HEPATITIS E VIRUS IN THE TERAII OF NEPAL:

COMMUNITY-BASED COHORT STUDY OF PREGNANT WOMEN IN SARLAHI DISTRICT

AND INVESTIGATION OF THE 2014 OUTBREAK IN BIRATNAGAR, MORANG DISTRICT

by

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ABSTRACT

In South Asia, hepatitis E virus (HEV) is an important cause of morbidity and mortality and poses a special threat to pregnant women. However, the burden of both infection and illness in the Terai of Nepal have received scant attention.

We enrolled 2363 pregnant women in a community-based cohort study in Sarlahi District and followed them through three months postpartum. Baseline anti-HEV IgG seroprevalence was 11.7% [95% confidence interval (CI): 10.2%-13.3%] and varied with age, socioeconomic factors, and meat consumption, but not with water source or latrine access. The incidence of HEV seroconversion was 32.3/1000 person-years (p*y) during pregnancy [95% CI: 14.8 to 61.3/1000 p*y] and 44.3/1000 p*y overall [95% CI: 27.1 to 68.4/1000 p*y]. One acute hepatitis E case (genotype 1) was confirmed, but most hepatitis-like illnesses were not attributable to hepatitis A, B, C, or E. Jaundice was linked to 1/2 maternal deaths. Earlier and extended serologic monitoring would permit improved surveillance of pregnancy outcomes and antibody kinetics. Environmental testing could help clarify exposure pathways and seasonal patterns.

A large outbreak of waterborne hepatitis E occurred in Biratnagar municipality in 2014. We tested sera collected from healthy adults in Biratnagar and Dharan before the outbreak and from patients seen at three hospitals in Biratnagar during the outbreak. The population seroprevalence of anti-HEV IgG was low in Biratnagar (~5.6%) before the outbreak. Among 450 outbreak patients, 42.2% were IgG+, 14.7% IgM+, and 2.9% HEV Ag+. Acute HEV markers varied geographically and by hospital. The outbreak underscores the need for access to clean water, and for improved epidemiologic surveillance and response.

Hepatitis E virus threatens public health in the Terai. Rural and urban populations in Nepal remain highly vulnerable to infection, disease, and mortality due to HEV. Improvements in water,
sanitation, and hygiene are needed to prevent both sporadic and epidemic hepatitis E, especially as development of the Terai increases. Targeted interventions may help alleviate the increased burden of disease borne by pregnant women, individuals with chronic medical conditions, and members of socially- and economically-disadvantaged groups.

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INTRODUCTION

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Epidemics of jaundice with severe consequences in pregnant women have been recognized for many decades. However, the virus that causes hepatitis E was not isolated until the early 1980s, when a Soviet virologist intentionally ingested virus excreted by ill patients in Central Asia and carried the incubating infection back to his laboratory in Moscow for identification by immune electron microscopy. Researchers in the next decade described the genome sequences of hepatitis E virus (HEV) strains from Burma, Pakistan, and Mexico, now identified as representing HEV genotypes 1 and 2, the strains implicated in large outbreaks in developing countries. Genotypes 3 and 4, with a zoonotic reservoir, have more recently been found to cause human infection in countries throughout the world. The discovery, sequencing, and phylogenetic analysis of Hepeviridae from an ever-expanding range of places and host species have provided important insights into the epidemiology and geographic patterns of HEV infection and disease, but have raised new questions as well.
EPIDEMIOLOGIC PATTERNS OF HEPATITIS E VIRUS INFECTION

Clinical Presentation of Hepatitis E

Hepatitis E is generally an acute, self-limiting illness, with full resolution of symptoms occurring within weeks (usually) to months (less commonly) of onset. Presenting symptoms are often non-specific, and resemble those seen in acute hepatitis A. Clinically, patients suffering from acute hepatitis E typically present with combinations of symptoms such as fever, anorexia, nausea and/or vomiting, lassitude/weakness, dark urine, light (clay/ash-colored) stool, and jaundice (yellowing of the skin and sclera). Pruritus and/or upper right quadrant pain may also be present.

Asymptomatic and subclinical HEV infections are common in both epidemic and sporadic transmission settings, and have been documented in diverse geographic regions. HEV infections without overt symptoms have been detected in organ donors and in contacts of case patients in industrialized countries.

Several case reports have noted neurological symptoms during or shortly after acute infection, such as meningitis, meningoencephalitis, acute transverse myelitis, Guillain-Barré syndrome, and other peripheral neuropathies. However, these presentations appear to be relatively infrequent.

While most cases of infection with HEV are uncomplicated and self-limiting, some individuals with hepatitis E progress to acute liver failure (ALF). ALF, also called fulminant hepatic failure if onset is within 6-8 weeks of first symptoms, is often fatal. A disproportionate number of these severe cases occur in pregnant women, though men and women with pre-existing chronic liver disease or other medical problems appear to be at increased risk as well. Rarely, HEV infections may be prolonged or chronic, though this phenomenon has been observed primarily
among patients with compromised immune systems – often transplant or cancer patients receiving immunosuppressive drugs.

**Human-Associated Genotypes 1 and 2**

*Outbreaks and sporadic cases in developing countries*

Genotype 1 (HEV-1) is the primary cause of epidemic and sporadic cases of hepatitis E in developing countries in Africa and Asia, where it is transmitted primarily through fecally-contaminated water supplies. [Figure 1] Frequent HEV-1 outbreaks affecting tens of thousands of people in Central, South, and East Asia have been documented since the 1950s; the largest known HEV epidemic to date, occurring from 1986-1988 in the Xinjiang region of China, sickened over 119,000 people and resulted in 707 documented fatalities, 414 of whom were pregnant women. ³ Although molecular data from Central and South America are limited, locally-acquired HEV-1 infections and small-scale outbreaks have been reported in Latin America as well. ⁵³, ⁵⁴

Genotype 2 (HEV-2) was first identified from cases in outbreaks in two rural towns in Morelos, Mexico, in 1986-1987. ⁹, ⁵⁵, ⁵⁶ Other HEV-2 strains have appeared in Africa, where they have also been implicated in outbreaks. ⁵⁷, ⁵⁸

*Age-specific incidence and patterns of transmission of HEV-1 and HEV-2*

Although, like hepatitis A virus (HAV), HEV-1 and HEV-2 are enteric viruses commonly spread through fecally-contaminated water, the age-specific incidence of hepatitis E differs markedly from that of hepatitis A in most of the world. While HAV causes near-universal childhood infection in endemic areas, hepatitis E cases in most of the world peak in early- to mid-adulthood, with both antibody prevalence and attack rates of clinical illness highest among adults and little overt disease in children. ⁵⁹, ⁶⁰ Intrafamilial transmission of HEV appears relatively uncommon, ⁶¹ though some evidence of household transmission has been reported. ⁶²-⁶⁴ Slums and refugee camps
are particularly vulnerable to outbreaks, as access to clean water and sanitary waste disposal facilities is often limited. In addition, transient or migrant populations may repeatedly replenish the pool of susceptible individuals and/or import new infections.

Animal-Associated Genotypes 3 and 4

Host range

Genotypes 3 and 4 (HEV-3 and HEV-4) have been found in other mammalian hosts, most notably wild and domestic swine, which serve as reservoirs for human infection. [Figure 2] HEV-3 is prevalent among swine worldwide as well as in other mammals, including deer and mongooses, though little is known about the sylvatic circulation of HEV.

The known animal reservoirs of HEV continue to multiply; novel members of the Hepeviridae have been detected in wild and farmed rabbits, wild rats, birds, bats, and trout. Of these, only rabbit HEV strains have been shown to share a close phylogenetic relationship and possess antigenic similarity to human and swine-associated HEV. Newly-identified rabbit HEV strains appear to share a common ancestor with HEV-3 based on whole-genome and ORF2 sequence comparisons. However, rabbit strains contain a ~90-nucleotide insertion in ORF1 and cluster separately from most human and swine HEV-3 strains.

Genotype 4 is indigenous to South and East Asia, where it has been detected in both wild and domesticated swine. Short HEV sequence fragments obtained from sheep and cattle fecal samples, clustering with genotype 4, have been reported in the Chinese-language literature, but evidence for this expanded host range requires replication. Serologic evidence of HEV infection obtained from a variety of mammals around the world, including dogs, goats, camels, cows, and buffalo, has yet to be followed by successful isolation and sequencing of HEV strains from these species.
Studies in eastern China have recently documented a shift in the HEV strains causing human illness; over the past decade or so, HEV-4 strains of zoonotic origin have overtaken HEV-1 strains as the major cause of hepatitis E in this region. More recently, both imported and locally-acquired HEV-4 strains have been detected in Europe and the Americas.

In the relatively wealthy nations of Europe, North America, and East Asia, human infections with HEV-3 and HEV-4 are often asymptomatic, though more virulent strains appear to exist. In these settings, autochthonous HEV-3 appears to pose the greatest threat to people with chronic medical conditions that require immunosuppressive therapy or that directly compromise the immune system and/or liver.

Routes of exposure to animal-associated HEV

Identified human exposures to HEV-3 and HEV-4 have occurred via consumption of undercooked pork or game meats, especially liver sausages or other organ meats, which are frequently contaminated with HEV. HEV-3 has also been detected in bivalves in sewage-contaminated waters, and shellfish consumption has been identified as a possible risk factor for infection.

However, cases without obvious food-linked exposures have been reported frequently, suggesting other environmental exposures may play a role. Occupational exposures to farm animals, especially swine, have been associated with HEV infection in some studies in Africa, Europe, and the United States but a recent study in Thailand found no difference in seroprevalence among swine workers compared with others in the local population. Inadequately-treated manure from swine farms represents another potential source of environmental contamination.
A cross-sectional study of the U.S. population found that anti-HEV seroprevalence was elevated among those with pets at home. In 2003, the pet cat of a Japanese man who developed hepatitis E was found to have anti-HEV antibodies; subsequent to this case, a substantial proportion of sampled Japanese pet cats were found to be seropositive. These findings suggest that some pet-related exposures – perhaps contaminated animal food, indirect contact with wild or domesticated animal reservoirs, or outdoor activity – could mediate some of the risk of human infection with zoonotic strains.

**HYPOTHESES REGARDING REGIONAL DIFFERENCES IN THE NATURE AND IMPACT OF HEV**

**Ecologic Influences on HEV Epidemiology Across Regions**

Limited exposures, typically low doses of virus, and better overall health may protect most otherwise-healthy people in industrialized nations from clinically-apparent hepatitis caused by HEV-3. In developing countries in Asia and Africa, where HEV-1 predominates, human infections with HEV-3 and HEV-4 are rarely reported, and no large outbreaks have been attributed to these strains. However, HEV-3 and HEV-4 cases have been confirmed sporadically in local populations, in travelers to these regions, and in resident animal populations and locally-obtained meat.

Broad environmental, dietary, and overall health differences across settings and limited information on the distribution of viral subgenotypes in many parts of the world make it difficult to discern whether HEV-1 and HEV-2 are inherently more virulent in humans than HEV-3 and HEV-4, or whether the occurrence of epidemics and the incidence of illness and death reflect primarily exposure- and host-related risk factors. Similarly, it remains unclear to what extent subtle subgenotypic differences in HEV-1 strains may contribute to the variability in patterns of illness in endemic areas, and what can instead be explained by host vulnerabilities (e.g., genotype, nutritional
status, coinfections, immunocompetence) or by environmental determinants of timing and dose of exposures (e.g., seasonal rainfall patterns, sanitation systems, animal exposures).

**Genotypic and Subgenotypic Variation and Virulence**

Despite the challenge of confounding by ecologic factors, there is some evidence to suggest that differences in circulating virus strains may influence patterns and severity of illness. Studies of circulating subgenotypes and strains within geographic regions have identified several variants with increased or decreased virulence.

Renou et al. have suggested that HEV-3 (subtype 3c), which is common in Europe, may be less likely to produce symptomatic illness than genotype 1; however, the better overall health status and typically non-waterborne exposures of individuals in industrialized countries may at least partly explain the relative lack of symptomatic cases in these settings. Cordoba and colleagues identified an attenuated swine HEV-3 strain from the United States with mutations in the sequences encoding the capsid protein that appeared to be associated with reduced viral replication.

On the other hand, successful *in vitro* cultivation of HEV-3 and HEV-4 strains with nucleotide substitutions associated with more severe illness was reported by Inoue and colleagues in Japan in 2009. In a comparison of isolates from patients with milder and more severe hepatitis E, several substitutions in the RNA helicase and capsid protein-encoding domains, in particular, were found to be associated with severe hepatitis E leading to liver failure. Mutations identified in HEV-3 isolates collected from several severe hepatitis E cases across Japan, including another substitution affecting the helicase domain, also suggest that these mutations may affect the pathogenetic potential of HEV strains. A series of cases in France, who presented with more severe disease than typical HEV-3 cases from the same area, were attributed to HEV-4 strains
closely related to a Belgian swine isolate; however, specific mutations related to virulence in these strains were not identified.\textsuperscript{123}

A recent report by Shukla and colleagues described the integration of a nucleotide sequence from human host S17 ribosomal RNA into the HEV-3 genome in a patient with chronic HEV infection who had both neurologic and hepatic symptoms.\textsuperscript{156} In contrast to other tested HEV viral strains, this recombinant virus (the Kernow-C1 strain) replicated in cell culture and also may have grown in extra-hepatic sites in the patient.\textsuperscript{156, 157} Similarly, Nguyen et al. isolated an HEV-3 strain from a chronically-infected U.S. liver-transplant patient that contained an insertion derived from the human ribosomal S19 sequence, and this strain, too, predominated in cell culture compared with isolates lacking the insertion.\textsuperscript{158} These observations suggest another source of genomic variation that may be important in the pathogenesis and epidemiology of HEV.
Figure 1. Global distribution of hepatitis E virus (HEV) genotypes 1-4 in humans.
Genotypes 1 and 2 circulate in human populations and are transmitted primarily through fecally-contaminated water supplies. Genotype 2 was first identified in Mexico, but has subsequently been found across Africa. Genotypes 3 and 4, with reservoirs in swine and other species, are the dominant genotypes affecting human populations in industrialized countries.
Figure 2. Phylogenetic tree of global hepatitis E virus (HEV) isolates, based on a portion of the nucleotide sequence encoding the capsid protein.

Genotypes 1 and 2 (HEV-1 and HEV-2) circulate among humans, primarily in Africa and Asia, while Genotypes 3 and 4 (HEV-3 and HEV-4) have animal reservoirs, and zoonotic, often foodborne, transmission has often been implicated where a source of infection can be identified. Recently-discovered rabbit strains appear to form a closely-related clade. Sequences from open reading frame (ORF) 2, homologous to the 350bp segment of the Burma B1 strain used by Lu, 12 were identified from within the GenBank database (accession numbers in parentheses). Alignments were performed using the Basic Local Alignment Search Tool (BLAST), 19 and the tree display was modified with MEGA5.2. 20
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HEPATITIS E IN PREGNANCY: MATERNAL HEALTH

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Epidemiology of Hepatitis E in Pregnant Women

Severe hepatitis E disproportionately affects pregnant women, with a high incidence of acute liver failure and reported case-fatality rates of ~15 to 30%. 1-3 This perplexing and grave feature, which is distinctive of hepatitis E epidemics, has been noted for decades (perhaps centuries) but remains poorly explained. 4 Recent work in Bangladesh suggests that acute hepatitis, with HEV the probable etiologic agent in a majority of cases, may underlie an astonishing ~10 to 25% of pregnancy-related deaths. 5-7 In addition to the effects on maternal health, fetal and newborn health may also be compromised, with elevated risk of miscarriage, stillbirth, premature delivery, and neonatal jaundice. 7-15 Vertical transmission appears to be common among mothers with symptomatic hepatitis E, with deleterious effects on fetal and neonatal health. 14, 16, 17 Because few studies have addressed the issue, it is unclear to what extent the fetuses and neonates of women with subclinical or asymptomatic infection are at risk. 18 There are not yet any reliable data on whether HEV is transmissible via breast milk. Geographic discrepancies in the frequency and severity of clinical hepatitis E in pregnant and postpartum women persist.
REGIONAL VARIATIONS IN OCCURRENCE AND SEVERITY OF HEPATITIS E IN PREGNANCY

Although severe liver disease among pregnant women, with high mortality, is the hallmark of epidemics of HEV-1 in Asia and Africa, there have been reports of severe hepatitis among pregnant women infected with HEV-3 in countries where locally acquired genotype 1 infections do not occur. Clinical hepatitis E attributed to HEV-3 was recently reported in a pregnant woman in France. HEV-3-linked acute liver failure also occurred in a nonpregnant Spanish woman whose medical history was devoid of known risk factors for severe hepatitis E. The woman reported long-term use of hormonal contraceptives (norgestrel/ethinyl estradiol) and was found to have hepatic adenomas upon examination, which the authors speculated may reflect elevated estrogen levels, mimicking pregnancy, prior to the onset of hepatitis E.

Whether these reports suggest that pregnancy or pregnancy-like states may represent a risk factor for more severe clinical disease even in high-income countries where HEV-3 and HEV-4 circulate, or whether the paucity of such reports in the face of 10 to 20% population seroprevalence supports the increased virulence of genotype 1 viruses for pregnant women, is not certain. Severe infections during pregnancy in developing countries may be primarily a result of greater virulence of the human-adapted HEV-1 and HEV-2 genotypes, increased susceptibility of pregnant women in developing countries where these genotypes are endemic, or the epidemiologic conditions of exposure to these viruses. Also, in many developed-country settings, awareness of hepatitis E as a plausible diagnosis may be low, leading to missed cases and underdiagnosis of this infection.

Also unexplained is the contrast between the apparently infrequent and unremarkable occurrence of hepatitis E in women in Egypt and the disproportionately severe hepatitis E observed in pregnant women sub-Saharan Africa and in South and Southeast Asia, despite the fact that HEV-1 predominates in all of these regions. The different patterns of illness seen among pregnant women, paralleling regional differences in age-specific attack rates and seroprevalence,
remain perplexing. [Table 1] The study of this range of possible outcomes to HEV infection is challenging, given that most HEV research occurs either in acute outbreak settings or in clinical contexts where only severely ill patients are typically referred. The prospective identification of HEV seroconverters and the study of subsequent disease outcomes require the longitudinal surveillance and follow-up of large pregnancy cohorts – an expensive prospect for a pathogen that has yet to achieve standing even as a “neglected” disease.

**Immune Function and Susceptibility to Viral Infection During Pregnancy**

Pregnancy is associated with changes in sex hormone levels and immune system function [Figure 3] that serve primarily to protect the fetus from attack by the maternal immune system but also exert strong effects on the immune response to pathogens. In addition to maternally mediated changes in the biochemical milieu, fetal hormones may also affect susceptibility to infection. Pregnancy-related changes in body chemistry and fetal and placental influences have been variously linked to increases in the severity of certain infections, including *Plasmodium falciparum* malaria, leprosy, influenza, varicella, viral hemorrhagic fevers, and measles and decreases in the severity of some autoimmune diseases, e.g., rheumatoid arthritis during pregnancy.

During pregnancy, a shift from T helper cell type 1 (Th1)-dominated to T helper cell type 2 (Th2)-dominated immune responses, or “Th2 bias,” has been hypothesized to help protect the fetus by suppressing macrophage activation. Th1-type responses are marked by increases in IFN-γ synthesis, and this type of response appears to help protect against parasitic infections. Th2 responses, on the other hand, are tipped toward anti-inflammatory cytokines, such as IL-4, IL-6, and IL-10, and increases in antibody production. These changes in the immunologic environment are not instantaneous, but vary with increasing estradiol and progesterone levels, becoming most apparent as the pregnancy approaches term.
Extrapolation from serial measurements of IFN-γ and IL-6 (as biomarkers of Th1- and Th2-type responses, respectively) in 35 healthy pregnant women in Quebec suggested that Th1-type responses prevail until the mid-second trimester, with IFN-γ decreasing and IL-6 increasing from the 10th to 40th weeks of gestation. Serial serum samples from a cohort of 50 healthy pregnant and postpartum women in New York City showed elevated levels of TNF-α throughout pregnancy compared with 6 months postpartum. These women also experienced increases in levels of β-defensins and IP10 and decreases in IFN-γ during gestation.

