Name</td>
<td>Head Name</td>
</tr>
</tbody>
</table>

Sex [ ] 1=Male 2=Female 3=Don’t Know

Date of Birth [ ] [ ] [ ]

Form Status [ ]
1=Form Complete
2=Not Met 3 Times
3=Refused Consent
7=Permanently Moved from Study Area
8=Died

Section B. CHRW Information
From Case Report Card:

1. Hepatitis Score:

2. Days since Onset:

Section C: Fingerstick Specimen

I will now collect a few drops of blood from the tip of your finger.

1. Specimen collected:
2=Collected from Virology Tube
6=Refused Specimen

FIELD TEAM NOTE: Remember to place ID LABEL on the TUBE (□):

Place FORM Label Here: [ ]
Appendix K2. Data Collection Instrument – Case Pre-Enrollment Form (CPE) (Bangla)
Appendix L. Data Collection Instrument – Virology Assessment (VA)

HEV VA v.1_August 14, 2004

HEV Virology Assessment (HEV - VA)

MO ID: ___________ MO Initials: ___________

Attempt: 1 - ___________ ___________ ___________ ___________ ___________

2 - ___________ ___________ ___________ ___________ ___________

3 - ___________ ___________ ___________ ___________ ___________

Date of Examination: ___________ ___________ ___________ ___________

Section A: Identifiers

Name: ___________ HEV Study ID: ___________ ___________ ___________

CID: ___________ ___________ ___________ ___________ ___________

Bari Name: ___________ Head Name: ___________

Sex: 1=Male 2=Female 9=Don't Know

Date of Birth: dd / mm / yy

Form Status: 1=Form Complete 2=Refused 3=Permanently Moved from Study Area

STOP EXAMINATION

Section B: Physician Examination

1. Pulse: ___________ bpm

2. BP: ___________ / ___________ mm Hg

3. Temperature: ___________ °F

4. Anemia?: ___________ 0=Yes 1=No

5. Jaundice?: ___________ 0=Yes 1=No

6. Liver Enlarged?: ___________ 0=Yes 1=No

Section C: Patient History

Section D: Specimen (If < 10 days since onset)

1. Blood collected: ___________ 0=No 1=Yes 6=Refused Specimen

2. Stool collected: ___________ 0=No 1=Yes 6=NA or Refused Specimen

MO NOTE: Remember to place ID LABEL on the TUBE / STOOL CONTAINER (☐):

Place “FORM” ID Label Here: ___________

MO NOTE: Remember to Complete HEV Case Pre-Enrollment Form (☐):
# Appendix M1. Data Collection Instrument – Case Control Questionnaire (CCQ)

## HEV Case Control Questionnaire (HEV - CCQ)

**Worker ID:** [ ] [ ] [ ] [ ]

**Attempt:**

1. [ ] [ ] [ ]
2. [ ] [ ] [ ]
3. [ ] [ ] [ ]

**Section A: Identifiers**

- **Name:**
- **HEV Study ID:** [ ] [ ] [ ] [ ]
- **CID:** [ ] [ ] [ ] [ ] [ ] [ ]
- **RID:** [ ] [ ] [ ] [ ] [ ] [ ]
- **Bar1 Name:**
- **Head Name:**
- **Sex:**
  - 1 = Male
  - 2 = Female
  - 0 = Don’t Know
- **Date of Birth:** [ ] [ ] [ ]

**Form Status:**

1 = Form Complete
2 = Not Met 3 Times / Not Met
4 = Not Eligible for Study (Controls Only)
6 = Refused
7 = Permanently Moved from Study Area
8 = Died

→ **STOP INTERVIEW**

---

**Field Team Note:**

If a Control is Not Eligible for Interview, place a ‘4’ in the Form Status Box.
If a Case or Control is eligible and agrees to participate, then staple the remaining form to this COVER PAGE.
Section B. Demographic and Socioeconomic Assessment

1. What is your marital status?
   1 = Single
   2 = Married
   3 = Divorced
   4 = Widowed
   5 = Refused
   6 = Don't Know
   7 = Muslim
   8 = Hindu
   9 = Tribal
   10 = Other

2. What is your religion?

3. How many members live in your household? (Members include all infants, children and adults who have lived in the household for at least 6 months. Infants under 6 months also count as members.)
   Male
   a. Adults (≥ 50 y)....................... [ ] [ ]
   b. Adults (> 18 y and < 50 y)........ [ ] [ ]
   c. Adolescents (13-18 y)............ [ ] [ ]
   d. Children (5-12 y).................. [ ] [ ]
   e. Children (< 5 y).................... [ ] [ ]

   Female
   a. Adults (≥ 50 y)....................... [ ] [ ]
   b. Adults (> 18 y and < 50 y)........ [ ] [ ]
   c. Adolescents (13-18 y)............ [ ] [ ]
   d. Children (5-12 y).................. [ ] [ ]
   e. Children (< 5 y).................... [ ] [ ]

4. What kind of work do you spend most of your time doing?
   0 = Homework
   1 = Work on own farm or co sharecropper
   2 = Day, unskilled labour (agricultural & migrant)
   3 = Fisherman/woman
   4 = Contracted labourer (long term, domestic, agricultural)
   5 = Own business (shopkeeper, vendor, itchiya van vendor, artisan)
   6 = Private service (unskilled, skilled factory and office workers, salesmen, skilled labourer)
   7 = Government service (all GOI-paid employee)
   8 = Other
   9 = Don't know
5. Do you do this work mainly indoors or outside?
   1 = Indoors
   2 = Outside
   9 = Don’t Know

6. Do you have a secondary occupation for which you are paid in cash or kind?
   If yes, what is it?
   0 = None (Go to 7)
   1 = Work on own farm or as sharecropper
   2 = Day, unskilled labor (agricultural & migrant)
   3 = Fisherman/woman
   4 = Contracted labor (long term domestic, agricultural)
   5 = Own business (shopkeeper, vendor, akocha/van puller, artisan)
   6 = Private service (maid, skilled factory and office workers, salesmen, skilled laborer)
   7 = Government service (all OCB-paid employees)
   8 = Other
   9 = Don’t know

6a. Is this work you do mainly indoors or outside?
   1 = Inside
   2 = Outside
   9 = Don’t Know

7. What was the highest class you completed in school?
   00 = No schooling
   01-09 = From class 1 to class 9
   10 = SSC passed
   11 = 11 years completed
   12 = HSC passed
   13 = 13 years completed
   14 = Degree or higher
   99 = Don’t know