A study of 111 pregnant, 126 postpartum, and 86 nonpregnant healthy women in Japan indicated that natural killer (NK) cell activity was markedly reduced by the third trimester of pregnancy but relatively high during the first trimester and the first month postpartum. Similarly, the Viral Immunity in Pregnancy study in New York City suggested a nadir in NK cell activity in the third trimester. Caution is warranted, however, in generalizing biomarker findings from healthy pregnant women in industrialized countries to women in resource-limited settings.

**Hypotheses Regarding Mediation of HEV Severity in Pregnant Women**

The interplay of these factors in hepatitis E during pregnancy is complex and not yet well understood. Pal et al. measured cytokine responses in cells from pregnant and nonpregnant women with HEV in India, as well as from healthy pregnant and nonpregnant controls. They found a Th2 bias among pregnant women with HEV, as well as an increased lymphoproliferative response to stimulation relative to that in healthy pregnant women. However, as their study was cross-sectional, infection-induced changes in cytokine and lymphoproliferative responses, as opposed to preexisting differences that predispose to disease, cannot be excluded.

In addition to mediating shifts in the immune system, pregnancy-associated hormones may also directly influence viral replication. Poorer outcomes in HEV-infected pregnant patients have
been associated with higher viral load,\textsuperscript{8,40} but not consistently.\textsuperscript{41} Elevated sex steroid hormones in women with HEV-associated acute liver failure, relative to those with milder illness, may suggest that higher estradiol and progesterone levels are a risk factor predisposing women to poorer outcomes; however, the elevated hormone levels may also be triggered by the infection state itself.\textsuperscript{39}

Host factors such as nutritional status, which may affect and be affected by pregnancy, may also contribute to the immune response to HEV infection in pregnant women. In contrast to the findings of Pal et al.,\textsuperscript{38} preliminary work in Bangladesh has suggested that the immune profiles of pregnant women who develop clinical hepatitis E may not reflect the Th1-Th2 shift observed in other cohorts of pregnant women, and it is possible that this is related to micronutrient deficiencies.\textsuperscript{42} Pregnant women elsewhere in South Asia are often deficient in several micronutrients, and seasonal food availability may exert a major influence on maternal micronutrient status.\textsuperscript{43} A proteome analysis conducted in a cohort of pregnant women in southern Nepal identified changes from the first to third trimesters in the expression of proteins, some of which had not previously been reported in studies of healthy pregnant women.\textsuperscript{44} Several proteins whose expression differed significantly between the first and third trimesters were associated with immune function and inflammation, including gelsolin, pregnancy zone, plasma protease C1 inhibitor, and various complement-related subcomponents, as well as other proteins whose functions remain uncertain.\textsuperscript{44} Environmentally and genetically mediated differences in immune system modulation over the course of pregnancy could help explain regional differences in the observed hepatitis E incidence and severity during pregnancy.

Hepatitis E as a catalyst for coagulopathy may also contribute to maternal morbidity and mortality. Hemorrhage, often postpartum hemorrhage (PPH), is the leading proximal cause of maternal death in developing countries.\textsuperscript{45,46} If HEV infection disrupts clotting, this may further
increase the likelihood of uncontrolled bleeding during the peripartum period. As PPH remains one of the major causes of maternal mortality globally, especially in areas of endemicity, it may be that some proportion of peripartum hepatitis E is misclassified as delivery-associated PPH; this and other manifestations of HEV infections in pregnancy may explain the underrecognition of HEV etiologies in emergency obstetric complications.

Women admitted to a tertiary care hospital in New Delhi, India, with acute HEV \( (n = 132) \) had significantly elevated prothrombin times relative to those of women admitted for other acute viral hepatitides \( (n = 88) \).\(^ {10}\) The HEV-infected women had a greater incidence of antepartum (but not postpartum) hemorrhage, including gastrointestinal bleeding, and elevated maternal mortality. In marked contrast to other studies, the women with acute HEV in this study were admitted with a lower average gestational age than women with other acute viral hepatitis.\(^ {10}\) Puri et al.,\(^ {47}\) working in the same hospital in New Delhi in subsequent years, conducted a case-control study of third-trimester pregnant women with acute HEV and coagulopathy, comparing those who did \( (n = 13) \) and did not \( (n = 25) \) suffer postpartum bleeding. Women with postpartum hemorrhage had approximately five times the odds of having experienced hepatic encephalopathy as women without PPH and had 20 times the odds of a gastrointestinal bleed, though only gastrointestinal bleeding remained a significant clinical predictor of PPH after controlling for other factors. Fully a third of women admitted to the hospital with acute HEV infections experienced postpartum hemorrhage.\(^ {47}\)

Work by Geng et al.\(^ {48}\) suggests possible mechanisms by which HEV infection may contribute to dysregulation of coagulation, which may pose a particular hazard to maternal health. Their study of HEV-1, HEV-4, and rabbit HEV ORF3 protein interactions with human liver cell proteins found that HEV ORF3 may interact with several clotting-related pathways. Specifically, they propose that down-regulation of fibrinogen production may precipitate hemostasis, eventually
depleting clotting factors, and that several other protein pathways may also be disturbed. Poliakova et al. also observed dysregulated coagulation among HEV patients with acute liver failure, although their study did not focus specifically on pregnant women.

**CHALLENGES FOR SURVEILLANCE AND PREVENTION**

Despite emerging evidence explaining the role(s) of hepatitis E in maternal and neonatal morbidity and mortality, HEV-related deaths in pregnant women, especially if unaccompanied by frank jaundice, may be not be identified or recorded as such. Careful examination of the proximal and distal causes of maternal death, in clinical studies and, importantly, in community-based studies, may help elucidate the contribution of HEV infection and associated risk factors to maternal death. Maternal verbal autopsy methods used in both rural and urban Bangladesh have revealed a higher-than-anticipated prevalence (9.8 to 25%) of jaundice and/or acute viral hepatitis-like illnesses among women who died from all pregnancy-related causes during the maternal period. Similar investigations, especially if supported by serologic and/or molecular confirmation of etiology, could help clarify the role of HEV in maternal death in other settings and suggest new avenues for prevention of maternal mortality.

The long-awaited release of a commercial vaccine against hepatitis E in China is a hopeful sign for prevention of HEV-associated morbidity and mortality among pregnant and postpartum women. However, the vaccine has not yet been tested for safety and efficacy in large populations of pregnant women or in places where HEV genotype 1 is the primary cause of human infection. Although a post hoc study of 34 pregnant women inadvertently included in the Chinese vaccine trial revealed no obvious differences in immunogenicity or safety, the “hormonal and immunological milieu of pregnant women is strikingly different from that of women who are not pregnant”, and
thus dedicated studies are needed to establish more clearly the risks, benefits, and optimal timing of vaccination in the context of pregnancy, for both the mother and her fetus. 52
### Table 1. Seroprevalence of IgG antibodies to HEV among asymptomatic pregnant women recruited from community-based cohorts or antenatal clinics.

Abbreviations: see table footnote b.
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Figure 3. Changes in hormone levels and immune function are associated with an increased risk of severe hepatitis E in late pregnancy.
(a) Changes in hormone levels and immune function over the course of pregnancy. Pregnancy is characterized by marked changes in hormone levels and corresponding shifts in immune status, which vary over the course of gestation. Levels of estradiol and progesterone are at their lowest early in pregnancy, while an increase in human chorionic gonadotropin (hCG) levels occurs and then begins to wane during the first trimester. As estradiol and progesterone levels continue to rise over the course of pregnancy, the dominant T helper 1 (Th1) immune regimen, associated with proinflammatory activity, is gradually superseded by T helper 2 (Th2)-biased responses associated with anti-inflammatory activity. Regulatory T-cell (Treg) activity also increases over the course of pregnancy. (Adapted from Fig. 2 of reference 52 with permission from Elsevier.)
(b) Distribution by trimester of hospital admissions of pregnant women with acute hepatitis during the historic Delhi hepatitis E virus (HEV) epidemic of 1954 to 1955. An association of greater hepatitis E severity with more advanced pregnancy is a frequent observation during outbreaks and among sporadic cases. Alterations in both hormone levels and immune function during pregnancy appear to contribute to these outcomes. Data are from reference 15. [Note that some early-pregnancy cases may not be classified as cases in pregnant women if the pregnancy is not detected, potentially biasing this apparent association to some extent.]
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FETAL AND NEONATAL HEALTH CONSEQUENCES OF VERTICALLY-TRANSMITTED HEPATITIS E VIRUS INFECTION

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ABSTRACT. Hepatitis E virus (HEV) infections lead to tens of thousands of deaths annually, mostly in developing countries. Hepatitis E poses a significant threat to the health of expectant mothers, a well-noted epidemiologic feature of the disease, but the contribution of vertically-transmitted HEV infection to fetal and neonatal morbidity and mortality has received limited attention. Evidence assembled to date suggests that mother-to-child HEV transmission may be frequent and deleterious to the fetus and newborn in pregnancies affected by hepatitis E. Further work is required to resolve key questions: 1. What risks do subclinical maternal HEV infections and infections early in pregnancy pose to fetal health and development? 2. Does vertical transmission occur during labor and/or breastfeeding and contribute appreciably to neonatal morbidity and mortality? 3. How do treatment decisions for severely-ill mothers affect fetal and neonatal outcomes? 4. Can maternal vaccination effectively prevent vertical transmission of HEV?
INTRODUCTION

Although hepatitis E virus (HEV) is sometimes referred to as an ‘emerging’ infectious agent, HEV is well-established as a major cause of acute viral hepatitis (AVH) worldwide. Of the more than 20 million infections estimated to occur globally each year ~70,000 result in death. The vast majority of these deaths occur in resource-poor countries in Asia, Africa and Latin America, where exposure to fecally-contaminated water results in outbreaks and sporadic cases of hepatitis E. Hepatitis E in these locations is nearly always attributable to the human-associated HEV genotypes 1 (in Asia, Africa, and Latin America) and 2 (in sub-Saharan Africa and Mexico). However, the globally-ubiquitous zoonotic HEV strains (genotypes 3 and 4) have more recently been identified as a cause of sporadic hepatitis in medically-vulnerable patients and in the general population in high-income countries.

High case-fatality ratios among pregnant women, particularly during the third trimester of pregnancy, remain an almost pathognomonic feature of hepatitis E epidemics caused by HEV genotype 1. There is mounting evidence that hepatitis E is an important contributor to maternal morbidity and mortality in South Asia, even outside of periodic large outbreaks. In the early literature on hepatitis E, there was much conjecture about the extent to which maternal hepatitis E threatened fetal and neonatal health, beyond catastrophic maternal illness or death. Emerging evidence from epidemiologic and clinical studies now suggests that vertical transmission of HEV may occur frequently among mothers ill with hepatitis E and contribute to serious perinatal health outcomes along with the effects of maternal morbidity and mortality.

HEPATITIS E AND MATERNAL-CHILD HEALTH

Epidemic jaundice marked by an excess of illness and death among pregnant women and their infants has been documented at least as far back as 18th century Europe. The first
(retrospectively) serologically-confirmed hepatitis E outbreak occurred in Delhi, India, in the mid-1950s,\textsuperscript{17,28,29} although molecular evidence suggests that HEV may already have been circulating in humans for several hundred years.\textsuperscript{30,31} A hospital-based study during the Delhi epidemic documented a $\sim$10\% maternal case-fatality rate, along with miscarriage, stillbirth, or neonatal death in 56\% of infants of women with HEV infection. In addition, jaundice was reported among both expiring and surviving infants.\textsuperscript{16}

Numerous studies conducted over the three decades since HEV was identified as a cause of infectious hepatitis\textsuperscript{32} have continued to support high rates of maternal, fetal, and neonatal illness and death in affected pregnancies. According to one recent model, HEV may be responsible for $\sim$2,400-3,000 stillbirths each year in developing countries,\textsuperscript{2,33} with many additional fetal deaths linked to antenatal maternal mortality.\textsuperscript{34} Pre-term delivery in mothers with hepatitis E is common and is associated with poorer neonatal survival.\textsuperscript{33,35} During a 2002 outbreak in the Central African Republic, all of the pregnant women with serologically-confirmed hepatitis E (n=7) delivered prematurely; three of these babies were stillborn (one macerated), and another died within minutes of delivery.\textsuperscript{36} Newborns whose mothers had acute hepatitis at the time of delivery comprised half (4/8) of the fatalities in a 1993-1994 outbreak in Islamabad, Pakistan.\textsuperscript{21} Pregnant women with jaundice during a 2008-2009 hepatitis E outbreak in Tongi, Bangladesh, were more than twice as likely as non-jaundiced pregnant women to miscarry or deliver a stillborn baby.\textsuperscript{37} In two separate, hospital-based prospective studies in New Delhi and Chennai, India, $\sim$15\% to $>$50\% of the live-born infants of mothers with hepatitis E died within the first week postpartum.\textsuperscript{33,38} During a 2010-2011 outbreak in Sudan, 14 intrauterine deaths and 9 premature deliveries were reported among 39 pregnant hepatitis E cases.\textsuperscript{39}

To date, only hepatitis E caused by HEV genotype 1 has consistently been observed to yield these effects in pregnancy. However, HEV genotype 2 was implicated in acute liver failure in a
pregnant woman during an outbreak in Namibia,\textsuperscript{40} and the potential of genotypes 2-4 to cause adverse outcomes in pregnant women, given exposure, remains uncertain.

**Miscarriage and Stillbirth: The Role of Antenatal HEV Infection**

Despite the ample epidemiologic evidence that maternal hepatitis E may result in adverse consequences to the fetus, few studies have been able to address whether these outcomes are solely the result of maternal health complications, or whether fetal HEV infection also plays a role. Etiologic studies of pregnancy loss and stillbirth are complicated by numerous logistical, technical, cultural, and ethical obstacles.\textsuperscript{41-44} The prospective design, sample size and frequency of perinatal observations and follow-up would require considerable resources and advanced clinical and research infrastructure. As a result, the task of demonstrating a contribution of fetal HEV infection to intrauterine or intrapartum death has rarely been attempted.

Early research in China failed to detect HEV antigen (HEV Ag) in any of 17 stillborn fetuses whose mothers were infected during a 1986-1988 outbreak.\textsuperscript{45} In a case-control study in Egypt, HEV Ag was detected in 5% of aborted fetal tissue samples, but absent from the cord blood of live-born, full-term infants.\textsuperscript{46} Anti-HEV IgM was detected in 3%, and HEV RNA in 16% of mothers who aborted, but in none of the mothers who delivered live infants.\textsuperscript{46} While these Egyptian results support an association of HEV with abortion, they fall short of implicating antenatal HEV transmission to the fetus as a cause of fetal death.

Results in other animals have been similarly inconclusive. Experimental inoculation studies of HEV genotype 1 in pregnant rhesus macaques\textsuperscript{47,48} and of genotype 3 in swine\textsuperscript{49} have failed to provide clear evidence of vertical transmission or of adverse pregnancy outcomes attributable to HEV. Of four pregnant macaques inoculated with an epidemic HEV strain isolated from an Indian patient in 1990, three delivered healthy infants (two of them following elevation of liver enzymes),
while the remaining macaque delivered a macerated fetus five days prior to the onset of hepatitis.\textsuperscript{48} Consistent with other experimental studies of HEV involving animal models,\textsuperscript{50} the disease provoked in these pregnant animals was less severe than that seen in most pregnant women who come to clinical attention.

Investigation of adverse gestational outcomes among naturally-infected animals has also yielded results that are challenging to interpret. HEV genotype 3 RNA was amplified from the livers of ~16\% of aborted porcine fetuses on two South Korean farms.\textsuperscript{51} Concomitant detection of porcine circovirus-2 (PCV2) in all of these fetal pig samples casts doubt on the role of HEV in these deaths, but also raises the important question of whether coinfection with immunosuppressive viruses may facilitate fetal infection with HEV.\textsuperscript{51}

In South and Central Asia and sub-Saharan Africa, where genotypes 1 and 2 predominate, no human studies have yet systematically evaluated the association of fetal HEV infection with miscarriage or stillbirth. Therefore, it remains unclear whether some of the increased risk of miscarriage and stillbirth reported in these diverse settings may be attributable to vertically-transmitted infection or whether it is the result of maternal complications of hepatitis E alone.

**HEV Infection and Outcomes Among Live-Born Infants**

Morbidity and mortality among neonates born to mothers with hepatitis E may be explained, to a large extent, by preterm delivery and other pre- and perinatal stresses caused by the maternal response to infection. However, a small body of evidence suggests that vertically-transmitted infections also contribute directly to infant morbidity and mortality in the early postnatal period.

A landmark study published in 1995 by Khuroo and colleagues was the first to document mother-to-child transmission of HEV using serologic and molecular methods.\textsuperscript{52} HEV RNA was
found in the cord blood of 5/8 Kashmiri infants whose mothers had serologic evidence of infection preceding delivery. All five of these infants had elevated alanine aminotransferase (ALT) at birth. Two infants died within a day of delivery. Serum viremia, IgM antibodies, and hepatitis persisting for several weeks in two of the surviving infants suggested that these results could not be explained solely by contamination of cord blood with maternal blood, and that vertically-transmitted HEV could, in fact, cause illness in neonates.

Subsequent studies have found a similarly high prevalence of vertically-transmitted HEV infection [Table 2], which frequently, but not always, results in disease. Hepatitis, either icteric or anicteric, may be present from birth in a substantial proportion of neonates born to mothers with hepatitis E. Recently, a second-trimester fetal HEV infection associated with ascites was reported to have resolved in utero, resulting in a healthy infant who was born at 38 weeks of gestation. However, evidence of severe necrosis in liver tissue samples from neonatal autopsies suggests that some babies, like their mothers, experience fulminant hepatic failure (FHF) as a result of HEV infection.

In contrast to perinatally-transmitted hepatitis B virus infections, which may be lifelong if preventive measures are not initiated at birth, there have been no reports of persistent HEV infection in infants born to mothers with hepatitis E. This is consistent with the natural history of hepatitis E in adults, which is typically self-limiting. Chronic HEV infections have been reported primarily in immunosuppressed and immunocompromised populations. They have not been documented in pregnant women or infants, though some authors have speculated that among immunocompromised pregnant women or neonates, such infections could, plausibly, be of concern. In addition, pregnant women may be coinfected with other hepatotropic pathogens, but how such coinfections influence vertical HEV transmission and outcomes has not been studied.
CHALLENGES IN UNDERSTANDING MOTHER-TO-CHILD TRANSMISSION OF HEV

Limited surveillance and reporting have presented obstacles to understanding the consequences of HEV infections on maternal, fetal, and neonatal outcomes. Misclassification of HEV infection due to barriers to medical care, failure to consider hepatitis E in differential diagnosis, or the use of insensitive assays may obscure the impact of HEV on pregnancy outcomes.

In addition, study populations in the reported literature are largely hospital-based and skewed towards women with more severe illness and thus a predisposition to having worse fetal and/or neonatal outcomes. Nonetheless, results from the largest prospective studies of women with hepatitis E paint a picture of relatively poor pregnancy outcomes even in women with milder illness, and not only among those with acute liver failure.

The extent to which asymptomatic infections influence pregnancy outcomes has not yet been studied systematically. In addition, there are no reliable data on whether HEV can be transmitted through breast milk. Population-based serologic surveillance of pregnant women and follow-up of pregnancy and neonatal outcomes may help to address these issues. Furthermore, studies are needed across a wider variety of settings. Differences in viral characteristics, exposure patterns, underlying population health and nutrition status, host genetics, and other host factors may modify the effects of maternal HEV infection on fetal and neonatal outcomes.