8. What is the highest class completed by the head of your household?
   00 = No schooling
   01-09 = From class 1 to class 9
   10 = SSC passed
   11 = 11 years completed
   12 = HSC passed
   13 = 13 years completed
   14 = Degree or higher
   90 = Not applicable (same as 7)
   99 = Don’t know
9. How many of the following animals are owned by your household?
   a. Cattle / Water Buffalo
   b. Goats / sheep
   c. Mature chickens
   d. Mature ducks
   e. Other (A)
   f. Other (B)

10. How much land owned by your household is cultivated for crops?  
    cultivated for crops
    kitchen garden
    fruit / bamboo groves
    used for fish pond
    house / home
    other land

11. House construction (look and record):
    a. Ground floor walls: 0 = No walls (≤ 2 walls) / Fence  
    1 = Thatch, grass, sticks, branches  
    2 = Katcha (bamboo with/without mud, packed mud)  
    3 = Tin/wood plank  
    4 = Pakka (concrete, brick, stone)  
    9 = Don't know
    b. Roof:
    0 = No roof/plastic  
    1 = Thatch or grass  
    2 = Tin  
    4 = Pakka (concrete/tile)  
    9 = Don't know
    c. Floor:
    0 = Earth  
    1 = Cement  
    2 = Tiles  
    9 = Don't know

Unit
1 = Biglu
2 = Decimal
3 = Katha
4 = Acre
12. What is your family’s average monthly income?
- No income (0)
- 1-100 taka (1)
- 100-499 taka (2)
- 500-999 taka (3)
- 1000-1999 taka (4)
- 2000-2999 taka (5)
- 3000-4999 taka (6)
- 5000-7999 taka (7)
- 8000-8999 taka (9)
- Don’t know / Refused (9)

13. Are you a member of any of the following micro-credit organizations?
   a. Grameen Bank
   b. BRAC
   c. BRDB
   d. Any other organization

Section C. Health and Sanitation Exposure Evaluation

1. Over the past three months how many times have you traveled to a town or city?
- 0-98 Number of Times (0)
- Don’t know (9)

2. In the past week, on average, how many times did you eat outside the home?
- 0-98 Number of Times (If > 98, Go to 2a)
- Don’t know (9)

   2a. When eating outside, do you eat food cooked at home or food from restaurants (hotels)?
   - Home (1)
   - Other Source (2)
   - Don’t know (9)

3. Are you currently suffering from any illness which has lasted for more than 30 days?
- No (0)
- Yes (Go to 2a)
- Don’t know (9)

   3a. What illness have you had?

4. Over the past 3 months have you had any contact with any person with jaundice or yellow eyes or yellow skin?
- No (0)
- Yes (1)
- Don’t know (9)
5. Over the past 3 months have you had any contact with any sick person?
   □ 0= No
   □ 1= Yes
   □ 9= Don't Know

6. Over the past 3 months has any doctor or medical person told you that you had jaundice or hepatitis?
   □ 0= No
   □ 1= Yes
   □ 9= Don't Know

7. Over the past 3 months have you had ________? 7a. IF YES, How many days did this last?
   i. extreme weakness
   ii. nausea or vomiting
   iii. fever
   iv. loss of appetite
   v. yellow eyes or skin
   vi. clay colored stools
   vii. dark colored urine
   viii. liver pain
   ix. constipation

   □ 0= No
   □ 1= Yes
   □ 9= Don't Know

8. Over the past 3 months have you had any injections?
   □ 0= No
   □ 1= Yes
   □ 9= Don't Know

9. Over the past 3 months have you had any blood transfusions?
   □ 0= No
   □ 1= Yes
   □ 9= Don't Know

10. Over the past 3 months have you had sought treatment from any ouja / fakir / kabiraj?
    □ 0= Self treatment
    □ 1= Home remedy
    □ 2= Shaman (Religious Healer)
    □ 3= Homopathic/Ayurveda
    □ 4= Medicine shop
    □ 5= Village doctor
    □ 6= Paramedic (PWV, MA, HA, SAC/W, SC, FWC/CC, CHW)
    □ 7= Doctor (MBBS) / Clinic / Subcenter
    □ 8= Hospital (THC)
    □ 9= Don't Know

11. When you are sick, to whom do you normally go for treatment?
    □ 0= Self treatment
    □ 1= Home remedy
    □ 2= Shaman (Religious Healer)
    □ 3= Homopathic/Ayurveda
    □ 4= Medicine shop
    □ 5= Village doctor
    □ 6= Paramedic (PWV, MA, HA, SAC/W, SC, FWC/CC, CHW)
    □ 7= Doctor (MBBS) / Clinic / Subcenter
    □ 8= Hospital (THC)
    □ 9= Don't Know
12. Do you regularly take medicine for “gastric” illness?

0 = No
1 = Yes
9 = Don’t Know

13. What is your blood group?

1 = A
2 = B
3 = AB
4 = O
9 = Don’t Know

14. MUAC Measurement: [ ] [ ] mm

15. What kind of latrine/toilet facility does your household use?

0 = None/field/bush
1 = Open/hanging latrine
2 = Pit latrine
3 = Water sealed/slab
4 = Flush toilet
9 = Don’t know

15a. Is this located within, adjacent to or far from your residence?

0 = Never
1 = Within
2 = Adjacent to
9 = Far from

15b. How often is your latrine cleaned?

0 = Never
1 = Once per month
2 = Once per week
3 = Once per day
9 = Don’t know

15c. Who cleans your latrine?

0 = Nobody / Not applicable
1 = Self
2 = Family member
3 = Non-family member
9 = Don’t know

16. Normally, do you wash your hands…….

IF YES, ……………………. IF YES, ……………………. 

a. before eating food?……

b. What do you use?……

c. How many hands do you wash?……

1 = Water only
2 = Water and Ash
3 = Water and Earth
4 = Water and Soap
5 = Other
9 = Don’t know

1 = One hand
2 = Both hands
9 = Don’t know

d. after defecation?……

e. What do you use?……

f. How many hands do you wash?……
17. Other than clothes-washing soap, do you currently have any other type of bar soap in your household?