Further investigation of the mechanisms of HEV pathogenesis in pregnant women would help in understanding the role of transplacental transmission in fetal loss and stillbirth. The timing of HEV infection relative to pregnancy may also be a critical variable. Although the preponderance of severe hepatitis E in pregnant women occurs during the third trimester, how this observed maternal response relates to vertical transmission and fetal viability has not yet been elucidated.
It has been suggested that in utero fetal infection may itself contribute to adverse maternal outcomes.\textsuperscript{53} Such “upside-down” vertical effects have been posited to occur with other viral infections as well. Studies of murine gammaherpesvirus 68 in mice, for example, have found that fetal inflammatory responses to viral replication in the placenta may predispose the mother to morbidity and reduce the mother’s capacity for sustaining the pregnancy.\textsuperscript{102,103} Hormone receptor-moderated inflammatory responses at the feto-maternal interface have been associated with pregnancy outcomes in human hepatitis E.\textsuperscript{97}

There are currently no adequate treatments for hepatitis E in pregnancy. Experimental use of ribavirin has recently shown promise in treating severe, acute hepatitis E in non-pregnant patients,\textsuperscript{104-106} but this drug is typically contraindicated in pregnancy due to “significant embryocidal and/or teratogenic effects” and fetal harm.\textsuperscript{107} Some researchers have suggested expedited delivery or pregnancy termination could be considered to preserve the life of the mother.\textsuperscript{53,108} Whether this approach would prevent death in women who present with severe disease has not been studied systematically. Given the high rates of miscarriage, stillbirth, and premature delivery in pregnancies affected by severe hepatitis E, the net impact of such a strategy on neonatal morbidity and mortality is also uncertain.

New HEV vaccines show promise in preventing hepatitis E,\textsuperscript{109,110} and they may help obviate the need for treatment of severe illness. However, follow-up data indicate that HEV infections can still occur among vaccinated adults,\textsuperscript{111} and only incidental data on safety and efficacy in pregnant women are available.\textsuperscript{112} Evaluating the effectiveness of these vaccines in preventing maternal disease and death, and in reducing the burden of fetal loss, premature delivery, and neonatal morbidity and mortality, should stand among global maternal-child health priorities.
### Table 2. Vertically-transmitted hepatitis E virus infection, morbidity, and mortality in live-born infants of mothers with laboratory-confirmed antenatal HEV infection.

AVH = acute viral hepatitis due to HEV, FHF = fulminant hepatic failure (acute liver failure) due to HEV.  
**"Laboratory-confirmed antenatal HEV infection" means detection in maternal serum of anti-HEV IgM, HEV RNA, or both, at any time during the pregnancy or at delivery; most cases occurred in the 3rd trimester.**

Note: Among mothers, serological testing was performed for HAV, HBV, and HCV infections in the Srinigar and New Delhi-based studies. No coinfections were reported by Khuroo and colleagues in Srinigar; 2 HBV + HEV coinfections and 1 HCV + HEV coinfection were reported by Kumar and colleagues and by Singh and colleagues in each of the two different New Delhi-based studies. The mother of the tested neonate in the Ghana study by Bonney and colleagues was initially suspected to have a malaria + viral hepatitis coinfection, but confirmation of a malaria diagnosis was not reported. Aside from the Ghana study, none of the papers provided sufficient detail to assign specific outcomes to the infants of mothers with coinfections.
Search strategy and selection criteria

We searched the PubMed database over the date range 1966 - September 2012 using the Medical Subject Headings (MeSH) terms ["Infant, Newborn" OR "Fetus" OR "Infectious Disease Transmission, Vertical" OR "pregnancy outcome") AND ("Hepatitis E" OR "Hepatitis E virus"). We performed free-text searches for variants of these terms as well as "jaundice" and "non-A, non-B hepatitis" in both PubMed and Google Scholar. After screening abstracts for broad relevance, we downloaded full-text papers and manually searched the references to identify additional sources. Several historic papers known to the authors were included for context. New papers received via topical HEV publication alerts from PubMed, and several suggested by reviewers, were also added. Although we did not explicitly restrict our search to English-language papers, all of the papers included were published in English. In this paper, we survey the broader literature, but emphasize those mammalian studies in which both maternal and fetal/neonatal diagnoses or outcome investigations were supported by immunologic and/or virologic assays.

Box 4.1. Search strategy and selection criteria.
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vaccine in healthy adults: a large-scale, randomised, double-blind placebo-controlled, phase 3 trial. 


Study Background and Evolution

Motivations and Framework

The preceding chapters have provided most of the context for the work described in the remainder of this dissertation, i.e.: hepatitis E is a major public health problem in developing countries and its effects in pregnancy may be especially serious, but many unanswered questions remain about global-scale, regional, and interpersonal variations in hepatitis E occurrence and outcomes.

That the era of hepatitis E vaccines had recently dawned – at least in theory – was another major motivating factor for initiation of the study. Two candidate vaccines had recently been tested and found efficacious in controlled trials, but neither had been made available to high-risk populations in South Asia. The fact that the first vaccine was tested in Nepali army conscripts and subsequently shelved added what many observers viewed as an ethical insult to the unmitigated injury of high HEV-associated morbidity and mortality in the region. We expected that the results of our work would help strengthen the evidence base (and perhaps galvanize the international resolve) needed to evaluate hepatitis E vaccine as a means for preventing maternal, fetal, and neonatal illness and death across South Asia.

An ongoing study in southern Nepal provided the ability to collect data from a large cohort of Nepali women prospectively, taking advantage of a well-established field research infrastructure. Johns Hopkins University investigators have a long history of work in Sarlahi District. The Nepal Nutrition Intervention Project Sarlahi (NNIPS) was established by Johns Hopkins in 1989 to investigate the potential reductions in mortality that obtained by Vitamin A supplementation in children. The research and organizational infrastructure have since been expanded to undertake work on a variety of maternal and child health concerns, and the organization is well-respected in
Sarlahi. Working within the organization and with the ongoing Mother’s Gift Maternal Influenza Immunization Field Trial (MaGIFT) would allow us to obtain detailed demographic, behavioral, and biological information over time.

In this way, not only would we be able to establish baseline prevalence and incidence of hepatitis E in pregnant women in this part of Nepal, but we could also begin to examine risk factors for infection and explore potential mediators and moderators of illness severity and the association of HEV infections with pregnancy outcomes. [Figure 4] Based on estimates from Bangladesh and preliminary data from an earlier phase of the MaGIFT study in Nepal, we expected to observe a large number of cases and of infections, giving us at least modest power to conduct nested case-control analyses of immunologic, hormonal, and nutritional biomarkers that might be associated with predisposition to better or poorer outcomes.

**ONE DOOR CLOSES...**

Once the cohort study finally got off the ground, it proceeded apace, with a few minor hiccups and changes of protocol. I spent a cumulative six months in the field, variously accompanying field staff to interviews and blood draws, helping to implement a biosafety / good clinical practice initiative [see Appendix 3], checking study forms, and practicing my limited Nepali with many patient residents of the town of Hariyon.

The study began winding down in the spring of 2014, bringing with it the very welcome revelation that not very many women had become seriously ill with hepatitis, and the corresponding (somewhat more challenging) realization that there would be far fewer cases for our nested biomarker studies than anticipated.

No sooner had I returned home from my final stint in Sarlahi than reports of a jaundice outbreak began appearing in Nepali news media, this time in Biratnagar, a large Terai city on the
Indian border, a few districts to the east. A return trip to investigate the outbreak first-hand was contemplated and contacts were made, but the trip was shelved at the last minute due to logistical and political concerns, and an inability to secure the necessary IRB approvals within a short time-frame. However, one of my contacts in Biratnagar directed a Nepali virologist who had been involved in the outbreak response to Johns Hopkins, and a collaboration was born. Undeterred by bureaucratic and administrative delays and logistical challenges, not to mention the magnitude 7.8 earthquake that devastated Nepal on April 25, 2015, we finally met in Baltimore in the summer of 2015 to analyze specimens obtained during the 2014 outbreak.

The remaining chapters detail the conduct and results of the cohort study in Sarlahi and offer additional description of, and perspective on, the Biratnagar outbreak.

**Figure**

Figure 4. Conceptual framework for the study of hepatitis E viral infections among pregnant women in Nepal.
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HEPATITIS E VIRUS IN PREGNANT WOMEN IN SARLAHI DISTRICT, NEPAL

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ABSTRACT

In South Asia, hepatitis E virus (HEV) has recently been recognized as an important contributor to maternal morbidity and to maternal, fetal, and infant mortality. A community-based, observational cohort study was conducted in Sarlahi District, Nepal, from 2012-2014, using existing clinical trial infrastructure to enroll 2363 pregnant women and follow them through delivery or three months postpartum. Active symptom surveillance and serologic testing were used to detect sporadic acute “hepatitis-like” illnesses and subclinical or asymptomatic viral hepatitis infections. Pregnant women in Sarlahi have low immunity to HEV and are vulnerable to hepatitis E during pregnancy. Baseline anti-HEV IgG seroprevalence was 11.7% [95% confidence interval (CI): 10.2%-13.3%] and varied with age, village, caste/ethnicity, land ownership, and diet (meat consumption). Water sources and latrine access were not associated with seropositivity. Incidence of HEV seroconversion was 32.3/1000 person-years (p*y) during pregnancy [95% CI: 14.8 to 61.3/1000 p*y] and 44.3/1000 p*y overall [95% CI: 27.1 to 68.4/1000 p*y]. Postpartum seroreversion was common following HEV seroconversion in pregnancy. One acute case of hepatitis E due to genotype 1 HEV was confirmed by serology, but most “hepatitis-like” illnesses could not be attributed to acute hepatitis A, B, C, or E. Jaundice of uncertain etiology was linked to one of two maternal deaths during the study period. Mid-to-late pregnancy HEV infection was not clearly associated with adverse pregnancy outcomes, but study design and sample size limited detection. Serologic monitoring beginning prior to (or earlier in) pregnancy and longer postpartum follow-up would permit improved surveillance of both pregnancy outcomes and antibody kinetics. Environmental testing could help clarify exposure pathways and seasonal patterns of infection in the Terai.
BACKGROUND

Community-based studies have recently established that sporadic hepatitis caused by the hepatitis E virus (HEV) are an important contributor to maternal morbidity and mortality in Bangladesh. Nearby, Nepal is no stranger to enterically-transmitted hepatitis outbreaks, nor their devastating consequences for some expectant mothers. The untimely death of a pregnant woman – one of many – from acute liver failure during a 1987 outbreak ultimately helped to demonstrate the etiologic role of hepatitis E virus in the recurrent Kathmandu Valley epidemics of enterically-transmitted non-A-non-B hepatitis.

Still, hepatitis E-related illness and death in Nepali pregnant women, especially in the lull between epidemics, has rarely been more than a blip on a very busy maternal health radar. A handful of hospital-based studies in Kathmandu have called attention to the poor outcomes of severely ill pregnant women with acute hepatitis E and attempted to quantify the role of hepatitis E in severe maternal illness and death. These hospital-based studies and case series capture some of the most concerning outcomes associated with hepatitis E, but provide only a limited glimpse of the broader epidemiology of HEV infection.

Previous population-based studies were conducted primarily within the densely-populated Kathmandu Valley. Results generally suggest high population exposure to HEV among adults, with recent seroprevalence estimates near or above 50%. Several older studies were conducted in the late 1990s in advance of the contentious Phase I testing in Nepal of a vaccine that was promptly abandoned by its commercial sponsor, despite its demonstrated efficacy. As Nepali Army and Police conscripts were offered up as study subjects, those data also have a decidedly male skew.
A new recombinant vaccine, tested and produced in China, has been approved there since 2012. Though this vaccine currently lacks World Health Organization pre-qualification for use in other countries, there have been calls to expedite the process in order to address public health crises. The vaccine has been subject to limited safety and efficacy testing in pregnant women, but pregnant women are among the potential targets if the vaccine is cleared for global use.

With this context in mind, and with the benefit of an established field study site in southern Nepal, we undertook this study to begin to address outstanding issues:

- What is the burden of hepatitis E virus infections during pregnancy in a community setting in Nepal, considering a range of maternal, fetal, and neonatal outcomes?
- Do documented rates of HEV infection and illness in the Kathmandu Valley reflect the epidemiologic patterns elsewhere in Nepal?
- What factors place women at risk of – or protect against – infection, disease, and death?

**METHODS**

**Study Setting - MaGIFT**

The Mother’s Gift Maternal Influenza Immunization Field Trial (MaGIFT; NCT01034254) was conducted in 9 village development committees (VDCs) of Sarlahi District, in the Terai (lowlands) of Nepal. MaGIFT and the Hepatitis Substudy were carried out in the field through the Johns Hopkins University-affiliated Nepal Nutrition Intervention Project – Sarlahi (NNIPS) and its field headquarters in Hariyon, Sarlahi District. [Figure 5]

The nine VDCs where the study took place had a combined population of just under 100,000 people in 2011. The study area has few improved roads; however, it is bisected by the East-West
(Mahendra) Highway, the major road traversing the Nepali Terai. In May 2014, several VDCs in the study area were merged and officially reclassified as municipalities as part of an urbanization initiative.\textsuperscript{31}

From April 2012 through April 2013, MaGIFT participants were individually randomized to receive influenza vaccine or saline placebo between 17-34 weeks of gestation. At the end of this controlled trial phase, all participants received the active vaccine. Women with new pregnancies identified from late April 2013 onward were also offered immunization (without randomization, at scheduled clinics) and followed through delivery or the end of the follow-up period in May 2014. The hepatitis substudy spanned the period from October 2012 until the end of follow-up in May 2014 and was open to all women eligible for participation in MaGIFT.

**Study Enrollment**

Married women ages 15-40 living in the 9 participating VDCs of Sarlahi District were identified through a baseline community census conducted in 2011 and ongoing reports by field workers assigned to sectors in each ward of the participating VDCs. With consent, field data collectors visited these married women on a 5-week schedule and offered pregnancy tests to women who reported a missed menstrual period.

Field data collectors reported pregnancies to the field headquarters in Hariyon. Each pregnant woman was then visited by a trained team leader-interviewer (TLI). Eligible women (≤34 weeks gestation, planned to deliver within the study area, had not received current flu vaccine, no allergies to vaccine components, not previously a MaGIFT participant) were invited to join MaGIFT and the hepatitis substudy. [Appendix 1]

Upon obtaining informed consent, [Appendix 2, Form 66] the TLI administered questionnaires concerning the woman’s health and reproductive histories, routine behaviors, and
living situation. Additional questions about possible hepatitis-related risk factors focused on water, sanitation, and hygiene practices; occupation; food preparation and consumption; travel history; and animal exposures. [Appendix 2, Form 79] All women were offered basic antenatal care packages, including iron supplements, deworming tablets, and a safe birthing kit.

**Routine Blood Collection (Enrollment, Delivery, and Postpartum)**

Auxiliary nurse midwives (ANMs) trained in phlebotomy collected ~5mL of venous blood from participants by at each of three event-contingent time-points: upon enrollment, after delivery, and at 3 months postpartum. Specimens were labeled and placed on ice packs in insulated coolers and transported from the field by motorcycle to the field laboratory in Hariyon within 4-6 hours of collection. At the field laboratory, specimens were centrifuged, and ~2.5mL of serum derived from each blood sample was aliquoted into four cryovials. These cryovials were labeled and placed in liquid nitrogen shippers for storage and shipment.

The aliquots were batched and transported in liquid nitrogen dewars to the Walter Reed/AFRIMS Unit Nepal (WARUN) in Kathmandu. The first set of aliquots was used at WARUN to conduct serologic testing for the hepatitis substudy. The other aliquots were shipped in liquid nitrogen shippers to Cincinnati Children’s Hospital for distribution to MaGIFT investigators in the United States. [Appendix 1]

**Symptom Surveillance and Follow-Up of Acute Hepatitis Cases**

As part of MaGIFT, field workers visited women weekly to ask about a limited set of symptoms. After enrollment, each woman was visited by a TLI at 30-day intervals to ask about broader symptoms of illness and any care sought, using the Monthly Pregnancy Follow-Up Form (PFF) [Appendix 2, Form 14]. The PFF recorded the number of days in the past 30 that a symptom was experienced, along with a code for type of provider (e.g., traditional healer, health post, medical
To adapt the PFF for the Hepatitis Substudy in this phase of MaGIFT, additional symptoms relevant to hepatitis were included.

Hepatitis-related symptoms (nausea or vomiting, fever, yellow eyes or skin, dark urine or light stools, upper abdominal pain, or strange/unappealing smell to food) reported at monthly visits yielded a weighted symptom score, calculated on the PFF. Scores above the threshold of 4, indicating a presumptive case of acute hepatitis, were reported by the interviewer to the field office by telephone at the end of the visit. [Table 3]

Upon receipt of a telephone report of a possible hepatitis case, auxiliary nurse midwives (ANMs) scheduled a case follow-up visit within 24 hours. At this visit, after obtaining informed consent for the follow-up procedure, [Appendix 2, Form 78] the ANMs complete a rapid anti-HEV IgM assay, a 5mL venous blood draw, and a brief questionnaire about treatments sought, and they provided a referral for local medical care if warranted. The Case Visit Form [Appendix 2, Form 80] included questions about previous care or advice sought for the reported symptoms, treatments recommended or administered (pills, herbs, teas, spiritual practices), and perceived effectiveness of treatments.

Two drops (~30µL) of whole blood from the venous blood draw were tested in the field with the Wantai Rapid HEV IgM test (Wantai BP Co., Beijing, China), carried out according to the manufacturer's instructions.

All symptomatic women were informed of the rapid HEV test result, and told about local medical care options. Women testing positive for acute HEV infection were also provided with a “Hepatitis E Report Card” (HERC) [Appendix 2, Form 81] that they could present to a local medical facility if they sought treatment. The ANMs explained the typical course of illness in simple terms,
and recommended seeking care at a medical facility if symptoms worsened or if the woman had concerns about her pregnancy.

Blood samples from symptomatic women were transported to the field laboratory and processed following the same procedures as the routine blood samples, but the entire volume was designated for the hepatitis substudy.

Serologic Surveillance and Testing

Routine serologic testing of baseline, post-delivery, and 3 month postpartum samples, and diagnostic serology for cases detected by syndromic surveillance, were performed by the WARUN under the terms of a collaborative research agreement.

All participants’ baseline (pregnancy enrollment/PE) samples were tested for antibodies to hepatitis A, B, C, and E, as well as for acute or recent infection with HEV using Wantai ELISA kits (Beijing Wantai BP Enterprise Co., Beijing, China). Specimens testing positive for anti-HBc were also tested for HBsAg to determine if the infection was likely recent or chronic. All women, regardless of baseline serostatus, were tested for antibodies to HEV at all three timepoints to capture seroconversions and possible seroreversions.

Hepatitis follow-up samples drawn from women identified through syndromic surveillance as presumptive cases underwent diagnostic screening. These samples were tested for anti-HAV IgM, HBsAg, anti-HCV, and anti-HEV IgG and IgM (Wantai HAV IgM ELISA, AiD HBsAg ELISA, AiD HCV ELISA, HEV IgG ELISA, and HEV IgM ELISA, Beijing Wantai BP Enterprise Co., Beijing, China) to identify hepatitis virus(es) causing the symptoms. Results of the Wantai Rapid HEV IgM assays performed by study staff in the field in Sarlahi were compared with the results of the Wantai HEV IgM assays conducted in the laboratory at WARUN in Kathmandu. Acute hepatitis E was diagnosed in a symptomatic woman when either the rapid field test or the laboratory test for anti-HEV IgM
returned a positive result. Personnel at WARUN also attempted to detect viral RNA in serum from women with acute hepatitis symptoms and/or positive anti-HEV IgM results using reverse transcriptase polymerase chain reaction (RT-PCR) methods described elsewhere and sent viremic samples to the Armed Forces Medical Institute of Research (AFRIMS) in Bangkok, Thailand, for sequencing.

**Ascertainment of Pregnancy Outcomes and Maternal and Infant Mortality**

In addition to limited weekly and extensive monthly symptom surveys and hepatitis case follow-up, maternal health, pregnancy outcomes, and infant health were monitored as part of the MaGIFT trial framework.

Upon notification of labor or delivery by a family member or field worker, a trained Birth Assessment Team was dispatched by motorcycle to the woman's residence. Maternal and infant birth assessment forms were used to collect information about the health of the mother-child pair prior to, during, and after delivery, and to record any complications or apparent birth defects. In the cases of multiple births, separate forms were completed for each infant.

Hospitalizations occurring during the study period were reported by field data collectors and by members of the birth assessment team to the study PIs. Maternal and infant deaths identified by field staff during follow-up were recorded, and verbal autopsy forms were completed with the aid of relatives or neighbors, when possible, to ascertain causes of death.

**Feasibility and Sample Size Considerations**

Conducting the proposed work through an ongoing field trial was intended to make efficient use of existing study infrastructure and minimize added burden to study participants. The project also stood to benefit from good community rapport and interest in research resulting from both
long-term engagement of the MaGIFT team in a number of other health interventions in the region, and also the employment of local men and women as field workers.

Target enrollment during the April 2012-April 2013 phase of MaGIFT was 1850 pregnant women. Based on prior NNIPS work in Sarlahi, fewer than 10% of eligible participants were expected to decline participation, and under 5% were expected to be lost to follow-up. There was some concern that higher-than-anticipated enrollment during the previous phase of the trial, due primarily to a large number of prevalent pregnancies enrolled into the cohort at study inception, could reduce yield in this second phase, as women were ineligible to contribute more than one pregnancy to the trial. (J. Tielsch, pers. comm.) The enrollment targets were increased to 2300 when the purely observational phase was added following completion of the randomization phase.

Based on population-based incidence rates of roughly 60 seroconversions / 1000 person-years (p*y) observed in a rural Bangladeshi cohort (overall and in women)\textsuperscript{34} and \textasciitilde{}64 infections / 1000 p*y observed in Kathmandu (in a primarily male population),\textsuperscript{23} and an assumption that each of 1000 participants would contribute \textasciitilde{}9 months of person-time over the study period, approximately 45-48 HEV seroconversions would have been expected if all participants were susceptible at enrollment. Assuming a 20\% prevalence of protective immunity at baseline,\textsuperscript{35} the number of seroconversions would instead have been be about 36-39. Of these, we estimated that \textasciitilde{}1/3 to 1/2 would be acutely symptomatic and detected by syndromic surveillance, yielding approximately 12-20 cases of acute hepatitis E. We expected to detect a similar number of non-HEV-attributable hepatitis-like illnesses through the symptom surveillance system. A sample size of 1000 women would be sufficient to estimate the incidence of seroconversion and symptomatic illness with a precision of \textasciitilde{}6.2\% (at the 95\% confidence level; with 1400 women, to within 5.3\%).\textsuperscript{36} We expected to detect viral RNA in one or more samples from confirmed cases, allowing us to examine HEV strain(s) circulating and causing illness in the Terai.
Were the study period to have coincided with a sizable outbreak of hepatitis E, our *a priori* predictions of seroconversion and clinical illness incidence would have underestimated the number of observed cases, but our incidence estimates would be higher than expected. Conversely, if only sporadic cases were detected and no outbreaks were to occur during the study period, our estimates of HEV infection and illness would likely be low relative to what one might expect over a longer period of observation that included both sporadic cases and occasional outbreaks.

**Ethical and Human Subjects Considerations**

Study protocols and forms for the hepatitis substudy were reviewed and approved by the Johns Hopkins Bloomberg School of Health IRB (#2458), the Nepal Health Research Council (NHRC, #92/2011), and Walter Reed Army Institute of Research (#1973). The parent study, the Mother's Gift Field Trial of Maternal Influenza Immunization in Asia (MaGIFT), is a registered Phase III clinical trial (clinicaltrials.gov ID #NCT01034254) with a data safety monitoring board and an independent monitor. Its principal investigators and study staff have had a long history of commitment to health projects in the region. Most field workers were women hired locally, reducing potential cultural or linguistic barriers.

The study was designed with local cultural practices and reported preferences in mind, and attempts were made to minimize the burden of participation. Venous blood collection was substituted for the fingerstick blood collection system used successfully in Bangladesh because venipuncture was reported to be more acceptable to participants in Sarlahi (J. Tielsch, pers. comm.). Using aliquots from venous blood draws instead of fingerstick samples also reduced the total number of needlesticks participants received. Confidentiality was maintained to the extent possible in this community setting. Data collection forms and biological samples were indexed by coded study ID number, and identifiers were kept for follow-up purposes in a secure location in the field office in Hariyon.
Participation in the hepatitis substudy was not designed to yield any direct benefits to participants, as no standard treatments are currently available for acute hepatitis E during pregnancy. Furthermore, due to off-site laboratory testing arrangements, detection of antibodies to other hepatitis viruses was delayed and results could not be provided in real time. Although a pentavalent vaccine including hepatitis B is part of the routine childhood immunization schedule in Nepal, its coverage in Sarlahi is lax (J. Katz, pers. comm.) and no monovalent hepatitis B vaccine is currently available in Nepal to treat infants born to mothers with chronic hepatitis B. Thus, unfortunately, we would have been unable to intervene to prevent vertical transmission of hepatitis B even if a chronic infection could have been detected in a pregnant woman in a timely manner. Women whose rapid test result was positive for HEV received information from study staff and a card they could take with them if they sought medical care. Study staff referred all ill women, regardless of test results, to the nearest medical clinic for supportive care, as indicated. Indirect benefits to participants included contribution to the empirical evidence base describing illness and death from HEV in their community, and helping to inform possible interventions, including the potential introduction of a vaccine that has been approved in China.

**Data Management, Quality Assurance, and Quality Control**

General forms and protocols for the study were field-tested and refined during the first phase of MaGIFT in Sarlahi. Hepatitis Substudy-specific forms and protocols, including the exposure assessment questionnaire and symptom scoring procedures, were adapted from those used successfully in the Matlab study areas in rural Bangladesh. From July-October 2012, all Hepatitis Substudy data collection instruments were translated into Nepali, pre-tested, and refined by study personnel in an iterative process to ensure clarity, usability, and sensitivity to local custom.
All field workers involved in the study were trained for their roles. Venipuncture was performed by auxiliary nurse-midwives (ANMs) trained in phlebotomy. All study personnel handling biological samples were trained in bloodborne pathogen precautions.

Training was provided in the use of Hepatitis Substudy-specific forms and protocols. Written instructions and examples were documented in the MaGIFT Manual of Operations. Weekly meetings of field workers, team leaders, and other personnel based in Hariyon provided a means for reviewing protocols, troubleshooting any problems, and providing feedback.

All forms contained participants' study identification number, date, and worker initials for tracking purposes. Paper forms completed in participants' villages were returned to the field office in Hariyon for preliminary review of completeness, legibility, and consistency by team leaders before being transported to the NNIPS Data Center in Kathmandu. Entry of form data into the MaGIFT database was done by staff at the NNIPS Data Center in Kathmandu. The staff at the Data Center in Kathmandu have historically had a low data error rate over time, and corrective action was taken when a database updating problem was discovered.

Laboratory results from WARUN and AFRIMS were sent to hepatitis substudy investigators for integration with the study database using participants' study identification number. WARUN used appropriate positive and negative controls for all assays performed.

**Data Analysis**

Stata v.11 (StataCorp, College Station, Texas) was for statistical analyses. Sessions were logged and archived.
Prevalence, Incidence, and Related Estimates

Seroprevalence for each of HAV, HBV, HCV, and HEV at a given time-point (enrollment, delivery, 3 months postpartum) was estimated as the number of women with seropositive assay results, divided by the total number of consenting women tested at that time-point.

To estimate the incidence of seroconversion during pregnancy, the number of women seronegative for anti-HEV IgG at baseline who subsequently tested positive at the post-delivery blood draw were divided by the amount of person-time contributed by susceptible women between those two points less one-half the person-time contributed by seroconverters. The incidence of seroconversion during the postpartum period was estimated as the number of women seronegative at the post-delivery timepoint (excluding women seropositive at baseline) who subsequently tested positive at the three month postpartum timepoint, divided by the amount of person-time contributed by susceptible women during this time period. We calculated incidence over additional intervals (e.g., baseline to 3 months postpartum) for comparison with published results from other studies. Person-time for susceptible women was calculated by subtracting the date of one blood draw from the date of the previous blood draw and converting the result to years.

We estimated the incidence of hepatitis-like illness using the number of cases defined by above-threshold Hepatitis Score as the numerator and the number of person-months ascertained by completed follow-up forms as the denominator. The proportion of acute viral hepatitis cases attributable to HEV was estimated by dividing the number of symptomatic women with lab-confirmed acute Hepatitis E by the total number of women with acute hepatitis-like illness. The case-to-infection ratio was estimated by dividing the number of confirmed hepatitis E cases by the total number of women with baseline anti-HEV IgM plus anti-HEV IgG HEV seroconverters detected through routine follow-up serology from baseline to the post-delivery timepoint.
RESULTS

Study Enrollment

A total of 2,363 women consented to participate in the hepatitis substudy within MaGIFT, and 5 women declined consent. Of these, 956 women joined the hepatitis study and 5 declined during the randomized, controlled influenza vaccine trial period of MaGIFT (8 October 2012-24 April 2013) and 1407 women enrolled during the post-randomization observational phase (25 April 2013–2 May 2014). Forms for 29 women exist but have not yet been entered into the study database, and forms for another 8 women are missing; these 37 women and their data are not included in the enrollment figures or in the data analyses. Enrollment of study participants relative to the total female population of each VDC and ward is shown in Figure 6.

Serologic Follow-Up

Blood collection over the course of the study is summarized in Figure 7. Baseline maternal blood specimens were collected from 1669 participants at a median gestational age (estimated from self-reported last menstrual period) of 19.4 weeks (range: 6.7-34.4 weeks). Of the 117 other women for whom baseline specimen collection attempts were recorded: 108 declined to provide baseline specimens, and 9 did not provide specimens for other reasons. The rest of the enrolled women (n=577) were not approached to provide a sample due to the transition between the RCT phase and the observational phase of the study or the end of the study period, or they had missing records.

Delivery specimens were collected from 811 women a median of 13 days following the end of the pregnancy, with >90% of these specimens collected within 4 weeks of the pregnancy outcome date. Of the 470 other women for whom delivery specimen collection attempts were recorded: one woman was deceased, 327 were not met, 50 declined to provide specimens, and 92
women did not provide specimens for other reasons. The remainder of the women (n=1082) did not deliver in time to give a blood sample prior to the end of the study period, or they had missing records.

Three-month postpartum specimens were collected only from women who joined the study as part of the randomized, controlled trial, and not from women who joined during the later observational phase. These specimens were obtained from 659 women. One additional woman was deceased, 149 were not met, 38 declined to provide specimens, and 46 did not provide specimens for unknown reasons. The remainder of the women either did not reach 3 months postpartum prior to the end of the study, or they had missing records.

A total of 792 women provided both baseline and delivery blood samples, 643 women provided both baseline and 3 month postpartum samples, and 480 women provided samples at all three timepoints (baseline, delivery, and 3 months postpartum).

**Baseline Serostatus and Risk Factors for HEV Seropositivity**

Among 1669 women with baseline specimens, 195 or 11.7% [95% confidence interval (CI): 10.2%-13.3%] were positive for anti-HEV IgG (one of whom was also anti-HEV IgM positive). Nearly all were positive for IgG antibodies to HAV, roughly one-quarter had anti-HBc antibodies and about 2% were positive for HBsAg, and none was anti-HCV IgG positive. [Table 4]

Characteristics of the full cohort, women who provided baseline specimens, and women seropositive for anti-HEV IgG at baseline are shown in Table 5, and Figure 6 depicts study enrollment relative to local population. Seropositivity for anti-HEV IgG was associated in univariate analyses with age, parity, education, caste, household land ownership, and meat-eating, but not with household latrine presence or type or ward-level latrine prevalence, nor with primary
drinking water source. [Figure 8] Village- and ward-level variation in baseline seroprevalence of anti-HEV IgG, in population by caste, and in distribution of latrines are shown in maps in Figure 9.

In multivariate logistic regression analysis, [Table 6] increasing age was associated with increased odds of baseline seropositivity. Both socioeconomic variables included in the final model were independently associated with serologic status, with traditionally lower caste/religious affiliation (especially Shudra, Muslim, and those with another or no reported caste) and lack of agricultural land ownership both linked to increased odds of seropositivity. Although residents of Hariyon VDC had the highest seroprevalence of anti-HEV IgG overall, only residence in Dhungrekhola (and no other VDCs) was statistically-significantly protective [OR 0.47; 95% CI: 0.23-0.95] after adjustment for other covariates.

Meat-eating [Figure 10] was consistently and fairly strongly associated with seropositivity in both univariate and multivariate analyses. Inclusion of individual meat consumption patterns in the multivariate models (i.e., specific meats eaten or frequency of consumption of specific meats) did not yield effects as strong as the general binary vegetarian/meat-eater variable. Eating fish appeared to underlie much of the association between meat-eating and seropositivity, as its inclusion in models produced the largest decrease in the general meat-eating effect size. However, addition of a “dose” variable based on frequency of fish consumption in the prior 30 days did not improve the models, whereas both pig consumption and buffalo consumption appeared to show some differences in effect with increasing consumption. While the direct association of frequency of pig consumption with seropositivity represented a statistically-significant trend on its own, it did not improve the models in multivariate analyses. The association of buffalo meat consumption frequency with seropositivity, while statistically significant at some levels, was inconsistent in direction, driven by a large number of seropositive women who had eaten buffalo exactly 2 times in the prior month. Very few women ate only a single type of meat, and no women ate only fish or
only pig; the collinearity of several of the specific binary meat-consumption variables and the unclear dose-response relationships for specific meat consumption frequencies were additional reasons for inclusion of the general vegetarian/meat-eater variable in the model rather than individual meat types.

**Serologic Estimation of HEV Incidence**

Excluding those women who had positive anti-HEV IgG serology at baseline, we observed 9 seroconversions during pregnancy (from baseline to delivery) and 8 seroconversions in the postpartum period (from delivery to 3 months postpartum). An additional 3 seroconversions occurred between enrollment and 3 months postpartum among women who did not provide post-delivery samples. The 9 seroconversions, occurring among 695 susceptible women who gave both baseline and delivery blood samples an average of ~20 weeks apart, represents an approximate incidence proportion of 1.3% (or 1.4% if the woman who was seropositive for anti-HEV IgM and HEV RNA at baseline were also counted as a seroconverter).

Accounting for actual person-time contributed by susceptible women during each of these periods, and subtracting ½ the person-time contributed by seroconverters, these observations yield seroincidence estimates of 32.3/1000 person-years (p*y) [95% CI: 14.8 to 61.3/1000 p*y] during pregnancy and 93.3/1000 p*y [95% CI: 40.3-183.8/1000 p*y] during the first three months postpartum. The incidence among women for whom only baseline and 3 month postpartum specimens were obtained (n=141) was 34.5/1000 p*y [95% CI: 7.1-100.9/1000 p*y].

Considering person-time contributed to serologic surveillance by the full cohort, regardless of distribution relative to pregnancy or absence of a specimen at a single time-point, we obtain an overall incidence estimate of 44.3/1000 p*y [95% CI: 27.1-68.4/1000 p*y] based on 20 seroconverters over 451.4 p*y. Looking at baseline and 3 month postpartum serostatus only (and
excluding women who failed to provide a specimen at either of these timepoints), we obtain an estimate of 26.3/1000 p*y [95% CI: 12.0-49.9/1000 p*y] based on 9 seroconverters over 342.2 p*y.

Seroconversion from anti-HEV IgG+ to anti-HEV IgG- was also observed in our cohort. Among 90 women seropositive at baseline who provided post-delivery samples, 6 were seronegative following delivery. Among 62 women seropositive at delivery who provided 3 month postpartum samples, 5 were seronegative at three months postpartum. Among the 9 women who seroconverted between enrollment and delivery, 5 provided samples at 3 months postpartum, and all 5 of these were seronegative; however, the woman who was viremic and seropositive for anti-HEV IgG and IgM at baseline maintained her IgG seropositivity at 3 months postpartum.

**Maternal Morbidity and Mortality**

Pregnant participants contributed a total of 8716 completed follow-up visits at roughly monthly intervals, each covering symptoms experienced during the prior 30-day period. Using the symptom-based case definition shown in Table 3, 15 pregnant women were identified as potential cases of hepatitis-like illness, for an incidence of approximately 20.7 / 1000 person-years.

Only one of these 15 symptomatic cases was confirmed to be hepatitis E, both by rapid field test (IgM ELISA) and by later serologic and molecular testing. [See case report for further details.] Two other symptomatic cases had anti-HEV IgG, but were anti-HEV IgM negative, and neither had a usable baseline sample to determine whether or not the positive IgG result was linked to the recent illness or represented infection in the more distant past. None of the acutely symptomatic women tested positive for anti-HAV IgM, anti-HBsAg, or anti-HCV antibodies.

If we count only the single lab-confirmed case to be caused by HEV, this yields a hepatitis E incidence of roughly 1.37 / 1000 person-years. If we relax the case definition and assume that all 3 HEV IgG+ cases represented hepatitis E, we obtain an estimate of 4.13 / 1000 person-years.
There were 2 maternal deaths recorded among women enrolled in the cohort. One of these deaths occurred on the day of childbirth, and no specific cause was recorded; the woman had reported dark urine but no other hepatitis-like symptoms at a follow-up visit three weeks earlier. The other death occurred in a woman who had delivered a child 7 weeks earlier, and this death was associated with “cough, body pain, and jaundice”. In the 3-4 months preceding her death, while still pregnant, she reported persistent nausea, weakness, upper abdominal pain, and olfactory disturbances, but never jaundice, and these combinations of symptoms never exceeded the threshold for hepatitis case detection used in the study.

Pregnancy and Infant Outcomes

Primary Birth Outcomes

Among the 2363 cohort participants, 1801 pregnancy outcomes were recorded through 2 May 2014. 1729 (96%) of these were live births (singleton or twin), 31 were stillbirths, 36 were miscarriages, and 5 were abortions. All of the nine mothers who seroconverted for HEV during pregnancy, as well as the one woman confirmed to have acute hepatitis E, gave birth to live infants.

Gestational Age and Birthweight

The median duration of pregnancy, based on self-reported last menstrual period, was 39.6 weeks in the 1801 women for whom pregnancy outcome was known. Among live births (n=1729), 196 (11.3%) were preterm (<37 weeks gestation). Two of the nine HEV seroconverters during pregnancy (22.2%) had preterm deliveries, one at 30.7 weeks and the other at 36.3 weeks; the association of preterm births with HEV seroconversion was not statistically significant (odds ratio 2.5, 95% confidence interval 0.5-12.3). In 1244 women with live births for whom baseline anti-HEV IgG testing was completed, maternal baseline HEV seropositivity was associated with increased odds of preterm birth compared with baseline seronegativity (odds ratio 1.9, 95% confidence interval 1.2-3.0). The size and statistical significance of this apparent association were
diminished slightly when socioeconomic factors (caste and household land ownership), maternal age, and parity were taken into account (odds ratio 1.6, 95% confidence interval 0.9-2.6).