☐ 0=No
☐ 1=Yes
☐ 9=Don't know

18. At what level is the household flooded in the rainy seasons?

☐ 0=Not flooded (Go to 19)
☐ 1=Partially
☐ 2=Completely
☐ 9=Don't know

18a. Is your household flooded now?

☐ 0=No
☐ 1=Yes
☐ 9=Don't know

19. Do you use attend a local barber for hair cutting or shaving?

☐ 0=Never
☐ 1=Sometimes
☐ 2=Always
☐ 6=Not Applicable
☐ 9=Don't know

Section D: Water Related Questions

1. In the past two months, what has been your main source of water for:
   a. Drinking 
   b. Cooking 
   c. Bathing 
   d. Washing dishes 
   e. Washing clothes 

2. Do you have a secondary source of water for ____? If YES, what is it?
   1= Tubewell
   2= Pond
   3= Rainwater
   4= River
   5= Bheel
   6= Khal
   8= Other
   9= Don't Know

3. How many glasses of water do you drink per day on average?

☐ 0= No
☐ 1= Yes (Go to 3a)
☐ 6= Number
☐ 8= 8 or more
☐ 9= Don't know

3a. Do you store drinking water in your household?

☐ 0= No
☐ 1= Yes (Go to 3b)
☐ 9= Don't know
3a. In what do you store it?

4. Is there a pond in your bari?

4a. How much water did you have in the pond last month?

5. Is there a tubewell in your bari?

5a. What is the arsenic status of the well?

6. Does your family use some method to purify the water before drinking?

6a. What method does your family use?
### Section E: Animal exposures

*I will now ask you if you spend time working with any animals such as cows, goats, chickens, ducks or other animals on a regular basis.*

<table>
<thead>
<tr>
<th>Do you work with _____ on a regular basis?</th>
<th>1. <strong>IF YES:</strong> What work do you do with _____s?</th>
<th>2. How many hours each week, on average, do you spend doing this?</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>0 = No</strong></td>
<td><strong>00 = Less than One Hour</strong></td>
</tr>
<tr>
<td></td>
<td><strong>1 = Bathing / Cleaning</strong></td>
<td><strong>01-08 = Number of Hours</strong></td>
</tr>
<tr>
<td></td>
<td><strong>2 = Feeding</strong></td>
<td><strong>99 = Don’t Know</strong></td>
</tr>
<tr>
<td></td>
<td><strong>3 = Milking</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>4 = Sacrificing</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>5 = Other</strong></td>
<td></td>
</tr>
<tr>
<td>i. Cow</td>
<td>[ ] [ ] [ ]</td>
<td>[ ] [ ]</td>
</tr>
<tr>
<td>ii. Goat / Sheep</td>
<td>[ ] [ ] [ ]</td>
<td>[ ] [ ]</td>
</tr>
<tr>
<td>iii. Chickens</td>
<td>[ ] [ ] [ ]</td>
<td>[ ] [ ]</td>
</tr>
<tr>
<td>iv. Ducks</td>
<td>[ ] [ ] [ ]</td>
<td>[ ] [ ]</td>
</tr>
<tr>
<td>v. Dogs</td>
<td>[ ] [ ] [ ]</td>
<td>[ ] [ ]</td>
</tr>
<tr>
<td>vi. Other: ________</td>
<td>[ ] [ ] [ ]</td>
<td>[ ] [ ]</td>
</tr>
</tbody>
</table>

3. Is the dung of any animal used in your household?

   0 = No
   1 = Yes (Go to 4a)
   9 = Don’t Know

3a. For what purposes?

   1 = Fertilizer
   2 = Fuel / Fire
   3 = House repairs
   4 = Cleaning
   5 = Other
   9 = Don’t Know

4. Have you noticed rats or mice or moles in or near the household area in the past 30 days?

   0 = No
   1 = Yes (Go to 4a)
   9 = Don’t Know
5. Have you ever noticed rats or mice or moles in the household area in the past year?

☐ 0= No
1= Yes (Go to 4a)
9= Don’t Know

Section F: For Female Participants > 12 yrs Only (If Age < 12, GO TO SECTION G)

1. Are you currently pregnant? ☐

1a. How many running months pregnant are you?

☐
0–< 1 month
01–99= Number of Months
99= Don’t Know

2. Have you used Injection or ‘Kathi’ contraceptive in the past one year?

☐ 0= No
1= Yes
9= Don’t Know

Section G: For Participants < 12 yrs Only. (If Age > 12, GO TO SECTION H)

1. Has this child played with or been near any sick children over the past 3 months?

☐ 0= No
1= Yes
9= Don’t Know

2. Is this child currently breastfeeding?

☐ 0= No
1= Yes
9= Don’t Know

Section H: Fingerstick Specimen (CONTINUE ONLY IF STUDY ID BEGINS WITH “CO”)

I will now collect a few drops of blood from the tip of your finger.

1. Specimen collected:

☐ 0= No
1= Yes
2= Collected from Virology Tube
6= Refused Specimen

FIELD TEAM / MO NOTE: Remember to place ID LABEL on the TUBE (☑): ☐

Place FORM Label Here: ☐
Appendix M2. Data Collection Instrument – Case Control Questionnaire (CCQ) (Bangla)

HEV Case Control Questionnaire (HEV - CCQ)

<table>
<thead>
<tr>
<th>Worker ID:</th>
<th>Worker Initials:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Attempt: 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Attempt: 2</td>
<td></td>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Attempt: 3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Section A: Identifiers

- Name ____________
- HEV Study ID ____________
- CID ____________
- RID ____________
- Bari Name ____________
- Head Name ____________
- Sex
  - 1 = Male
  - 2 = Female
  - 9 = Don't Know
- Date of Birth: ____________, ____________, ____________
- Form Status
  - 1 = Form Complete
  - 2 = Not Met 3 Times
  - 3 = Not Eligible for Study (Controls Only)
  - 4 = Refused
  - 5 = Permanently Moved from Study Area
  - 6 = Died

Field Note:

- If the Form Status of a Control is not completed, move to Box 4. If Case or Control Form Status is not completed, then mark it as not completed. If Household Case or Control is not completed, move to the next Form Status.