Among all singleton live births visited within 48 hours of delivery (n=1164), the prevalence of low birth weight (<2500g) was 21.6%. This was similar to the 25.0% prevalence of low birth weight among babies born to mothers who seroconverted for HEV during pregnancy, and the difference was not statistically significant ($\chi^2=0.02, p=0.88$). None of the babies born to seroconverting mothers had very low birth weight (<2000g). No association was found between maternal baseline HEV serostatus and low birth weight.

**DISCUSSION**

**Prevalence and Incidence of HEV Infection and Illness**

This community-based, longitudinal study evaluated sporadic hepatitis E virus infection and illness among pregnant and postpartum women in the Terai of Nepal, where hepatitis E has not previously been well-characterized. The baseline anti-HEV IgG seroprevalence of 11.7% in pregnant women in Sarlahi is considerably lower than among adults living in the Kathmandu Valley$^{15,16,23}$ and in some areas of India and Bangladesh$^{35,42,43}$ where estimates ranging from 25% to >75% have been reported with comparably sensitive assays. However, it is similar to reports of 13.7% and 10% in two recent cohorts of pregnant women drawn from the JiVitA study area in northern Bangladesh.$^{44,79}$ It is also comparable to seroprevalence estimates in healthy adults in the eastern Terai cities of Dharan and Biratnagar (cf. Biratnagar outbreak chapter).

Our finding of relatively low baseline seroprevalence and incidence in pregnant women in a country where very high rates of infection with HEV have previously been documented is somewhat surprising. Typically higher seroprevalence estimates among men, which tend to elevate general population estimates if not adjusted for sex distribution, likely explain some of the
discrepancy. A 1994 study conducted in Kathmandu showed divergence of seroprevalence by sex with increasing age, with an increase in men’s seroprevalence but a decrease in women’s seroprevalence from ages 20-30 and overall seropositivity in 16% of men and 9.5% of women over age 19.²² That study used an older, considerably less-sensitive assay,⁸⁰ so the absolute seroprevalence estimates they obtained are not directly comparable to ours (and may underestimate seroprevalence by as much as 200%); however, the observation of greater seroprevalence in men and the different age-specific seroprevalence patterns are still of interest. The men involved in the Kathmandu study were mostly members of the military or police forces,²² who may have had increased risk of HEV exposure relative to men in the general population due to their outdoor occupations and housing in barracks, biasing the population estimates. However, the decline in seropositivity with increasing age among women in Kathmandu,²² which also contributed to the differences by sex and age, is not a common finding. By contrast, in our cohort of pregnant women, seropositivity was greater in older age groups through the second and third decades of life. A more recent study comparing age-specific population seroprevalence estimates in Nepal (Kathmandu), Bangladesh, and France did not report results by sex, precluding consideration of sex-by-age interactions.¹⁵

The decentralized nature of the water supply in rural areas of the Terai, with most women obtaining water for drinking and other household uses from local tubewells or ring wells, may also help explain the relatively low exposure in Sarlahi as compared with urban areas in both the Kathmandu Valley and in northern India, where water is more commonly supplied by pipe and has ample opportunities to become contaminated as it is distributed. Rural-urban differences in seroprevalence among pregnant women have been studied elsewhere, with seroprevalence in rural women lower in Gabon,⁸¹ higher in Turkey, ⁸² and similar to that in urban women in Qingdao and
Weihei, China. Major differences in study populations and geographic factors make it difficult to generalize findings from these studies to the Nepali setting.

Jaundice (representing both icterus and a variety of non-icteric complaints) is reported frequently in Bangladesh, but it was uncommon in our southern Nepali cohort. Even among the 15 women who met the criteria for our case definition of hepatitis-like illness, only 4 reported jaundice as a symptom. As has been noted in other settings, the case-to-infection ratio in our cohort was low, with only one symptomatic case of HEV occurring for 20 apparently-asymptomatic seroconverters. Although we kept our case definition intentionally broad so that it would be more sensitive to milder hepatitis E cases without frank jaundice, we did not capture any additional seroconverters this way.

The incidence of serologically-detected, asymptomatic or subclinical HEV infection in the cohort was 44.6/1000 person-years (p*y), substantially less than the 99/1000 p*y incidence reported in young adult populations in Kathmandu and the 64/1000p*y in a general population in southern Bangladesh. Although our estimate at first appears comparable to the 46/1000 p*y incidence recently estimated in northern Bangladeshi pregnant women, Kmush et al. defined seroconverters as women who were seropositive at 3 months postpartum but seronegative at earlier points (excluding from their pool of seroconverters those women who may have seroconverted and subsequently seroreverted within that timeframe). Applying a similar definition of seroconversion to the Sarlahi cohort, we obtained a much lower estimate of 26.3/1000p*y.

As in northern Bangladesh, where only one of 40 detected seroconversions occurred between early and late pregnancy and the rest occurred postpartum, we observed a much greater incidence of seroconversion from delivery to 3 months postpartum than from baseline enrollment to delivery. The reasons for this difference in rate are unclear.
In the Sarlahi cohort, 5/5 women who had seroconverted between baseline and delivery were again seronegative when re-tested at 3 months postpartum. Seroreversion could perhaps explain a small portion of the discrepancy observed by Kmush et al., as their testing for antibodies at earlier points was conditioned on anti-HEV IgG seropositivity at 3 months postpartum. Thus, some women who seroconverted during early pregnancy could have seroreverted during the late pregnancy/early postpartum period, and those infections would have gone undetected. Transience of seropositivity would not explain the differences in rates observed in the Sarlahi cohort, however, as specimens collected from all participants at all three study timepoints were tested prospectively for IgG.

Completeness of serologic follow-up with the Nepali women was limited considerably by several factors: the MaGIFT study dates and changes to protocol during the study period; Nepali women’s greater participation in activities outside the home relative to women in Bangladesh, such that a large number of women were not met on multiple occasions when the ANMs visited to collect samples; and participants who declined to provide samples for various reasons. Women who reported staying in their homes all day were slightly more likely to be seronegative at baseline than women who regularly went out. If engagement in activities outside the home were a risk factor for infection, more non-seroconverters than seroconverters might have been at home to provide follow-up specimens, artificially depressing our incidence estimates. Whether or how this would have a differential effect on women providing samples following delivery vs. at 3 months postpartum is not clear, but missing data (if differentially missing by serostatus and/or season) could affect the validity of our incidence estimates.

Behavioral changes associated with pregnancy could potentially change HEV exposure patterns. Some of these factors (such as reduced outdoor exertion) might be expected to decrease potential exposure to HEV, while others (such as increased water intake and/or more frequent
need to urinate) might be expected to increase it. Our behavioral exposure data were collected only at enrollment and may not adequately capture changes over the course of the pregnancy and postpartum periods. Changes in food intake patterns during pregnancy resulting from morning sickness, increased nutritional needs, or cultural traditions are likewise poorly reflected in our data.

Seasonal patterns of infection could also potentially account for some of the incidence difference in the Sarlahi cohort, as follow-up periods were not evenly distributed over the calendar year due to MaGIFT study dates. During the 1981-1982 hepatitis E epidemic in Kathmandu, hospital-based case counts were highest during the monsoon, and in 1980, prior to the epidemic, a peak in cases from July-September was still evident. In southern Bangladesh, a higher incidence of seroconversion was detected during the monsoon than during the pre-monsoon season. In Vellore and Lucknow, India, HEV genotype 1 RNA was detected substantially less frequently in sewage samples collected during the monsoon season than in other seasons. While these environmental observations could conceivably reflect dilution of the sewage stream by rainwater during the monsoon rather than a true reduction in viral shedding in the human population, fewer symptomatic hepatitis E cases presented to the hospital in Vellore during the monsoon as well.

In the Sarlahi cohort, postpartum follow-up person-time was more likely than pregnant person-time to occur during the monsoon season in the Sarlahi cohort due to the study's October 2012 start and the gap in new enrollments from May-July 2013. A slight majority of intervals during which participants seroconverted fell entirely outside the monsoon season (even with a 2-4 week lag to allow for delayed development of antibodies). The single confirmed acute hepatitis E case occurred during the winter season. Determining seasonality of infection in our study with a relatively small number of seroconverters was not possible to do with high confidence, and infrequent and dynamically-scheduled serologic testing made it more difficult to pinpoint when infections were most likely to occur. If a seasonal pattern could be detected, both exposure-related
and host-related factors might play a role. Micronutrient levels vary by season among pregnant women in Sarlahi.\textsuperscript{86,87} Zinc deficiencies and anemia (which peak in summer in Sarlahi\textsuperscript{86}), as well as vitamin D deficiencies (which peak in winter in Sarlahi\textsuperscript{86}), were associated with HEV seroconversion in Bangladeshi pregnant women.\textsuperscript{44}

**Maternal Mortality**

Neither of two maternal deaths reported during the hepatitis substudy, one peripartum and one postpartum, was conclusively attributable to hepatitis E. Verbal autopsy for the participant who died 7 weeks postpartum mentioned jaundice, and that woman had a history of symptoms consistent with hepatic illness (including dark urine and upper abdominal pain) in the months prior to death. Although a small number of delayed cases have been documented in non-pregnant adults,\textsuperscript{46} liver failure resulting from acute hepatitis E typically occurs within days to a few weeks of symptom onset. Although we cannot rule in or out HEV as a potential contributor to this woman's death, other causes of liver dysfunction in pregnancy and adulthood, many of which present with similar symptoms,\textsuperscript{47} may be more likely.

To determine whether reports of jaundice or hepatitis as causes of death may have been unusually low during the study period, and to consider a larger sample, we reviewed all maternal deaths that had been documented by verbal autopsy in an extended MaGIFT cohort from January 2011 onward (n=22). Among the 22 recorded maternal deaths, 6 were attributed to suicide or accidental injuries, 4 to postpartum bleeding or abdominal abscess, 2 to jaundice, 2 to preexisting conditions (heart valve defect, asthma), 2 to tuberculosis, 1 to liver cancer, 1 to fever and kidney failure, 1 to fever and edema of unknown etiology, 1 to neurologic dysfunction associated with a fall and bleeding, and 2 to unknown causes.
The overall prevalence of jaundice as either a cause or as a precursor of death (as mentioned in free narrative or in response to questions about prior jaundice diagnosis and yellow eyes) was 13.6% (3/22). This was somewhat higher than the 9.8% noted in a population in rural Bangladesh; however, the proportion of jaundice-related deaths attributable to hepatitis E is unclear, and may be lower than in that Bangladeshi population. Of the 3 jaundice-associated maternal deaths, one was part of our hepatitis substudy cohort, as described above, and two were not. One of the deceased women had a 5-month history of jaundice that did not respond to treatment, possibly a result of hepatitis B virus infection or another condition. The other woman’s death was attributed to a diagnosed-but-unrepaired heart-valve defect, and the jaundice may have been secondary to heart failure or even to treatments prescribed by the traditional healers or hospitals she visited.

The recorded deaths were distributed unevenly over time, with more reported in 2011 than in 2012, and more in 2012 than in 2013, suggesting possibly-incomplete ascertainment of the more recent maternal deaths, but jaundice-associated deaths were not obviously underrepresented in the hepatitis substudy cohort. While we cannot definitively determine all of the causes contributing to these deaths, and we cannot rule out some contribution of HEV infection, jaundice-associated mortality in this population of pregnant women is attributable to multiple causes.

**Pregnancy Outcomes**

We were unable to robustly evaluate the potential impact of hepatitis E on pregnancy outcomes because of the systematic exclusion from the MaGIFT clinical trial of women who miscarried prior to randomization, poorer postpartum sample collection among women who did not have live births, and relatively few seroconversions and even fewer clinical cases among enrolled women. A greater proportion of infants born to seroconverting mothers than to seronegative mothers were delivered preterm, but this association was not statistically significant.
Otherwise, in our small sample of women infected with HEV during pregnancy, most of whom were not ill, and none of whom developed life-threatening disease, we observed no greater occurrence of adverse fetal or neonatal outcomes.

**Risk Factors for Exposure to HEV**

Socioeconomic factors, including traditionally lower caste / non-caste affiliation and lack of family land ownership, were strongly associated with increased seroprevalence of anti-HEV IgG as has been observed elsewhere. The relationship of lower status and lack of land ownership to elevated seroprevalence in Sarlahi did not appear to be explained, as might be expected, by differences in household water sources, nor by individual access to latrines, nor by ward-level access to latrines. Few women purified their drinking water in any way prior to consumption, and those who did mostly used a cloth filter to remove sediment. Soap was widely available in homes and nearly all women reported good hand hygiene.

We did not ascertain all potentially-relevant details of water sources, such as tubewell depth, that may vary with economic means and be associated with likelihood of water contamination. It is also unclear whether either individual- or ward-level lack of latrine access is an appropriate proxy for potential exposure to infectious human waste resulting from open defecation, as these variables were neither strongly nor statistically-significantly associated with HEV seropositivity. Local sanitation customs, geographic controls on water and waste runoff, or ward boundaries that do not correspond well to natural “neighborhood” boundaries may partly explain the apparent lack of association between water, sanitation, and hygiene-related variables and anti-HEV IgG serostatus in our analyses.

Many of the young women in the study were recently-married, and some may have only recently moved into the study area; thus, it is possible that local environment at the time of study
enrollment was a poor proxy for lifetime exposure. However, many of the women did reside within the study area or within nearby villages prior to marriage, and given strong economic, caste, and ethnicity-based influences on marriage customs, it is not clear that their exposures would change markedly.

Among participants who self-identified as belonging to one of the four broad Hindu varna-based (caste) groups, seroprevalence increased steadily from Brahmin (5.9%) to Chhetri (8.0%) to Vaishya (10.4%) women, before rising sharply to 17.6% among Shudra women. The bounds of the “Shudra” designation have been defined variously, with the label traditionally applying to the lower-status groups still considered “pure” under the varna system and others extending it to groups identified by tradition as “impure”, “water-unacceptable” (meaning ), or “untouchable” and now more often called “Dalit” (“oppressed”). Only four women identified themselves as belonging to groups other than those defined by the four-varna system (or religion, in the case of Islam), and we lacked caste-identification data for another 69 women, who were grouped with them in an “other/unknown category” which had the highest seroprevalence of anti-HEV IgG (24.7%). Some of the “other” groups may include Dalit communities as well as “Adivasi Janajati” (“indigenous peoples”). Janajati are those belonging to ethnic groups tied to various regions of Nepal, some of which are not predominantly Hindu, and others of which practice Hinduism but which, for historical reasons, were not as tightly bound to the formal varna system. Both Dalit and Janajati groups have historic legacies of “social exclusion and discrimination” in Nepal, and these groups remain underrepresented in government and in other positions of influence. In tandem with their disenfranchise, Dalit and Janajati communities continue to lag on a variety of indicators of economic development and health, including latrine access, antenatal care, and infant mortality. In our cohort, however, the prevalence of open defecation was higher among Shudra, Muslim, and

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Vaishya women than among women of “Other/Unknown” caste, and access to latrines appeared to show little association with HEV seropositivity, in any case.

Muslims in Sarlahi (who, as non-Hindus, also do not fit within the traditional varna system), like the Shudra and “other” groups, had a greatly-elevated seroprevalence of anti-HEV IgG (20.9%) relative to higher caste-based groups, even after adjustment for several other covariates. This stands in contrast to an observation in Balinese pregnant women, where Muslims had a lower seroprevalence of anti-HEV IgG than Hindus.48 Muslims in Bali, as in Sarlahi, are a religious minority in an area with a large, caste-stratified Hindu majority. Further investigation is needed to determine what specific socioeconomic, cultural, phylogeographic, or other factors might account for these observed differences in anti-HEV IgG seroprevalence in Nepali vs. Balinese Muslim women.

In both Indonesia and Nepal, as in India, waterborne HEV-1 has been implicated as the predominant cause of outbreaks and clinical illness.4,49-55 An earlier study in western India found that even where genotype 4 HEV was prevalent among swine, human cases from the same region were all attributable to genotype 1.56 Thus, exposure in these regions South and Southeast Asia might be expected to be less heavily dependent on dietary practices or animal exposures than in places like North America, Europe, and urban East Asia, where HEV-3 and HEV-4 are the dominant circulating genotypes and there is no sustained, local HEV-1 transmission.

In the context of direct animal-to-human foodborne transmission, Muslims and others (including some Hindu Brahmins) who eschew pork might be less frequently exposed to HEV than the general population. In Bali, HEV genotype 4 has been detected in swine,57 and foodborne transmission via pork is suggested by the authors of the Bali study as an explanation for the lower seroprevalence of HEV antibodies among Muslim women on that island.48 In Nepal, HEV antibodies
(of unknown genotype) have been detected in swine near Kathmandu, though a more recent survey detected no HEV in manure runoff at 41 small pig farms along Dhobi Kholu in the Kathmandu Valley. [Kerry Morrison et al, unpublished data] No human HEV-3 or HEV-4 cases have yet been reported in Nepal, however, and it is unclear what role, if any, zoonotic reservoirs may play in HEV transmission in South Asia.

Despite the high seroprevalence of anti-HEV IgG in Muslim women in Sarlahi, we still observed a modest (but not statistically-significant) association of HEV seropositivity with pig meat consumption, as well as a strong association of strict vegetarianism with seronegativity that persisted even after controlling for caste and other socioeconomic indicators (land ownership and literacy). It is plausible that there could be concurrent circulation of both waterborne (genotype 1) and zoonotic HEV in southern Nepal, with exposure to the latter having gone previously undetected due to limited molecular surveillance and/or lower virulence of those strains. Scant (and murky) regional data exist on potential HEV reservoirs in domesticated animals other than swine, with one report of ubiquitous but not specifically reactive antibodies in sera obtained from goats, buffalo, and even chickens in North India. HEV has, however, been documented in buffalo, cattle, sheep, and goats in other regions, as well as in milk from infected cows.

The segregation of genotypes by host species observed in India by Arankalle and colleagues would seem to suggest that separate patterns of circulation and transmission may exist among livestock and among humans there. They initially speculated that amino acid substitutions present in swine HEV-4 isolates might reduce the potential for cross-species transmission, but moderated their stance when they subsequently demonstrated that these same strains produced intermittent serum viremia and provoked an immune response in experimentally-inoculated rhesus macaques. Human HEV-4 infections have been documented elsewhere, including recently in India, so it cannot be generally assumed that South Asian swine HEV strains pose no threat of
infection to humans. The much higher seroprevalence of anti-HEV IgG in swine handlers than in other, comparable adults in Vellore, India,67 is also suggestive of zoonotic transmission within a South Asian context. The possibility of HEV transmission directly from pigs or from buffalo deserves further attention.

Another possible mediator of the association of meat-eating with seropositivity in this setting is the consumption of fish, shellfish, or snails, likely harvested from contaminated local surface waters. HEV has been detected in shellfish in coastal waters off Korea,68 China,69 Spain,70 and Italy,71 most likely as a result of untreated effluent from swine farms reaching coastal estuaries. Some bivalves may contain high concentrations of pathogens, including HEV, because they filter microbial particles from the surrounding water.71

While Nepal is a landlocked country with no coastal estuaries, the Terai is crossed by numerous streams and rivers, many seasonally-fed by meltwater from the Himalayas and by monsoonal rains, with heavy sediment burdens. A large irrigation channel diverted from the Bagmati River --also used for livestock and recreation-- traverses the study area. Although an earlier dietary survey conducted by NNIPS found that fish and snails were infrequently consumed by women in Sarlahi District on a year-round basis,72 44% of the women in our cohort reported eating fish or snails at least once in the prior month and 7% reported eating these meats roughly once a week or more (≥4 times in the past 30 days), consistent with an independent survey in 2010 that found that the vast majority of households had consumed fish in the prior year.73 In Sarlahi, where industrial-scale pig farms are rare but open defecation is common, human waste containing HEV-1 virions may contaminate the surface waters from which fish and snails are harvested. It is plausible that foodborne transmission could occur if fish and snails from these surface waters are not handled carefully and cooked thoroughly. If exposure to HEV via fish, shellfish, and snails were
common, however, one might expect to see an even higher seroprevalence of anti-HEV IgG in this population.

It is important to note, in considering the observed association of meat-eating with seropositivity, that there is a strong possibility of residual confounding by unmeasured or inadequately measured factors such as ethnicity and religious affiliation* or extended family relationships that may be associated both with dietary customs and with possible human-waste exposures related to neighborhood of residence.

**Conclusions**

Hepatitis E virus circulates and causes illness among pregnant women in Sarlahi District, Nepal. While pregnant women in Sarlahi appear to be at reduced risk of HEV relative to populations in the Kathmandu Valley, the incidence of HEV infection remains substantial and represents a threat to maternal health in southern Nepal. As rates of HEV infection and hepatitis E elsewhere in Asia, including in the Kathmandu Valley, vary seasonally and from year to year,\cite{34,55,76} we cannot be certain whether the 1.5-year snapshot captured by this study is wholly representative. Our estimates may be also affected by the exclusion of women with early pregnancy loss and by incomplete serologic and symptomatic follow-up data; the true prevalence, incidence, and impact of HEV on pregnancy in Sarlahi may be somewhat greater than our estimates suggest.

Furthermore, the risks of maternal HEV infection and illness in Sarlahi appear to be borne unequally by populations of different social and economic standing. Given the limited access to improved sanitation and water sources in this area, the links between these factors and individual

* Some “1,250 ethnic/caste and 207 religious groups” were documented during the 2011 Census in Nepal.\cite{75}
and community-level HEV exposure must be examined further. The dual (and seemingly contradictory) findings of increased anti-HEV IgG seroprevalence among both Muslim women and among non-vegetarians, especially pork and buffalo consumers, may hint at parallel HEV-1 and zoonotic HEV transmission pathways in the Nepali Terai. If this were true – and particularly if the production and persistence of antibodies varied with nature or frequency of exposure – it would have important implications for modeling population-level HEV dynamics and the occurrence of outbreaks. However, our study did not capture the full range of exposures that may lead to the higher seroprevalence of HEV antibodies among both Muslim and non-Muslim women from disadvantaged groups relative to women from traditionally higher Hindu castes. Environmental sampling of surface waters and groundwater sources and testing of commonly-encountered animals in Sarlahi (including fish and snails) could help clarify where and how exposures to HEV occur in this population and what measures might be most effective in reducing the risk of infection.

The increasing urbanization of this part of Sarlahi brings with it the unwelcome potential for wider spread of hepatitis E (e.g., through development of contamination-prone community water supply systems, greater waste runoff into multi-use irrigation channels, or increased reliance on surface water when local wells run dry due to high demand and inadequate groundwater recharge). In this population, with its apparently low immunity to hepatitis E, many women would be susceptible to infection and illness in the face of greater population exposure to HEV. Thus, although hepatitis E does not currently appear to be as grave a threat to pregnant women and their infants in northern Sarlahi as it is in Kathmandu, exposure patterns may change as Sarlahi itself changes. Ensuring that communities have access to clean water and sanitary facilities, and rectifying the disparities that currently exist, will help prevent hepatitis E (and other waterborne diseases) from becoming a greater threat to the residents of Sarlahi and other Terai districts.
ACKNOWLEDGMENTS

We are grateful to Kenrad Nelson, Alain Labrique, the MaGIFT PIs, the staff of NNIPS in Sarlahi and in Kathmandu, WARUN, and the study participants for their contributions to the study. The research was funded by Grant #50274 from the Bill and Melinda Gates Foundation.
Figure 5. Location of study area in Sarlahi District, Nepal.
Top left: Sarlahi District (shaded light yellow) in the Terai region of southern Nepal, bordering India;
Center: MaGIFT study area (shaded light yellow) in north-central Sarlahi District;
Top right: Nine participating Village Development Committees (VDCs) comprising MaGIFT study area along
East-West Highway (H1), with Nepal Nutrition Intervention Project Sarlahi (NNIPS) field headquarters in
Hariyon marked by red dot;
Bottom right: NNIPS field headquarters and eye clinic in Hariyon VDC, prior to 2012-2013 addition of student
and staff lodging above.
### Presence in Past 30 Days of...

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nausea and/or vomiting</td>
<td>1</td>
</tr>
<tr>
<td>Fever</td>
<td>1</td>
</tr>
<tr>
<td>Jaundice (yellow eyes/skin)</td>
<td>4</td>
</tr>
<tr>
<td>Dark urine and/or light-colored stool</td>
<td>2</td>
</tr>
<tr>
<td>Pain in upper right abdomen</td>
<td>1</td>
</tr>
<tr>
<td>Smell of food strange or unappealing</td>
<td>1</td>
</tr>
</tbody>
</table>

**TOTAL HEPATITIS SCORE**  (sum of score column)

If Total Hepatitis Score ≥4, case is reported by phone to auxiliary nurse-midwife at field headquarters, who schedules follow-up visit as soon as possible (within 24-48 hours).

Table 3. Case detection algorithm for acute hepatitis-like illness based on symptoms assessed at monthly follow-up visits.
Figure 6. Enrollment of study participants relative to the total female population of each village development committee (VDC) and ward.

Top: Total female population of each VDC (left) and ward (right) from 2011 Nepal Population Census. 77, 78 Center: Number of study participants enrolled by VDC (left) and ward (right). Bottom: Study participation as a proportion of the total female population of each VDC (left) and ward (right). Note: total female population includes females of all ages, regardless of pregnancy status, while study enrollment was limited to pregnant women; bottom maps thus reflect variations in both study enrollment yield and local pregnancy prevalence.
Figure 7. Weekly specimen collection by specimen type over the duration of the study period.
Specimen collection began 8 October 2012 and ended 2 May 2014. The gap in baseline specimen collection in summer 2013 was due to the transition from randomized, controlled vaccine trial (RCT) enrollment to open vaccine clinics / observational enrollment and follow-up in the main MaGIFT study. This change in protocol necessitated institutional review and approval of new consent forms and retraining of staff. The low number of specimens collected one week each October reflects a break for the holiday of Dashain. Strikes that occurred around the time of Nepal’s Constituent Assembly Election, held on 19 November 2013, also caused brief disruptions in sample collection.
### Table 4. Baseline serologic results for markers of infection with hepatitis A, B, C, and E viruses (HAV, HBV, HCV, and HEV).

### Baseline characteristics of full cohort, all women providing baseline blood samples, and women seropositive for anti-HEV IgG at baseline.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All Enrolled Women</th>
<th>All Women Tested at Baseline</th>
<th>Anti-HEV IgG+ Women at Baseline</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n =</strong></td>
<td>2363</td>
<td>1669</td>
<td>195</td>
</tr>
<tr>
<td><strong>Age (yrs)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median 22.2</td>
<td>1697</td>
<td>195</td>
<td>195</td>
</tr>
<tr>
<td>Mean 23.0 (SD 4.9)</td>
<td>1697</td>
<td>195</td>
<td>195</td>
</tr>
<tr>
<td>&lt;20</td>
<td>707 (29.9%)</td>
<td>497 (29.8%)</td>
<td>45 (23.1%)</td>
</tr>
<tr>
<td>20-24</td>
<td>931 (39.4%)</td>
<td>670 (40.1%)</td>
<td>67 (34.4%)</td>
</tr>
<tr>
<td>25-29</td>
<td>514 (21.8%)</td>
<td>358 (21.5%)</td>
<td>47 (24.1%)</td>
</tr>
<tr>
<td>30-34</td>
<td>149 (6.3%)</td>
<td>100 (6.0%)</td>
<td>26 (13.3%)</td>
</tr>
<tr>
<td>35+</td>
<td>62 (2.6%)</td>
<td>44 (2.6%)</td>
<td>10 (5.1%)</td>
</tr>
<tr>
<td><strong>Parity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primipara</td>
<td>1170 (49.5%)</td>
<td>835 (50.0%)</td>
<td>74 (37.9%)</td>
</tr>
<tr>
<td>Multipara</td>
<td>1192 (50.5%)</td>
<td>834 (50.0%)</td>
<td>121 (62.1%)</td>
</tr>
<tr>
<td><strong>VDC</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sasapur</td>
<td>172 (7.3%)</td>
<td>118 (7.1%)</td>
<td>14 (7.2%)</td>
</tr>
<tr>
<td>Hariyon</td>
<td>527 (22.3%)</td>
<td>365 (21.9%)</td>
<td>59 (30.3%)</td>
</tr>
<tr>
<td>Dhungrekholi</td>
<td>323 (13.7%)</td>
<td>230 (13.8%)</td>
<td>18 (9.2%)</td>
</tr>
<tr>
<td>Karmaiya</td>
<td>219 (9.3%)</td>
<td>154 (9.2%)</td>
<td>17 (8.7%)</td>
</tr>
<tr>
<td>Ghurkauli</td>
<td>282 (11.9%)</td>
<td>215 (12.9%)</td>
<td>22 (11.3%)</td>
</tr>
<tr>
<td>Jabdi</td>
<td>225 (9.5%)</td>
<td>174 (10.4%)</td>
<td>19 (9.7%)</td>
</tr>
<tr>
<td>Netraganj</td>
<td>240 (10.2%)</td>
<td>162 (9.7%)</td>
<td>15 (7.7%)</td>
</tr>
<tr>
<td>Lalbandi</td>
<td>231 (9.8%)</td>
<td>162 (9.7%)</td>
<td>20 (10.3%)</td>
</tr>
<tr>
<td>Raniganj</td>
<td>144 (6.1%)</td>
<td>89 (5.3%)</td>
<td>11 (5.6%)</td>
</tr>
<tr>
<td><strong>Caste</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brahmin</td>
<td>236 (10.0%)</td>
<td>170 (10.2%)</td>
<td>10 (5.1%)</td>
</tr>
<tr>
<td>Chhetri</td>
<td>298 (12.6%)</td>
<td>200 (12.0%)</td>
<td>16 (8.2%)</td>
</tr>
<tr>
<td>Vaishya</td>
<td>1296 (54.8%)</td>
<td>946 (56.7%)</td>
<td>98 (50.3%)</td>
</tr>
<tr>
<td>Shudra</td>
<td>238 (10.1%)</td>
<td>165 (9.9%)</td>
<td>29 (14.9%)</td>
</tr>
<tr>
<td>Muslim</td>
<td>174 (7.4%)</td>
<td>115 (6.9%)</td>
<td>24 (12.3%)</td>
</tr>
<tr>
<td>Other/Unknown</td>
<td>121 (5.1%)</td>
<td>73 (4.4%)</td>
<td>18 (9.2%)</td>
</tr>
<tr>
<td><strong>Main drinking water source</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubewell/Tap</td>
<td>2024 (85.7%)</td>
<td>1435 (86.0%)</td>
<td>175 (90.2%)</td>
</tr>
<tr>
<td>Protected Ring Well</td>
<td>338 (14.3%)</td>
<td>233 (14.0%)</td>
<td>19 (9.8%)</td>
</tr>
<tr>
<td><strong>No latrine (open defecation)</strong></td>
<td>1115 (47.2%)</td>
<td>785 (47.0%)</td>
<td>100 (51.3%)</td>
</tr>
<tr>
<td><strong>Washes hands before eating</strong></td>
<td>2352 (99.6%)</td>
<td>1661 (99.5%)</td>
<td>195 (100.0%)</td>
</tr>
<tr>
<td><strong>Strict vegetarian</strong></td>
<td>221 (9.4%)</td>
<td>163 (9.8%)</td>
<td>6 (3.1%)</td>
</tr>
<tr>
<td><strong>Eats meat</strong></td>
<td>2142 (90.6%)</td>
<td>1506 (90.2%)</td>
<td>189 (96.9%)</td>
</tr>
<tr>
<td><strong>Eats pig meat</strong></td>
<td>585 (24.9%)</td>
<td>403 (24.2%)</td>
<td>62 (31.8%)</td>
</tr>
<tr>
<td><strong>Eats buffalo meat</strong></td>
<td>916 (38.8%)</td>
<td>648 (38.8%)</td>
<td>98 (50.3%)</td>
</tr>
<tr>
<td><strong>Eats goat/mutton</strong></td>
<td>1989 (84.2%)</td>
<td>1387 (83.1%)</td>
<td>173 (88.7%)</td>
</tr>
<tr>
<td><strong>Eats fowl</strong></td>
<td>1968 (83.3%)</td>
<td>1380 (82.7%)</td>
<td>177 (90.8%)</td>
</tr>
<tr>
<td><strong>Eats fish/shellfish</strong></td>
<td>2012 (85.2%)</td>
<td>1413 (84.7%)</td>
<td>182 (93.3%)</td>
</tr>
<tr>
<td><strong>Stays home all day</strong></td>
<td>633 (26.8%)</td>
<td>464 (27.8%)</td>
<td>42 (21.5%)</td>
</tr>
<tr>
<td><strong>Jaundiced contact (past month)</strong></td>
<td>51 (2.2%)</td>
<td>44 (2.6%)</td>
<td>8 (4.1%)</td>
</tr>
</tbody>
</table>

Table 5. Baseline characteristics of full cohort, all women providing baseline blood samples, and women seropositive for anti-HEV IgG at baseline.

VDC = village development committee.
Figure 8. Baseline seroprevalence of anti-HEV IgG antibodies by selected exposures. Unadjusted odds ratios are shown at the top of each bar; bars labeled "OR:" represent reference categories.
Figure 9. Geographic variation in anti-HEV IgG seroprevalence and distribution of selected covariates within the study area.

Top left: Study area map showing VDCs (village development committees) and wards. Top right: distribution of participants by caste and VDC. Center: Baseline anti-HEV IgG seroprevalence by VDC (left) and ward (right). Bottom: Prevalence of open defecation (no household latrine) by VDC (left) and ward (right). There was no association at the individual, ward, or VDC level of latrine access with anti-HEV IgG seropositivity.
**Table 6. Crude and adjusted odds ratios for the association of selected covariates with baseline anti-HEV IgG seropositivity.**

Dashes indicate reference group for categorical variables. Adjusted odds ratios were derived from a 1556-observation-based logistic regression model that included all other covariates as shown in the table, with the exception of “purifies water” and “washes [hands] before eating”, which were removed due to the extremely small number of women who purified water or reported not washing hands prior to eating. In univariate analysis, water purification was associated with reduced odds of seropositivity, but this association was not statistically significant; all of the 8 women who reported not washing hands before eating were seronegative. Age (years) and land ownership (# of household-owned khattas of khet and/or bari land) were modeled as a linear variables because these fit better than categorical variables with a range of cut-points.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Unadjusted OR (95% CI)</th>
<th>Unadj. P-Value</th>
<th>Adjusted OR (95% CI)</th>
<th>Adj. P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (Yrs)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unadjusted OR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Literate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Yes</td>
<td>0.48 (0.35-0.66)</td>
<td>0.000</td>
<td>0.87 (0.59-1.28)</td>
<td>0.469</td>
</tr>
<tr>
<td><strong>Stays Home</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Yes</td>
<td>0.68 (0.48-0.98)</td>
<td>0.039</td>
<td>0.69 (0.43-1.11)</td>
<td>0.124</td>
</tr>
<tr>
<td><strong>VDC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hariyon</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Sasapur</td>
<td>0.70 (0.37-1.30)</td>
<td>0.259</td>
<td>0.87 (0.40-1.85)</td>
<td>0.708</td>
</tr>
<tr>
<td>Dhungrekhola</td>
<td>0.44 (0.25-0.77)</td>
<td>0.004</td>
<td>0.44 (0.22-0.89)</td>
<td>0.023</td>
</tr>
<tr>
<td>Karmiya</td>
<td>0.64 (0.36-1.14)</td>
<td>0.134</td>
<td>1.09 (0.54-2.17)</td>
<td>0.811</td>
</tr>
<tr>
<td>Ghurkauli</td>
<td>0.59 (0.35-1.00)</td>
<td>0.048</td>
<td>0.66 (0.36-1.23)</td>
<td>0.192</td>
</tr>
<tr>
<td>Jabdi</td>
<td>0.64 (0.37-1.10)</td>
<td>0.108</td>
<td>0.85 (0.40-1.71)</td>
<td>0.662</td>
</tr>
<tr>
<td>Netraganj</td>
<td>0.53 (0.29-0.96)</td>
<td>0.038</td>
<td>0.83 (0.40-1.71)</td>
<td>0.610</td>
</tr>
<tr>
<td>Lalbandi</td>
<td>0.73 (0.42-1.26)</td>
<td>0.259</td>
<td>1.16 (0.63-2.12)</td>
<td>0.635</td>
</tr>
<tr>
<td>Raniganj</td>
<td>0.73 (0.37-1.46)</td>
<td>0.374</td>
<td>1.26 (0.58-2.74)</td>
<td>0.558</td>
</tr>
<tr>
<td><strong>Caste</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brahmin</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Chhetri</td>
<td>1.39 (0.61-3.15)</td>
<td>0.429</td>
<td>1.38 (0.59-3.22)</td>
<td>0.452</td>
</tr>
<tr>
<td>Vaishya</td>
<td>1.85 (0.94-3.62)</td>
<td>0.073</td>
<td>1.92 (0.93-3.97)</td>
<td>0.080</td>
</tr>
<tr>
<td>Shudra</td>
<td>3.41 (1.60-7.25)</td>
<td>0.001</td>
<td>3.32 (1.43-7.68)</td>
<td>0.005</td>
</tr>
<tr>
<td>Muslim</td>
<td>4.22 (1.93-9.22)</td>
<td>0.000</td>
<td>3.10 (1.23-7.79)</td>
<td>0.016</td>
</tr>
<tr>
<td>Other/Unknown</td>
<td>5.24 (2.28-12.03)</td>
<td>0.000</td>
<td>29.96 (3.94-227.64)</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Land Owned</strong> (Khattas)</td>
<td>0.97 (0.95-0.98)</td>
<td>0.000</td>
<td>0.98 (0.96-0.99)</td>
<td>0.003</td>
</tr>
<tr>
<td>% in Ward Lacking Latrine</td>
<td>1.00 (1.00-1.10)</td>
<td>0.673</td>
<td>1.00 (0.99-1.01)</td>
<td>0.711</td>
</tr>
<tr>
<td><strong>Water Source</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubewell/Tap</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Ring Well</td>
<td>0.64 (0.39-1.05)</td>
<td>0.077</td>
<td>0.86 (0.46-1.61)</td>
<td>0.627</td>
</tr>
<tr>
<td><strong>Purifies Water</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>---</td>
<td>---</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Yes</td>
<td>0.70 (0.21-2.32)</td>
<td>0.564</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td><em>Washes Before Eating</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>---</td>
<td>---</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Yes</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td><strong>Eats Meat</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Yes</td>
<td>3.76 (1.64-8.61)</td>
<td>0.002</td>
<td>3.03 (1.19-7.73)</td>
<td>0.020</td>
</tr>
</tbody>
</table>
Figure 10. Patterns of baseline anti-HEV IgG seroprevalence by meat consumption habits and frequency in pregnant women in Sarlahi District, Nepal.

Top: Baseline anti-HEV IgG seroprevalence among vegetarians, meat eaters (generally), and eaters of specific types of meat. The number of women who reported consumption of each type of meat is listed in parentheses. Note that among meat-eaters, the categories are not mutually-exclusive (i.e., a woman who ate both fish and goat would be counted in the denominator of both of those two bars, as well as in the “any meat” category).