STOP INTERVIEW

Page 1
Section B. Demographic and Socioeconomic Assessment

1. ইন্দোনেশিয়ান কাছা:  
   1 = ইন্দোনেশিয়ান  
   2 = বিনডিয়ান  
   3 = বাংলাদেশী  
   4 = বিনডিয়ান  
   6 = ইন্দোনেশিয়ান আন্তর্জাতিক  
   9 = ইন্দোনেশিয়ান

2. বংশ  
   1 = মূলধার  
   2 = ফন্ডা  
   3 = উলুমচার  
   6 = ইন্দোনেশিয়ান আন্তর্জাতিক  
   8 = জাতিরা  
   9 = জাতিরা

3. আসুপাতিক বংশ কুটিলতা (সাধারণ সংখ্যার মধ্যে) সূচিত পাতিক নিচে, নামক হোল নম্বর, নিবন্ধন-বিনডিয়ান ও বাংলাদেশী বংশ কুটিলতা নিকো মাদে শেষ পর্যন্ত শেষ হলেও বাংলাদেশী মাদে শেষ হলে (যখনের শব্দ কুটিলতা নিউক্লিয়ার নামে কুটিলতা তে সংরক্ষিত হবে)

<table>
<thead>
<tr>
<th>প্রশ্ন</th>
<th>উত্তর</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. বয়স (১৫ বছর)</td>
<td>☐ ☐ ☐</td>
</tr>
<tr>
<td>b. বয়স (১৬ বছর এবং &lt;৫০ বছর)</td>
<td>☐ ☐ ☐</td>
</tr>
<tr>
<td>c. বিশ্ববিদ্যালয়িত্ব (১৩-১৬ বছর)</td>
<td>☐ ☐ ☐</td>
</tr>
<tr>
<td>d. নিয়ন্ত্রণ (২-১২ বছর)</td>
<td>☐ ☐ ☐</td>
</tr>
<tr>
<td>e. নিয়ন্ত্রণ (&lt;৫ বছর)</td>
<td>☐ ☐ ☐</td>
</tr>
</tbody>
</table>

4. জাতিরা ও জন্মস্থান তথ্য ডাটান্নি বেশিরভাগ সময় পার্থক্য করেন

<table>
<thead>
<tr>
<th>নম্বর</th>
<th>উত্তর</th>
</tr>
</thead>
<tbody>
<tr>
<td>00 = পূর্ববর্তী নাম</td>
<td>☐ ☐ ☐</td>
</tr>
<tr>
<td>01 = বিনডিয়ান জন ধর্ম জনহিত সংস্থা</td>
<td>☐ ☐ ☐</td>
</tr>
<tr>
<td>02 = বেশি জনক, বাংলাদেশী নাম এবং বাঙালি নাম (নাম)</td>
<td>☐ ☐ ☐</td>
</tr>
<tr>
<td>03 = জাতি</td>
<td>☐ ☐ ☐</td>
</tr>
<tr>
<td>04 = বিনডিয়ান শিক্ষা</td>
<td>☐ ☐ ☐</td>
</tr>
<tr>
<td>05 = বিনডিয়ান (বিনডিয়ান, সশস্ত্র, বিনডিয়ান জনি, বিনডিয়ান)</td>
<td>☐ ☐ ☐</td>
</tr>
<tr>
<td>06 = জাতিরা সংজ্ঞা (বাংলাদেশী, বাংলাদেশী, বাংলাদেশী, বাংলাদেশী)</td>
<td>☐ ☐ ☐</td>
</tr>
<tr>
<td>07 = জাতিরা সংজ্ঞা</td>
<td>☐ ☐ ☐</td>
</tr>
<tr>
<td>10 = বিনডিয়ান</td>
<td>☐ ☐ ☐</td>
</tr>
<tr>
<td>11 = নির্দেশনা</td>
<td>☐ ☐ ☐</td>
</tr>
<tr>
<td>12 = নির্দেশনা</td>
<td>☐ ☐ ☐</td>
</tr>
<tr>
<td>88 = জাতিরা</td>
<td>☐ ☐ ☐</td>
</tr>
<tr>
<td>99 = জাতিরা</td>
<td>☐ ☐ ☐</td>
</tr>
</tbody>
</table>
5. আপনি কে করে নিয়োজিত নেটি ফি মজের কিছু না বাঁচিয়ে করুন?

☐ 1 = মজের ফিন্টার
☐ 2 = বাঁচিয়ে
☐ 9 = আমি না/বহুমূর্ত নয়

6. আপনি কে করে নিয়োজিত নেটি হাতাকাঁ অন্য বোলার পাশের নিয়োজিত আছেন নিঃ?

☐ 0 = অন্য একজন বোলার নাই (Go to 7)
☐ 1 = বিনা জন অন্যরা একজন বলভাবে সমালোচনা
☐ 2 = বিনা জন একজন (বিনামূল্যে বলসেবার বলভাবে)
☐ 3 = বলসেবার
☐ 4 = বিনা জন প্রথম (বিনামূল্যে বলসেবার বলসেবার)
☐ 5 = বিনা জন বাহ্য (সরলরেখায় বিনামূল্যে বলসেবার, বিনা জন বাহ্য, বৃত্ত বহিঃস্থিত)
☐ 6 = বাহ্য বিনা জন (বিনামূল্যে বলসেবার বলসেবার বলসেবার বলসেবার, বাহ্য বিনা জন বাহ্য, বৃত্ত বহিঃস্থিত)
☐ 7 = বাহ্য বিনা জন
☐ 8 = গুলাকাঁ
☐ 9 = আমি না

6a. এই কাউন্টি হতে ফিন্টার না সমালোচনা করুন?

☐ 1 = মজের ফিন্টার
☐ 2 = বাঁচিয়ে
☐ 9 = আমি না/বহুমূর্ত নয়

7. আপনি কোন ক্রান্ত পর্যন্ত পড়ান্তে রয়েছেন?

☐ ☐ 00 = বহুমূর্ত কিন্তু বিনা জন বোলার
01-09 = প্রথম প্রথম হতে বহুমূর্ত ক্রান্ত পর্যন্ত
10 = এমন-এমন বোলার
11 = একন্যার ক্রান্ত পর্যন্ত
12 = একন্যার এক বা দুটি বোলার
13 = ১০ চাপ বাহ্য
14 = অন্তর্গত বল বা তার উপরে
99 = আমি না

8. আপনার পরিস্থিৎের হিসেবে কোন ক্রান্ত পর্যন্ত পড়ান্তে রয়েছেন?