Bottom: Baseline seroprevalence of anti-HEV IgG by frequency of exposure to specific meats in the past 30 days. Vegetarians are included in the denominator of the zero-frequency categories. Both when grouped as shown, and when treated as linear variables, a test for trend was statistically-significant only for frequency of pig consumption.
# References


CASE REPORT: HEPATITIS E VIRUS (HEV) IN AN ACUTELY-ILL PREGNANT WOMAN IN NEPAL, A PRELIMINARY PHYLOGENETIC ANALYSIS, AND A CAUTIONARY TALE

Lisa J. Krain

ABSTRACT

Hepatitis E virus (HEV) is a major cause of morbidity and mortality in South Asia, especially among pregnant women. Few cases have been described outside of hospital settings, and little is known about the strains of HEV causing sporadic disease in Nepal, particularly outside of the Kathmandu Valley. Here we describe a case of sporadic, self-limiting hepatitis E in a late-second-trimester pregnant woman in southern Nepal. To help characterize the strains causing illness in pregnancy, we isolated hepatitis E virus (HEV) RNA at two time-points, one prior to the onset of symptoms and one during the acute symptomatic phase. Phylogenetic analysis of the isolates, based on a 291-nucleotide sequence flanking the junction of open reading frames 1, 2, and 3, placed this virus in a cluster with a genotype 1d strain from Morocco, only more distantly related to other HEV isolates from the Indian subcontinent. This solution, while intriguing, is almost certainly spurious, as genotype 1d HEV has not previously been reported outside of North Africa. Further analysis of longer, more informative regions of the genome may help clarify the evolutionary relationship of this HEV strain to others circulating in the region.
BACKGROUND

Hepatitis E virus (HEV) is a pathogen with global reach. HEV genotypes 1 and 2, associated with human disease, circulate in areas where sanitation and access to clean drinking water are inadequate. Genotypes 3 and 4 are common in swine worldwide; they are increasingly recognized as a cause of illness in industrialized countries, often transmitted via undercooked meat.

In South Asia, both sporadic cases and waterborne outbreaks caused by HEV genotype 1 are common. The first documented outbreak in Nepal, in 1973, affected ~10,000 people in the Kathmandu Valley.1 Through the 1970s and 1980s, Kathmandu Valley was hit by recurring epidemics.2,3 In 2014, an outbreak in the Terai (lowland) of Nepal gripped the city of Biratnagar for several months, sickened ~7000, and killed more than 15 people, including at least two pregnant women.4-6

Analyses of historic hepatitis E epidemics have highlighted the elevated risk of severe illness and death in pregnant women.2,7 More recently, studies in Bangladesh have demonstrated the contribution of sporadic hepatitis E to maternal morbidity and mortality in South Asia.8,9 Even in women who survive the illness, HEV is also associated with adverse pregnancy outcomes, including premature delivery and fetal loss.10-12

Despite the heavy public health and economic impact13 of hepatitis E in Nepal, little is known about the strains of HEV causing illness in the country, particularly outside of the Kathmandu Valley. The complete genome sequence of only one HEV isolate from Nepal, collected from a pregnant woman in the Kathmandu Valley in 1992, has been published to date.14

In this report, we describe a case of sporadic hepatitis E in a pregnant woman in Sarlahi District, in the Terai of Nepal, and discuss a preliminary phylogenetic analysis of the HEV strain with which she was infected.
METHODS

Study Overview

We conducted a community-based, observational cohort study of ~1700 pregnant women in nine villages along the East-West Highway in Sarlahi District, Nepal, [Figure 11] from October 2012 – April 2014. The study protocol received ethical review and approval from the Nepal Health Research Council (Reg. #92/2011), Johns Hopkins University (IRB #00002458), and the Walter Reed Army Institute of Research (WRAIR #1973).

The cohort study was conducted in conjunction with an ongoing influenza vaccine trial15 carried out under the auspices of the Nepal Nutrition Intervention Project – Sarlahi (NNIPS). Married women in the nine villages were visited monthly by field staff. Women who had missed menstrual periods were offered pregnancy tests and, if pregnant, were recruited into the study with their informed consent. Questionnaires covering household, demographic, educational, occupational, and behavioral factors were administered at the time of enrollment. Blood samples were collected from all participants at enrollment, within one week of delivery, and at three months postpartum. Pregnancy and birth outcomes were recorded by a dedicated team from NNIPS.

Identification of Acute Hepatitis Cases

Women were visited monthly during pregnancy by trained team leader-interviewers (TLIs) and asked questions about symptoms of illness. Those women meeting minimum threshold criteria for symptoms of acute hepatitis (e.g., jaundice and/or a combination of fever, nausea/vomiting, dark urine/pale stools, weakness, and dysosmia) were classified as potential cases of viral hepatitis [cf. previous chapter, Table 3] and visited by a team of auxiliary nurse-midwives (ANMs). At these visits, the ANMs inquired about symptom onset, duration, and management; drew an additional
blood sample; performed a rapid HEV IgM assay (Wantai BP Enterprise Co., Ltd., Beijing, China) in the field; and provided referral information for local hospitals.

**Laboratory Analyses**

All blood samples were processed in the field lab at NNIPS’ headquarters in Hariyon, Sarlahi District, Nepal. Sera were aliquoted and shipped in liquid nitrogen to Kathmandu, where they were tested for antibodies to hepatitis E virus and other hepatitides at the Walter Reed Research Unit – Nepal (WARUN).

WARUN attempted to isolate RNA from anti-HEV IgM-positive samples, and isolates were forwarded to the Armed Forces Research Institute of Medical Science (AFRIMS) in Bangkok, Thailand, for molecular analysis.

**Molecular and Phylogenetic Analysis**

AFRIMS amplified and sequenced a 291-nucleotide segment of HEV RNA, flanking the junction of open reading frames (ORFs) 1, 2, and 3 of the HEV genome. This region corresponds to nucleotides 5011-5301 of the Burma strain (GenBank accession # M73218).

We queried GenBank using the Sarlahi sequence data to identify highly homologous sequences. We also obtained sequences representing a variety of genotype 1 HEV isolates from Asia, as well as genotypes 2, 3, 4, and avian HEV, from the database.

To conduct phylogenetic analysis, we used CLUSTALW\textsuperscript{16} in MEGA software (v.6)\textsuperscript{17} to align the sequences. We generated phylogenetic trees using the maximum-likelihood method\textsuperscript{18} with 1000 bootstrap replicates.
CASE DESCRIPTION

*PG,* a primagravida in her mid-20s living in a village in northern Sarlahi District, was a participant in the observational cohort study. [Figure 12] Based on the self-reported date of her last menstrual period, she was estimated to be ~25 weeks pregnant at the time of enrollment. At enrollment, she reported no recent (30-day) history of fever, pain, jaundice, vomiting, or anorexia. A routine baseline blood sample was collected 2 weeks after initial enrollment.

At her first monthly symptom follow-up visit, 30 days after enrollment, *PG* reported jaundice, anorexia, and dark urine of several weeks’ duration. This triggered a visit by auxiliary nurse midwives (ANMs) one day later. With *PG’s* consent, the ANMs collected a blood sample, and they confirmed acute hepatitis E in the field via rapid anti-HEV IgM assay. They provided *PG* with information on hepatitis E and local medical facilities, as well as a printed card noting the field diagnosis of hepatitis E.

*PG* recovered fully from hepatitis E without additional complications. At her second monthly follow-up visit, 60 days after enrollment, *PG* reported no lingering symptoms of hepatitis E, though she had lost 1.7 kg of body weight during her illness. She regained the weight by the third monthly follow-up visit.

She went on to give birth to a full-term, healthy baby boy weighing 3560 g and measuring 51.3 cm in length. *PG* could not be contacted to provide a routine post-delivery blood sample, but she provided one additional scheduled blood sample three months post-partum. Both mother and infant remained healthy through the end of the study follow-up period, 6 months after delivery.

*PG* is a pseudonym used to protect the participant’s privacy.
FURTHER INVESTIGATION

Exposure History

PG’s responses to the questionnaire administered at enrollment indicated that she spent most of her time in or near her home: performing housework, tending crops, and gathering fuel for the stove. She reported no recent history of travel (within 30 days prior to enrollment) outside of her village.

The household’s primary source of water for drinking, cooking, bathing, and washing dishes and clothing was a shared tubewell, and some drinking water was stored in a communal covered container shared with other families. PG also frequently (9 times in the 30 days prior to enrollment) consumed fish, shellfish, or snails, likely sourced from local rivers. PG stated that she had not eaten any foods away from home, nor consumed prepared foods bought from the haat bazaar (market) or from street vendors, within the 14 days prior to enrollment.

Her household, like many others in the area, lacked a latrine; she practiced open defecation. PG reported regular hand-washing with soap and water following defecation and prior to handling food. No other household members, including another pregnant relative, were reported to have symptoms prior to or concurrent with PG; however, we could not collect blood from other family members to determine whether or not they may have had evidence of asymptomatic infection.

Serology

In the laboratory, all three available samples from PG (baseline at 27 weeks gestation, symptomatic case visit at 29 weeks, and 3 months postpartum) tested positive for anti-HEV IgG, while only the baseline and case visit samples had detectable levels of anti-HEV IgM. HEV RNA was isolated from both the baseline sample and from the case visit sample, collected 2 weeks apart. The
woman had antibodies to HAV IgG and HBc at enrollment, but was negative for anti-HAV IgM, HBsAg, and anti-HCV IgG and IgM. [Table 7]

**Molecular Analysis and Phylogenetics**

Preliminary molecular sequencing and phylogenetic analyses were performed by the Armed Forces Research Institute of Medical Sciences (AFRIMS) in Bangkok, Thailand.

Two 291nt sequences were isolated from samples taken two weeks apart. These two sequences, flanking the junction region of HEV open reading frames (ORFs) 1, 2, and 3, were 99.7% identical, differing only in a C-T polymorphism at position 286/291. The Sarlahi sequences were 98-99% identical to the closest matches in GenBank.

Phylogenetic analysis of these 291nt sequences performed by AFRIMS placed them in a cluster with a genotype 1d HEV isolated from Morocco in 1994. We were able to independently replicate this clustering using the 291nt sequences provided by AFRIMS, but this solution had poor bootstrap support. [Figure 13] Additional trees produced using the neighbor-joining method, restricted to HEV genotypes 1-4, and with different subsets of sequences included, also showed this clustering, though with different overall topologies.

**DISCUSSION**

We have described a sporadic, uncomplicated case of acute hepatitis E in a 2nd-trimester pregnant woman. Based on the woman’s lack of travel, the infection appears to have been acquired locally in the Terai of Nepal during the winter season. She may have been exposed by drinking untreated water, or by eating fish, shellfish, or snails from local waters contaminated with human waste. She may also have come into contact with infectious human waste in the fields near her home, where open defecation is routinely practiced. It is worth noting, however, that none of these exposures is remarkable; in the larger cohort of ~2300 pregnant women from Sarlahi District, fully
98% regularly drank untreated well water, 47% lacked access to a latrine at home, and 94% routinely consumed locally-caught fish or shellfish.

The HEV genomic sequence isolated from the woman’s serum is of genotype 1. This viral strain appears to be distinct from the HEV strain that caused an outbreak in Biratnagar, in the eastern Terai of Nepal, approximately one year later. [Phylogenetic results including the Biratnagar sequences have been omitted by agreement with Akbar et al, who provided an unpublished full-length sequence from Biratnagar for comparison.]

Preliminary phylogenetic analyses of a small segment of the HEV genome from Sarlahi suggested a closer evolutionary relationship with hepatitis E viruses isolated in Morocco and Algeria in the 1970s-1980s (sometimes referred to as subgenotype 1d) than with any of the HEV strains known to be circulating in Asia. However, in the absence of corroborating evidence, it would be imprudent to accept this conclusion. How such a geographically-divergent strain might materialize in the Terai of Nepal is unclear; the possibilities would appear to be direct or indirect import by a foreign visitor or a returning citizen. International tourism is Nepal’s primary source of income, with ~800,000 foreigners arriving annually at Tribhuvan International Airport in 2012 and in 2013, but tourism from Africa is minimal. Remittances from Nepali migrant laborers (often in the Middle East) make up the second-largest (and an increasing) share of Nepal’s gross domestic product, but a North African HEV strain would certainly be an unusual “remittance”! Dozens of Nepalis from communities in the Terai join the ~1.6 million Muslims worldwide, including some from North Africa, who go on hajj (pilgrimage) to Mecca each year, where crowded and often unsanitary conditions increase the risk of enteric disease. In any of these scenarios, however, the plausibility of someone being exposed to a North African HEV strain and importing it into Nepal appears vanishingly small, given the chain of contingencies that such an encounter would require.
Another possible explanation for this result is contamination of the sample in the laboratory. Relatively few North African isolates exist, and we are unaware of such specimens having been analyzed in AFRIMS’ Bangkok laboratory, but we cannot rule out the possibility.

Perhaps the most likely explanation for the unexpected phylogenetic result we obtained is related to the structure of the HEV genome itself in the sequenced region. The junction region of the three open reading frames is a highly-conserved region of the genome. On the one hand, this means that we would expect few differences between strains of the same genotype, even from different regions. On the other hand, it means that any (convergent?) mutations within this region may be given undue weight by a phylogenetic tree-generating algorithm. Two uncommon polymorphisms are shared by both the Sarlahi specimens and the Morocco strain: a G < A mutation at position 48/291 (also present in a Chinese hepatitis E patient from Xinjiang, GenBank accession #D11093) and a T < C mutation at position 200/291 (also present in a 2007 isolate from an acute liver failure patient in Pune, India, GenBank accession #JF443726, which is 98-99% identical to the Sarlahi specimens and the closest match to both Sarlahi specimens in GenBank). [Figure 14] These two mutations, along with limited variation in the sequenced segment overall, may explain the surprising clustering we observed.

Sequencing of HEV genomic material from PG’s remaining specimens (which were sent directly from Nepal to the United States and have not been opened since they were aliquoted) would help clarify the phylogenetic position of the Sarlahi HEV strain. Sequencing by an independent laboratory would help ensure that our results are not attributable to contamination of the Sarlahi sample with a North African strain. Obtaining better coverage of the genome will provide better resolution of the phylogeny and help guard against statistical flukes like the one probably responsible for the tree we obtained.
The pathogenesis of hepatitis E virus in pregnancy – and in general – has been challenging to unravel. Hormonal and immunological changes,\textsuperscript{26} viral replication in the placenta,\textsuperscript{27} and other pregnancy-specific factors may predispose some women to poorer outcomes, though disentangling cause and effect can be difficult.\textsuperscript{28-32} Immunogenetics,\textsuperscript{33,34} nutritional status,\textsuperscript{35} toxic exposures,\textsuperscript{36} and coinfections,\textsuperscript{37} among others, seem to influence susceptibility to HEV infection and illness. Genomic variability over time and space in circulating virus strains may also play a role, likely in interaction with these myriad host and environmental factors. Recent studies have begun looking at the association of viral genomic variability with the development and course of hepatitis E in pregnant and nonpregnant individuals,\textsuperscript{38-40} but limited geographic coverage and small sample sizes afford poor resolution and ability to detect clear signals.

This case, which occurred against a background of relatively low HEV seroprevalence and low hepatitis E incidence in the Terai of Nepal, ultimately resolved with a good outcome for both \textit{PG} and her infant. Many women who become infected with hepatitis E during pregnancy are not so fortunate. Improvements in access to clean water and sanitary facilities (and possibly vaccines) will ultimately reduce the risk of water-borne diseases like hepatitis E in developing countries. Meanwhile, efforts to characterize the diversity of hepatitis E viral strains may help shed light on the evolution, geographic transmission patterns, and pathogenesis of this formidable viral foe.

\section*{Acknowledgments}

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sincere appreciation to PG and the ~2300 other pregnant women in Sarlahi who participated in the study.
FIGURES AND TABLE

Figure 11. Location of the study area in Sarlahi District, Nepal.
Left: Location of Sarlahi District in the Terai (southern lowlands) of Nepal.
Center: Study area within Sarlahi District.
Right: Nine participating village development committees located along the East-West Highway in Sarlahi.
Figure 12. Timeline of PG’s hepatitis symptoms and serologic results relative to her pregnancy and study participation.

Abbreviations: + = positive for biomarker, - = negative for biomarker, IgG = anti-HEV immunoglobulin G, IgM = anti-HEV immunoglobulin M, RNA = HEV RNA detected by polymerase chain reaction.

Trimester was estimated from PG’s self-reported last menstrual period.
Table 7. Serologic results of viral hepatitis assays for participant PG at three time-points.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Anti-HAV IgG</th>
<th>Anti-HAV IgM</th>
<th>Anti-HBc</th>
<th>HBsAg</th>
<th>Anti-HCV IgG</th>
<th>Anti-HCV IgM</th>
<th>Anti-HEV IgG</th>
<th>Anti-HEV IgM</th>
<th>HEV RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline (~27 weeks)</td>
<td>+</td>
<td>nt</td>
<td>+</td>
<td>nt</td>
<td>–</td>
<td>nt</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Symptomatic Case Visit (~29 weeks)</td>
<td>nt</td>
<td>–</td>
<td>nt</td>
<td>–</td>
<td>nt</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3 Months Postpartum</td>
<td>nt</td>
<td>nt</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>nt</td>
<td>+</td>
<td>–</td>
<td>nt</td>
</tr>
</tbody>
</table>

PG had evidence of prior infection with HAV and HBV at baseline, but was negative for markers of acute infection with hepatitis viruses other than hepatitis E virus. Notation: + = positive test result, – = negative test result, nt=not tested, IgG = immunoglobulin G, IgM = immunoglobulin M, HAV = hepatitis A virus, HBc = hepatitis B virus core antigen, HBsAg = hepatitis B virus surface antigen, HCV = hepatitis C virus, HEV = hepatitis E virus. Serologic assays used were Wantai HAV IgG ELISA, HAV IgM ELISA, AiD HBsAg ELISA, AiD HCV ELISA, HEV IgG ELISA, and HEV IgM ELISA (Beijing Wantai BP Enterprise Co., Beijing, China). HEV RNA was detected by RT-PCR. Gestational age in weeks, estimated from PG’s self-reported last menstrual period, shown for baseline and acute symptomatic case visit specimens.
Figure 13. Preliminary phylogenetic tree of hepatitis E virus (HEV) isolated from pregnant woman in Sarlahi District, Nepal, based on 189 nucleotides at the junction of open reading frames 1, 2, and 3. Tree produced with MEGA v.6.06 software using the maximum-likelihood method. Caveat: This tree topology may be “maximally-likely” given the sequences included and the assumptions built into the algorithm, but it is also likely illusory!
Figure 14. Alignment of 291-nucleotide sequences showing uncommon variants shared by Sarlahi and Morocco hepatitis E virus (HEV) strains at positions 48 and 200.