☐ ☐ 00 = বহুমূর্ত কিন্তু বিনা জন বোলার
01-09 = প্রথম প্রথম হতে বহুমূর্ত ক্রান্ত পর্যন্ত
10 = এমন-এমন বোলার
11 = একন্যার ক্রান্ত পর্যন্ত
12 = একন্যার এক বা দুটি বোলার
13 = ১০ চাপ বাহ্য
14 = অন্তর্গত বল বা তার উপরে
99 = আমি না
9. যাঙ্গাদার পজিশনের মাধ্যমে নির্মিত কাগজে কথা লিখে আছে।
   a. সাপ ও মাসন.........................
   b. মানে ও মান্ত্র........................
   c. মুক্তি (ক্রম)
   d. বল (ক্রম)
   e. অফিস (A)
   f. অফিস (B)

00 = সাপ
01-97 = মাসন
98 = মানে অথবা
99 = মান্ত্র

10. যাঙ্গাদার অধিকার মাধ্যমে কথা লিখতে হবে কি কিনা জানা যায়?
   
   000 = নাহি
   001-997 = একক
   998 = ৬৬৮ একক না যেকী?
   999 = যেকী না

   a. জনসংখ্যা তথ্য
   b. গবেষণাপ্রতিষ্ঠান তথ্য
   c. কল এবং কলকেন্দ্র
   d. সাক্ষাৎ তথ্য
   e. দৌহিত্র তথ্য
   f. আদালত তথ্য

11. (নাহি দে কথা কোনো নাহি তথ্য)
   a. একক অথবা বৃহদে যেকী ব্যক্তি না
   b. ব্যক্তি
   c. কোনো
12. আপনার প্রজিয়েট থেকে মাসিক আয়/যেজনার কর্তা?

<table>
<thead>
<tr>
<th>নম্বর</th>
<th>অর্থ তালিকা</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>এক কয়েক তালিকা</td>
</tr>
<tr>
<td>1</td>
<td>&gt;৫০০ টাকা</td>
</tr>
<tr>
<td>2</td>
<td>৫০০-৬০০ টাকা</td>
</tr>
<tr>
<td>3</td>
<td>৬০০-৭০০ টাকা</td>
</tr>
<tr>
<td>4</td>
<td>৭০০-৮০০ টাকা</td>
</tr>
<tr>
<td>5</td>
<td>৮০০-৯০০ টাকা</td>
</tr>
<tr>
<td>6</td>
<td>৯০০-১০০০ টাকা</td>
</tr>
<tr>
<td>7</td>
<td>১০০০-১১০০০ টাকা</td>
</tr>
<tr>
<td>8</td>
<td>&gt;১২০০০ টাকা</td>
</tr>
<tr>
<td>9</td>
<td>জানি না</td>
</tr>
</tbody>
</table>

13. আপনি কি মাত্রে যেকোনো প্রতিযোগিতা প্রদর্শনীর সংখ্যা?

<table>
<thead>
<tr>
<th>উপায়</th>
<th>জবাব</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. প্রাতন ব্যাংক</td>
<td>0 = না</td>
</tr>
<tr>
<td>b. নীল</td>
<td>1 = হা</td>
</tr>
<tr>
<td>c. দিনি, আবার, বিয়</td>
<td>9 = জানি না</td>
</tr>
<tr>
<td>d. অন্য কোন পদ্ধতি</td>
<td></td>
</tr>
</tbody>
</table>

Section C. Health and Sanitation Exposure Evaluation

1. গত বছরে আপনি কত বার ভাইর বা বন্দরের জন্য খাঁচা করেছেন?

<table>
<thead>
<tr>
<th>জবাব</th>
<th>জবাব</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-98</td>
<td>99</td>
</tr>
</tbody>
</table>

2. গত এক বছরে আপনি কত বার আঘাত বা বাহিয় থাকেন?

<table>
<thead>
<tr>
<th>জবাব</th>
<th>জবাব</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-98</td>
<td>99</td>
</tr>
</tbody>
</table>

3a. বন্দর বাহিয় থাকার প্রথম বার আঘাত বা বাহিয় থাকার প্রথম বার কী হয়েছিল?

<table>
<thead>
<tr>
<th>জবাব</th>
<th>জবাব</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

3. গত ৩০ দিনের বেশি জুটি কত বার আঘাত বা বাহিয় থাকার প্রথম বার কী হয়েছিল?

<table>
<thead>
<tr>
<th>জবাব</th>
<th>জবাব</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

4a. আপনি যে অবস্থা আছে যেখানে আপনি কোন বৌদ্ধিক/সার্জন প্রয়োগ, বৌদ্ধিক সাথে সংযুক্ত হয়েছিল?

<table>
<thead>
<tr>
<th>জবাব</th>
<th>জবাব</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

199
5. গত তিন মাসে আপনি কি কোন অসুস্থতা সংঘের এবং ছিলেন?
   0 = না
   1 = যা
   9 = কাজের

6. গত তিন মাসে আপনার কোন ডাক্তার হিসেবে কাজ করা কাজ করেছেন তারা অসুস্থতা হিসেবে "Hepatitis" হিসাবে?
   0 = না
   1 = যা
   9 = কাজের

7. গত তিন মাসে আপনার ------------------ ছিল?
   7a. মাস্তি রয়েছে না, কাজের কোন কাজ করেনা কীভাবে মনে করেন?

<table>
<thead>
<tr>
<th>সংখ্যা</th>
<th>ক্রিয়া/চিহ্নিত করুন</th>
</tr>
</thead>
<tbody>
<tr>
<td>i. পুনরুদ্ধিত রোগের কারণ কবল করা</td>
<td>0 = না</td>
</tr>
<tr>
<td>ii. কসং বা বাকি পরিস্থিতি</td>
<td>1 = যা</td>
</tr>
<tr>
<td>iii. শুক্র</td>
<td>9 = কাজের</td>
</tr>
<tr>
<td>iv. সকলকে বোঝানি পরিস্থিতি নাই</td>
<td>0 = না</td>
</tr>
<tr>
<td>v. স্থানীয় রোগের কারণ বোঝানি</td>
<td>1 = যা</td>
</tr>
<tr>
<td>v. বাঁকুর রোগ</td>
<td>9 = কাজের</td>
</tr>
<tr>
<td>vi. ক্যাশার গ্রহণ করার সাথে</td>
<td>0 = না</td>
</tr>
<tr>
<td>vii. দুই বাঁকুর রোগ তারকের কারণে</td>
<td>1 = যা</td>
</tr>
<tr>
<td>viii. উপর পাওয়া নিয়ন্ত্রণ করার কারণে</td>
<td>9 = কাজের</td>
</tr>
<tr>
<td>ix. ল্যাপ প্ল্যাটফর্ম</td>
<td>0 = না</td>
</tr>
</tbody>
</table>

8. গত তিন মাসে আপনি সিঁড়ি বা কেনাবাঁকা নিয়েছেন?
   0 = না
   1 = যা
   9 = কাজের

9. গত তিন মাসে আপনি কি কোন ডাক্তার নিয়েছেন?
   0 = না
   1 = যা
   9 = কাজের

10. গত তিন মাসে আপনি কি কোন ওমা/বর্তিমাকাত নিয়েছেন?
    0 = না
    1 = যা
    9 = কাজের

11. আপনি কিনা অসুস্থতা সংক্রান্তে আপনি সিঁড়িকে ঝড় বাঁকুর নিয়েছেন?
     0 = নির্দেশিত
     1 = যা
     2 = যা যা নির্দেশিত
     3 = যা নির্দেশিত, অসুস্থতার নির্দেশিত
     4 = অন্য কোন সংক্রান্ত
     5 = বাঁকুর সিঁড়িকে নির্দেশিত
     6 = সিঁড়িকে (EWW, MA, HA, SACMO, SC, FWO, CC, CHR,W)
     7 = বাঁকুর (MBBS)/ক্যাশার/সাবেক সিঁড়ি কর
     8 = জনসাধারণ (ICDDR, B, THC)
     9 = কাজের
12. আপনি কি নির্দিষ্ট গায়েকের ওজন খালি?
   0 = না
   1 = খুব খালি
   9 = কারীনা

13. আপনার হাতের ধরন কি?
   1 = A
   2 = B
   3 = AB
   4 = O
   9 = কারীনা

14. MUAC Measurement: [ ] [ ] mm

15. আপনি কি চক্রের পাঠকেরা বাদাম দিড়ি?
   0 = না
   1 = খুব কম
   2 = বাদাম আর মূল্যবান
   9 = কারীনা

15a. কি বাদামের ফিকে, সাথে ভালো রাইকে?
   0 = না
   1 = খুব কম
   2 = কারীনা তাছাড়া

15b. দেই পাঠকের কাঠিন পার পরিশীলন কর করে?
   0 = না
   1 = কমই না
   2 = বাদাম একটু
   3 = মূল্যবান একটু
   9 = কারীনা

15c. দেই পাঠকের দেশীয় জন সমস্ত কে পরিশীলন করে?
   0 = কমই না/আকার তোয়
   1 = কমই না
   2 = পাঠকের দেশীয় জন
   3 = মূল্যবান আকার
   9 = কারীনা

16. সম্ভাব্য আলোক কি .......................................................................................................................................................................................... আলোকের হাতে খেলা?
   a. ডান মাথা আলোক
   b. কি পিছনে হাত খেলা?
   c. কি মাথা খেলা?
   d. পাঠকের কাচ খেলা?
   e. কি পিছনে খাতে খেলা?
   f. কি মাথা খেলা?
   
   0 = না
   1 = খুব
   9 = কারীনা

   1 = নুজ গায়েক
   2 = কর্ম ও বাড়ি
   3 = পাঠক ও বাড়ি
   4 = পাঠক ও গায়েক
   5 = গায়েক
   9 = কারীনা
17. কলকুড়া থেকে সড়ক ছাড়া আনাগোনা বাগান কি ধরণের কলিকুড়া বাড়িতে বসবাস করেন আছে?

0 = না
1 = হীরামা
9 = অন্য কোন প্রকারের বাড়ি

18. কলকুড়া আনাগোনা বাগানের কলিকুড়া পানি উদ্ধার?

0 = বাণিজ্য (19 দিন পর)
1 = অনুমোদন
2 = অনুমোদন এবং
9 = অন্য কোন প্রকার

18a. কলকুড়া আনাগোনা বাগানের পানি উদ্ধার?

0 = না
1 = হীরামা
9 = অন্য কোন প্রকার

19. আনাগোনা কি জলের সাহায্য করাতে যুক্তি দিতে কোন আনাগোনা পানি যোগ্যতা রয়েছে?

0 = না
1 = জল নোট প্রকাশ
2 = সন্ধান
6 = Not Applicable
9 = অন্য কোন প্রকার

Section D: Water Related Questions

1. গত ২ মাসে আনাগোনা বাগান বিদেশের পানি ব্যবহার করেন কতক্ষণ?

II. একোশাল ও তলা হিসেবে পানি ব্যবহার করতেন কতক্ষণ?

(যদি কোন চিহ্ন আপনার বিদেশের পানি)

a. বাণিজ্য পানি
b. বাণিজ্য পানি
c. বাণিজ্য পানি
d. পানি ব্যবহার করেন
e. বাণিজ্য ব্যবহার পানি

2. আনাগোনা বাণিজ্য করা পানি একোশাল নয় পানি পান করেন?

0-7 = গভীর
8-9 = মুন্না

3. কলকুড়া পানি কত পেন পান করা যায়?

0 = একোশাল
1 = তলা
9 = অন্য কোন প্রকার
3a. আপনি পানি কোথায় জমিয়ে রাখেন?

4. আপনার বাড়িতে কি পেয়ালা পুর্বী আছে?

4a. গতহারে আপনার পুরুষ কি পানি রইল হয়?

5. আপনার বাড়িতে কি বোমা টিকে আছে?

5a. এই গিটর প্রেক্ষাপটে কি পানীর মূল্য?

6. আপনার পাত্রের দামগাছ পানি পাল করার পূর্বে কি পরিমাপ করেন?

6a. আপনার পাত্রের দামগাছ কি পক্ষ ব্যাবসার কলে?

---

1 = সরকারী আয়ুধনিয়ার গাড়ি
2 = লোক আয়ুধনিয়ার গাড়ি
3 = সরকারী গাড়ি
4 = লোক গাড়ি
5 = সরকারী সম্পদ (সেকর)
6 = সন্তোষের সম্পদ (সেকর)
7 = অন্য ধরনের
8 = অন্য ধরনের
9 = না
Section E: Animal exposures

<table>
<thead>
<tr>
<th>Question</th>
<th>0 - No</th>
<th>1 - Yes</th>
<th>2 - Yes</th>
<th>3 - Yes</th>
<th>4 - No</th>
<th>5 - No</th>
</tr>
</thead>
<tbody>
<tr>
<td>i. Vaxx</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ii. Vaccination</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>iii. Malaria</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>iv. Dengue</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>v. Typhoid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vi. Other</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3. For each question, circle the answer:

3a. The animal had one of the following:

<table>
<thead>
<tr>
<th>0 - No</th>
<th>1 - Yes</th>
<th>2 - Yes</th>
<th>3 - Yes</th>
<th>4 - No</th>
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4. For this question, circle the answer:

4. 0-9

5. For this question, circle the answer:

5. 0-9
Section F: Identification and Registration of Children

1. Are any of the children documented?
   - 0 = No
   - 1 = Yes (linked to household)
   - 9 = Not sure

2. Are any of the children documented (linked to household)?
   - 0 = No
   - 1 = Yes
   - 9 = Not sure

Section G: Children 12 or younger who have lost parents

1. Are any children under 12 who have lost parents?
   - 0 = No
   - 1 = Yes
   - 9 = Not sure

2. Are any children under 12 who have lost parents?
   - 0 = No
   - 1 = Yes
   - 9 = Not sure

Section H: Fingerstick Specimen

The child is identified as "CO" by the team leader, and the specimen is collected. The specimen is collected:

- 0 = No
- 1 = Yes
- 9 = Not sure

FIELD TEAM MEMO NOTE: The ID Label was placed on the specimen. (☑):

The child's name should be added here: 

---

11
## Appendix N. Study Management Forms – Daily Activity Diary (DAD)

### Daily Activity Diary - TEAM 02

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**SFIRG:** Complete these sections from Control Data Request Form & FS Out HEY COG

**Notes:**

Visit: Eligible: Interviewed: Blood Drawn: Comments:
**Appendix Q. Study Management Forms – Lab Processing Logbook**

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Appendix R. Study Management Forms – Freezer Temperature Logbook

Temperature Log

Freezer No.: [ ]  Location: [ ]

Directions: Freezer temperatures should be recorded each day in the morning and in the evening.

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### Appendix S. Study Management Forms – Refusal Logbook

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<th>Result</th>
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- Refusal Log: 2-0, September 4, 2003
## Weekly Field Report - BL, 12MO, 18MO

### Date: [ ] / [ ] / [ ]  
Week: [ ]  
BL: [ ]  
12MO: [ ]  
18MO: [ ]

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**SFRO / MO Comments**

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Prepared by: __________________________

ID Number: __________________________

Signature: __________________________
# Weekly Field Report - Case Control / Virology

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**SFRO / MO Comments**

Prepared by: ____________________________

ID Number: ____________________________

Signature: ____________________________
References Part IV: Literature Cited


(9) Demographic and Health Survey, Bangladesh. 1994. 1994. Ref Type: Internet Communication


(53) Takahashi K, Kitajima N, Abe N, Mishiro S. Complete or near-complete nucleotide sequences of hepatitis E virus genome recovered from a wild boar, a deer, and four patients who ate the deer. Virology 2004 December 20;330(2):501-5.


(80) Kainer M. Hepatitis E Study in Nepal. 1998. Victoria, Australia, Travelers Medical and Vaccination Center Pty. Ltd. and the Victorian Infectious Diseases Service. Royal Melbourne Hospital. Ref Type: Generic


(87) Hassan M.S. HEV in Bangladeshi Clinic Patients. 1998. Ref Type: Personal Communication


(100) Bradley DW, Krawczynski K, Cook EH, Jr., McCaustland KA, Humphrey CD, Spelbring JE et al. Enterically transmitted non-A, non-B hepatitis: serial passage of disease in cynomolgus macaques and tamarins and recovery of disease-


(142) Arankalle VA, Chobe LP, Joshi MV, Chadha MS, Kundu B, Walimbe AM. Human and swine hepatitis E viruses from Western India belong to different genotypes. J Hepatol 2002 March;36(3):417-25.


(222) Vitral CL, Pinto MA, Lewis-Ximenez LL, Khudyakov YE, dos Santos DR, Gaspar AM. Serological evidence of hepatitis E virus infection in different animal species from the Southeast of Brazil. Mem Inst Oswaldo Cruz 2005 April;100(2):117-22.


(257) Staff correspondent. Hepatitis E main cause of jaundice at DU. The Daily Star 2004 Dec 5;1.


(283) Khuroo MS, Kamili S, Yattoo GN. Hepatitis E virus infection may be transmitted through blood transfusions in an endemic area. J Gastroenterol Hepatol 2004 July;19(7):778-84.


Brief Curriculum Vitae
Alain Bernard Labrique, MHS, MS, BS

BIOGRAPHIC DETAILS
Belgian Citizen
Born in Bangladesh, May 25, 1974
Wife: Kimberly Hoyle Labrique
Children: David Alexander Labrique

EDUCATION AND TRAINING
The Johns Hopkins Bloomberg School of Public Health
Doctor of Philosophy (Epidemiology - Infectious Disease) February 2007
Master of Health Science (Epidemiology - Infectious Disease) May 1999

University of North Carolina at Chapel Hill
Master of Science (Molecular Biology) June 1997
Bachelor of Science (Biology) May 1996
With Commendation for Undergraduate Research

HONORS AND AWARDS
Johns Hopkins Bloomberg School of Public Health Technology Transfer SEED Awardee (2005-2006)
The David and Elinor Bodian Scholarship (JHU SPH) 2001-2002
Ruth Rice Puffer Award for International Students (JHU SPH) 2001
Charlotte Silverman Award for Outstanding Community Service (JHU SPH-Epidemiology) 2001
Delta Omega (Alpha Chapter) Scholarship Recipient 2001
JHSPH Infectious Disease Program (Epidemiology) Tuition Scholarship 1999-2000, 2000-2001
JHSPH Student Volunteer InterAction Certificate of Appreciation for Exemplary Service
University of North Carolina Dean's List
Office of North Carolina Fellows Leadership Program

PROFESSIONAL EXPERIENCE
Johns Hopkins Bloomberg School of Public Health
Department of International Health – Center for Human Nutrition
•Research Associate Faculty / Epidemiologist – 2001 to Present
Resident Project Scientist and Country Representative for the JiVitA Research Program in Northern Bangladesh for the past 5 years. Responsible for the day-to-day scientific, operational, administrative, financial and logistical oversight/ maintenance. Specifically, responsible for ensuring the scientific and operational integrity of two large double-masked, randomized, micronutrient trials while leading senior field staff in planning a third in a surveillance population of over a half-million people covering an 800km² area and implemented by over 850 staff. The trials are assessing:
1. The efficacy of vitamin A in improving nutritional status and reducing maternal and infant mortality (N=67,000 pregnant women and 45,000 infants);
2. The efficacy of newborn vitamin A supplementation in improving infant status and reducing neonatal and post-neonatal mortality (N=20,000 newborns);
3. The efficacy of preconceptional, and antenatal through postnatal multiple micronutrient supplementation in improving reproductive health and infant health and survival, presently in its planning and scaling-up stages, due to begin in late 2006 (expected N=>100,000 women of reproductive age).

Johns Hopkins Bloomberg School of Public Health
Department of Epidemiology
•Epidemiologist / Co-Principal Investigator – 2001 to Present
Co-investigator and Project Scientist for an NIH R01-funded study designed to assess the natural history and risk factors of hepatitis E viral infections in rural southern Bangladesh, in collaboration with the ICDDR,B. This study, developed in 2001, began field data collection in April 2003. A baseline seroprevalence study of HBV, HCV and HEV was followed by a 12-, and 18-month HEV seroincidence study. A prospective surveillance of 23,500 individuals to identify community-based acute hepatitis E disease was carried out from August 2004 to June 2006, based on which an exploratory case-control study of hepatitis E disease risk factors was carried out. Responsibilities included:

- Writing grants and securing funding for this research
- Developing the study protocols, data collection instruments, and QC guidelines
- Training, standardizing and supervising the field data collection for this study
- Analysis, interpretation and writing periodic reports and final research findings

**Graduate Research Assistant – 1999 to 2001**
Teaching assistant for Epidemiology department courses. Assisted course faculty with teaching research methods. Designed epidemiologic and virologic studies to investigate hepatitis E virus infections. Coordinated and wrote grants (federal and private) to obtain funding for epidemiologic research. Conducted epidemiologic research on HIV in Burma.

**STARS - Students Teaching and Reaching Students – May 1999 to Present**
Assisted with the development and creation of teaching modules to introduce Epidemiology and Public Health to 9th grade high school students in Baltimore City. Helped formulate project goals, mission and strategic plan.

**Epidemiology 604 Course Development - December 2000 to March 2001**
Directed and managed the digital conversion of on-site Epidemiology course to a digital format. Trained faculty and staff in use of appropriate software for the course.

**Distance Education Division – May 1999 to May 2000, February 2001 to May 2001**
Assess current and develop new and effective internet-based epidemiology and biostatistics units for distance teaching.

**Molecular Biology/Genetics Research on Saccharomyces cerevisiae**
- **July 1996 to August 1997**
  Graduate Research Assistant for Dr. K. Bloom (UNC Department of Biology)
  • Real-time high resolution digital microscopy and imaging of Green Fluorescent Protein labeled chromosome dynamics in mutant yeast.
  • Characterized a novel kinetochore mutant.
- **August 1994 to May 1996**
  Undergraduate Research Assistant for Dr. K. Bloom (UNC Department of Biology)
  • Analysis of centromere chromatin structure in mutant strains of *S. cerevisiae*.
  • Described *S. cerevisiae* mutants with unique centromere histone proteins.
  • Studied conditional dicentric *S. cerevisiae* strains.
- **January 1994 to May 1994**
  Laboratory assistant for Dr. K. Bloom (UNC Department of Biology)

**PROFESSIONAL ACTIVITIES**
- Global Health Council – Associate Member (2006 to present)
- American College of Epidemiology – Member (2000 to present)
- Students Teaching and Reaching Students - Executive Committee (2001 to 2002)
- American College of Epidemiology - Communications Committee (2000 to 2004)
- Society for Epidemiologic Research (1999 to present)
- Tropical Medicine Dinner Club, Baltimore - Board Member (2000 to 2002)
- International Society for Infectious Diseases (2000 to present)
JHSPH School-Wide Committee on Information Technology (1998 to 2000)
JHSPH Student Outbreak Response Team (1998 to 1999)
JHSPH Special WWW Steering Committee - Student Representative (1998)

TEACHING

• **January 2004 to present**
  Mentoring and supervision of JHBSPH Master’s and PhD students conducting their field practica within the JiVitA Project.
  
• **January 2005 to July 2005**
  Designed and taught a short course on Epidemiology and Biostatistics to JiVitA Science Team.
  
• **January 2000 to March 2000**
  Teaching Assistant for STDs: Their Epidemiology and Control
  (Dr. K.V. Shah – Department of Molecular Microbiology and Immunology)
  
• **November 1999 to January 2000**
  Course Coordinator & Teaching Assistant for Infectious Disease Epidemiology
  (Dr. K.E. Nelson – Department of Epidemiology – Infectious Disease Program)
  
• **September 1999 to November 1999**
  Teaching Assistant for Introduction to Epidemiology I
  (Dr. J. Samet – Department of Epidemiology)
  
• **April 1997**
  Infectious Disease Epidemiology Comprehensive Exam Review. Outbreak investigation
  
• **July 1996 to August 1997 (2 Semesters)**
  Teaching Assistant for Introduction to Biology
  (Dr. J. DeSaix – UNC Department of Biology)
  
• **August 1995 to December 1995**
  Physiology Laboratory Intern   (Dr. P. Merrick – UNC Department of Biology)

INTERNSHIPS

• **May 1995 to July 1995**
  Oblate Father’s Clinic - Lokhipur, Bangladesh
  
• **May to August 1992, 1993, 1994**
  Damien Leprosy and Tuberculosis Foundation Jalchatra Hospital - Bangladesh

PRESENTATIONS AND CONFERENCES

Bangladesh Breastfeeding Foundation (2006)
Commonwealth Association of Pediatric Gastroenterology and Nutrition (2006)
Asian Conference on Diarrheal Disease (ASCODD X 2002)
Society for Epidemiologic Research, Baltimore, MD (1999)
February 9, 2007

Jennifer Moran  
Permissions Department  
The New England Journal of Medicine  
860 Winter Street Waltham, MA 02451-1413

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Sincerely,

Alain B. Labrique, MHS, MS, PhD  
Epidemiologist / Research Associate Faculty  
Center for Human Nutrition

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