Sequence alignment produced using ClustalW within MEGA v6.06 software.
REFERENCES


THE 2014 BIRATNAGAR, NEPAL HEPATITIS E OUTBREAK IN CONTEXT

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ABSTRACT

A large outbreak of hepatitis E occurred in Biratnagar, a metropolitan area in southern Nepal, from April-July 2014. We tested human serum samples collected from healthy adult populations in Biratnagar and nearby Dharan a few months prior to the outbreak, as well as serum samples collected during the outbreak (week of April 27 - May 3, 2014) from individuals presenting with hepatitis-like symptoms at one public hospital, one non-profit teaching hospital, and one private hospital in Biratnagar. We found a low population seroprevalence of IgG antibodies to hepatitis E virus (HEV) in healthy adults (college faculty, students, and staff) in Biratnagar (~5.6%) and Dharan (~12.3%) in January 2014, prior to the outbreak. Among 450 ambulatory patients presenting at Biratnagar hospitals during the epidemic, 42.2% (190/450) were IgG+, 14.7% (66/450) were IgM+, and 2.9% (13/450) were HEV Ag+. Seroprevalence of acute infection markers (anti-HEV IgM and HEV Ag) varied geographically and by hospital. Neither IgM nor Ag positivity was uniquely associated with the presence of RNA. HEV RNA recovered from epidemic patients was of genotype 1, but subgenotype could not be consistently determined. The outbreak underscores the need for improved access to water, sanitation, and hygiene in the Terai – where population susceptibility to HEV is high – and the need for improved epidemiologic surveillance and response.
BACKGROUND

Hepatitis E virus (HEV) is spread primarily through fecally-contaminated water and food in developing countries. The virus has an incubation period of roughly 3-6 weeks. HEV infections can be asymptomatic or subclinical, but they can also result in acute hepatitis E, with classic viral hepatitis symptoms including jaundice, vomiting, and elevated liver enzymes. Acute hepatitis E is usually self-limiting and without sequelae, with full recovery within one to two months of symptom onset. Severe hepatitis E, however, can lead to acute liver failure and death.\textsuperscript{1,2} In contrast to hepatitis A virus, which is similar to HEV in mode of transmission and clinical presentation, historical data indicate that HEV tends to cause more clinical illness in adults than in young children. Pregnant women, as well as individuals with pre-existing liver disease, are particularly susceptible to adverse outcomes and death due to hepatitis E. A 3-dose recombinant HEV vaccine has been commercially available in China since 2012, but it has not been prequalified by the World Health Organization for use elsewhere.\textsuperscript{3-6}

Nepal and India are considered endemic zones for hepatitis E virus in humans. Major epidemics in Kathmandu,\textsuperscript{7-10} Kanpur,\textsuperscript{11,12} Delhi,\textsuperscript{13,14} and other cities\textsuperscript{15,16} have sickened >150,000 people over the last century,\textsuperscript{17} and every year smaller outbreaks and sporadic cases are reported.\textsuperscript{18,19} Despite this, hepatitis E epidemics have not been well-documented in the populous Terai (lowland region) of Nepal, which borders India. In 2005-2006, in the midst of the country's Maoist uprising, outbreaks of jaundice – assumed to be hepatitis E – occurred in Birgunj, Parsa District.\textsuperscript{10,20} The April-June 2014 outbreak in Biratnagar is the first confirmed and largest known hepatitis E epidemic in the Terai to date.

Biratnagar is a city of over 200,000 people\textsuperscript{21} located in Morang District, Nepal. It is the largest city in the Terai and an important national center of commerce and education. The city
straddles the Koshi Highway, a paved north-south road that serves as a major truck route to and from India, which borders Biratnagar to the south-southwest. [Figure 15]

Development of a large-scale, piped water supply system in Biratnagar began in the 1970s, with aid from the United Nations and the World Bank. The water supply system was expanded in several phases. The Nepal Water Supply Corporation – Biratnagar Branch (NWSC-B) serves a majority of households in the city. Municipal water is pumped from deep wells to overhead tanks, minimally treated with chlorine, and supplied to the population for ~12 hours/day on a schedule.

The Mangadh Water Supply and Sanitation Users Committee, a community-run organization, supplies treated water to wards in northern Biratnagar and environs. Many residents also use shallower (20-30m deep) local tubewells to supplement the municipal supply.

Biratnagar still lacks a system of closed sewers and a wastewater treatment facility, and many residents still do not have access to sanitary latrines. Numerous organizations have worked to improve sanitation in the city via latrine construction in poorer areas, hygiene education, and even local filming of an anti-open-defecation television comedy, "Sugandhapur" ("Fragrant Village"). However, major municipal and regional infrastructure development projects have been hindered by financial and political conditions. Major water supply pipes dating to the first wave of development in the 1970s have not aged well. Many of these deteriorating water supply pipes lie within open waste water channels. Surveys conducted between 2008 and 2012 found microbial contamination, including fecal coliform bacteria, in the majority of tapwater samples. Wastewater runoff from the city contaminates surface water supplies and shallow tubewells. In November 2013, the Asian Development Bank awarded a US$24 million contract for the long-awaited construction of sewerage and storm water drainage systems and a wastewater treatment plant in Biratnagar.
Against this backdrop, in mid-April 2014, people with jaundice began appearing in increasing numbers at Biratnagar health facilities, and in late April the epidemic began receiving media and government attention. Hospitals were deluged with hepatitis patients and lacked the beds and supplies needed to attend to all of the ill. The radius of reported cases grew to encompass areas outside the municipality, the district prison in Biratnagar was affected and schools were closed. Cases peaked by early May and diminished considerably by the arrival of the monsoon season in late June. [Figure 16] A total of 2466 hepatitis cases and 12 deaths were officially recorded, but as many as ~7000 may have been ill. At least two pregnant women, both from outlying villages, were reported to have died from hepatitis E during the outbreak, contrary to earlier reports.

We undertook this study to better characterize the 2014 Biratnagar hepatitis E epidemic in terms of prior population susceptibility, burden and geographic distribution of cases presenting at area hospitals, correspondence between different markers of infection, and molecular characteristics of the virus.

**METHODS**

The research was approved by the Nepal Health Research Council (Reg. No. 153/2014) and the Johns Hopkins School of Public Health Institutional Review Board (IRB#00005879).

**Study Populations**

*Pre-outbreak samples.* As part of an independent study conducted to establish standard biochemical values for lab tests in a healthy Nepali population (NHRC Reg. No. 57/2014), serum samples were collected in January 2014 from 110 consenting adults from three sites in Biratnagar, Morang District, and from 88 adults in Dharan, Sunsari District (pop. ~120,000). De-identified
sera, along with basic demographic data (age and sex) and results of liver function tests (LFTs), were released to the authors for use in this study.

**Epidemic-period samples.** Serum samples were collected during the outbreak (week of April 27 - May 3, 2014) from one government hospital (Koshi Zonal Hospital), one non-profit teaching hospital (Nobel Medical College Teaching Hospital), and one private hospital (Biratnagar Aspataal, Pvt.) in Biratnagar. Participants were selected sequentially from among ambulatory patients referred for liver function tests by hospital clinicians. Consenting patients’ sera were collected from the hospital laboratories following completion of LFTs. LFT results, symptom duration, and demographic data (age, sex, neighborhood) were abstracted from medical records and indexed by specimen number to protect participants’ privacy.

**Sample Storage and Shipment**

Samples collected during the epidemic were stored in hospital laboratory freezers after completion of liver function testing. The samples were transported on ice from Biratnagar to the Central Department of Biotechnology of Tribhuvan University, Kathmandu, Nepal, where they were stored frozen at 0°C. Pre-epidemic samples were transported from Biratnagar and Dharan to Kathmandu on ice and frozen at 0°C after biochemical evaluation. Both sets of samples were shipped on dry ice to the Johns Hopkins Bloomberg School of Public Health in Baltimore, Maryland, USA, and stored at 0°C until processed.

**Serologic Testing**

We tested 450 epidemic-period specimens and 180 pre-epidemic specimens using highly sensitive and specific anti-HEV IgG, anti-HEV IgM, and HEV Ag assay kits (Wantai Biological Pharmacy Enterprise, Beijing, China). The assays were carried out according to manufacturer instructions, except that we substituted two each of 1:16 and 1:32 dilutions of the manufacturer-
provided positive control for the undiluted positive control, and we did not repeat the tests. Plates were processed using an automated ELx50 plate washer (BioTek, Winooski, Vermont, USA) and optical densities were read using a VERSAmax ELISA microplate reader with SoftMax software (Molecular Devices, Sunnyvale, California, USA).

Molecular and Phylogenetic Analyses

A subset of 27 epidemic-period specimens with positive antigen tests and/or high anti-HEV IgM titers, as well as two IgM-positive pre-epidemic specimens, were selected for molecular analysis.

Viral nucleic acids were purified from 200µL of serum plus internal-control bacteriophage MS2\textsuperscript{47,48} using the MagNA Pure LC 2.0 robotic instrument with the MagNA Pure LC Total Nucleic Acid Kit - High Performance (Roche Diagnostics, Indianapolis, Indiana, USA). HEV RNA was then amplified from total nucleic acid eluates using qRT-PCR as well as RT- and nested PCR protocols. We amplified segments of ORF1 using two sets of primers (as described by Wang et al.\textsuperscript{49} and Dong et al.\textsuperscript{50}), and we used two sets of primers to amplify segments of ORF2 (as described by Wang et al.\textsuperscript{49} and John R. Ticehurst [pers. comm.]).

Amplicons were sent to the Johns Hopkins Genetic Resources Core Facility (GRCF), where they were sequenced on an Applied Biosystems 3730 DNA Analyzer (Life Technologies Corp., Grand Island, NY, USA) using the Sanger method.\textsuperscript{50,51} Contiguous sequences from ORF1 and ORF2 of the HEV genome were assembled from sequence fragments returned by the GRCF using CodonCode Aligner (CodonCode Corp., Centerville, MA, USA).

BLASTN v.2.3.0\textsuperscript{52} was used to identify highly homologous sequences to the Biratnagar epidemic strains in GenBank. We also searched GenBank using keywords to locate and obtain sequence data for other Nepali and South Asian HEV isolates of HEV, as well as more distant HEV
isolates, including representatives of the four genotypes known to affect humans. We used MEGA v.6 software\textsuperscript{53} to align the sequences and to carry out phylogenetic analyses using the maximum-likelihood method.

**Data Analysis**

We produced descriptive statistics using age, sex, geographic information, and laboratory values for liver function tests extracted from participant records, as well as results of our serologic and molecular analyses. Demographic, laboratory, and serologic results were analyzed using Stata 11 (StataCorp LP, College Station, TX). Geographic locations for pre-epidemic participants were limited to city name (Biratnagar or Dharan). Locations within Biratnagar for epidemic-period participants were given as neighborhood or street/intersection names in patient records. Patients from each neighborhood were randomly assigned to locations within polygons approximating the neighborhood boundaries, both due to the low resolution of residence data and also to preserve participant confidentiality. The resulting geographic coordinates were mapped using ArcMap 10.3 (ESRI, Redlands, CA) for illustrative purposes.

**RESULTS**

Demographic and serologic results for both the pre-epidemic healthy adults and the outbreak patients are summarized in Table 8. Comparisons of liver enzyme levels by serostatus are summarized in Table 9.

**Pre-Epidemic Healthy Adults**

Among healthy adults sampled in January 2014, the seroprevalence of anti-HEV IgG was 5.6% (6/107) in Biratnagar and 12.3% (9/73) in Dharan. The six IgG+ adults in Biratnagar were substantially older, on average, than the IgG- population, while in Dharan, the age distributions of
IgG+ and IgG- populations were similar. [Figure 17] There were two IgM+ specimens obtained from healthy adults in Dharan (2.7%); both were anti-HEV IgG+ but HEV Ag-.

**Biratnagar Outbreak Patients**

Among the 450 patients seen at three Biratnagar hospitals during the week of April 27-May 3: 190 (42.2%) were seropositive for anti-HEV IgG, 66 (14.7%) were seropositive for anti-HEV IgM, and 13 (2.9%) were seropositive for HEV Ag. A total of 72 (16%) patients were seropositive for at least one marker of acute infection (IgM and/or Ag). All but four of these 72 specimens were seropositive for anti-HEV IgG; these four were Ag+ and IgM-. Only about half (7, or 54%) of the 13 HEV Ag+ individuals also had detectable anti-HEV IgM. [Figure 18]

**Demographics.** The age distribution of outbreak patients presenting to the three hospitals was similar to that of prior hepatitis E outbreaks in South Asia, with the largest numbers of patients in the 11-40 year age groups, and relatively fewer young children or older adults. [Figure 19] The seroprevalence of HEV infection markers among the patients tested did not vary markedly by age stratum. IgG antibodies were present in 42.2% of outbreak patients overall (190/450), peaking in the 21-30 year old age group, and lowest among patients above age 60. IgM antibodies were detected in 14.2% of patients (64/450) and HEV Ag was detected in 2.7% of patients (12/450) overall. More male (54.4%) than female (45.6%) patients were seen at the three hospitals, though the sex ratio varied markedly by hospital.[ Table 9]

All serologic markers of HEV infection were slightly more common among female patients (IgG: 42.6%, IgM: 16.7%, Ag: 2.9%) than among male patients (IgG: 41.9%, IgM: 13.0%, Ag: 2.8%). Neither these differences in seroprevalence by sex, nor in seroprevalence by sex when stratified by age group, were statistically-significant by Pearson's $\chi^2$ (IgG, IgM) and Fisher’s exact test (Ag).
**Geography.** Of the 450 outbreak patients, 405 provided the neighborhood or street name of their residence in Biratnagar. The other 45 (all at Koshi Zonal Hospital) were simply recorded as medical staff or staff relatives. We were unable to uniquely match residential addresses to city wards because some of the streets and neighborhoods lie along or overlap ward boundaries. Confirmed acute (anti-HEV IgM+ and/or HEV Ag+) cases were concentrated primarily in the densely-populated areas along and east of the Koshi Highway and north of the Devkota water tank. [Figure 20] In addition to the higher absolute numbers of cases in this northeast quadrant of the city, the seroprevalence of acute markers among patients from these areas was also elevated relative to other areas of the city. Seropositivity for HEV markers varied considerably by hospital, [Table 10] and the three hospitals’ patient populations came from different residential areas. At Koshi Zonal Hospital (n=153), where there were no IgM+ or Ag+ specimens, only 13.1% of patients reported living in the northeast quadrant of the city, while 57% of patients came from the less-densely-populated western and southern parts of the municipality, and another 29.4% were medical staff or staff relatives whose home address was unknown. In contrast, the northeast quadrant of the city was home to 63.5% of patients at Biratnagar Aspataal and 89.7% of patients at Nobel Medical College Teaching Hospital, where acute HEV markers were found in 16.2% (12/74) and 26.9% (60/223) of patients, respectively.

**Clinical and biochemical measures.** Outbreak patients reported median symptom duration of 4 days (range 1 – 9+ days); this did not vary by serostatus. All measured liver enzymes were significantly elevated in outbreak patients as a group relative to the healthy adult reference population, regardless of serostatus. [Table 9] There were no significant differences in liver enzyme levels between seronegative outbreak patients and outbreak patients seropositive only for anti-HEV IgG, with seronegative patients actually having slightly higher values. Outbreak patients with acute HEV infection markers (anti-HEV IgM and/or HEV Ag) had slightly higher values than
outbreak patients who were IgG+ only or seronegative for all markers, but only SGOT was statistically significantly higher.

**Molecular and Phylogenetic Characterization of HEV Isolates**

No HEV RNA was detected in two IgM+ pre-epidemic samples from Dharan. Of 27 epidemic specimens with HEV antigen and/or high anti-HEV IgM titers, 12 had detectable HEV RNA. Five of these were Ag+ specimens. Interestingly, all 12 of the RNA-positive specimens were also seropositive for anti-HEV IgG. [Table 10] Eight of the 12 specimens had sufficient RNA to attempt to isolate material for sequencing, of which three yielded expected products with at least one ORF1 primer and six yielded expected products with at least one ORF2 primer.

We were able to assemble a 530 nucleotide (nt) ORF1 sequence for three samples and a 191nt ORF2 sequence for six samples, corresponding to nt 80-609 and 6369-6559, respectively, of the Burma (B1) strain of HEV (GenBank Accession #M73218). Over the length of the 530nt ORF1 segment, the three Biratnagar isolates were 99% identical to each other, but shared <95% identity with the closest matches in GenBank. The shorter ORF2 sequence, 100% identical among the six Biratnagar isolates, was 96% identical to the closest match in the database.

Phylogenetic analysis of the 540nt ORF1 sequence [Figure 21] suggested that the Biratnagar HEV isolates share a common ancestor with HEV strains from India, China, and Pakistan identified elsewhere as genotypes 1b and 1c. However, phylogenetic analysis of the 191nt ORF2 sequence placed the Biratnagar HEV isolates in a cluster with HEV strains identified in other publications as subgenotype 1a, including the Burma strain and a strain isolated during an outbreak in Madras, India (GenBank accession #X99441), while still placing the previously-identified 1b and 1c strains as outgroups to the 1a strains, albeit with low bootstrap support. [Figure 22]
DISCUSSION

The low (5.6%) seroprevalence of anti-HEV antibodies in an adult reference population in Biratnagar in January 2014 stands in marked contrast to the higher seroprevalence typically observed in adults in Kathmandu (24.6%-66%)56-58 and elsewhere in South Asia (33.7% in pregnant women in New Delhi,59 ~ 25-40% in Pune,60 29.5% and 14.9% in urban and rural Vellore, and 22.5% in rural Bangladesh61); however, serosurveys of other Terai populations have also yielded lower –than-anticipated seroprevalence (12.3% in adults in Dharan, and 11.7% in pregnant women in Sarlahi District [cf cohort study chapter]). Assuming that the reference population is representative of adults in Biratnagar, the low seroprevalence suggests relatively low exposure to HEV in Biratnagar prior to the outbreak, and corresponding limited immunity and high susceptibility to hepatitis E.

Among 450 ambulatory, symptomatic patients seen at three Biratnagar hospitals during the week of April 27-May 3, we detected anti-HEV IgG in 42.2%, IgM in 14.7%, and HEV Ag in 2.9%, far lower than the 94%+ seroprevalence of the same three HEV markers detected in 48 patients at Koshi Zonal Hospital by Shrestha et al.10 It is likely that this reflects differences in selection criteria; our patient population was ambulatory, and we did not limit our sample to individuals with frank jaundice.

The lack of precise residence information for each case precluded formal geospatial analysis. Among confirmed acute cases (IgM+ and/or Ag+), 86% came from neighborhoods in the northeast quadrant of the city, with the remainder from a single neighborhood extending west a few blocks from the Koshi Highway. In our sample, acute cases were most concentrated in the vicinity of the Tinpaini water tank, which failed to meet water quality standards in the midst of the outbreak.62 However, as data on individuals’ water sources at home or at their workplaces were unavailable, we cannot conclusively link the outbreak to this water tank. Furthermore, during the
outbreak, the water tank at Devkota Chowk was also reported to have dispensed contaminated water. Our sample included few patients and no confirmed acute cases from areas south of Devkota Chowk, including Ward 11, but news media reported cases in the prison, as well as several deaths from hepatitis E, in that area.

The outbreak patients, including those seronegative for all markers of HEV, had elevated liver enzyme levels relative to the healthy reference population. Our failure to detect markers of HEV infection in many of these patients with elevated liver enzymes may reflect natural differences in the timing or quantity of appearance of anti-HEV antibodies across individuals (i.e., they may have been infected, but without having mounted a detectable immune response and without circulating HEV antigen), or it may reflect the presence of other hepatotrophic pathogens. Some news reports claimed that hepatitis A was co-circulating in Biratnagar, but we were unable to test for other hepatitides. We also cannot rule out dengue, malaria, or leptospirosis as possible causes of illness in some patients, but these are unlikely to have been major causes of illness during the hot, dry season.

Our phylogenetic analysis of a 191nt portion of HEV ORF2 was broadly consistent with the tree published by Shrestha et al., which identified the Biratnagar outbreak strains as subgenotype 1a on the basis of a non-overlapping 412nt ORF2 segment. However, our phylogenetic analysis of a 540nt segment of HEV ORF1 yielded a different tree topology, one which suggests that the Biratnagar outbreak strain is more closely related to strains from China, India, and Pakistan (classified elsewhere as subgenotypes 1b and 1c) than to the Burma strain. Cases of HEV in Nepal have historically been attributed to subgenotypes 1a and 1c, and 1a-1c coinfections have been identified. Whole-genome molecular analysis of the Biratnagar outbreak strain could help clarify whether our results reflect a natural recombination event between different HEV strains (as has
been inferred in other cases\textsuperscript{69-72}, distinct evolutionary trajectories of different portions of the genome, or simply poor resolution of tree topology at the subgenotype level.\textsuperscript{73}

The hepatitis E outbreak in Biratnagar, which sickened thousands and killed more than a dozen people, resulted from widespread exposure to fecally-contaminated water. The epidemic curve suggests a relatively abrupt population exposure to HEV in Biratnagar, which the head of Nepal’s Epidemic and Disease Control Division (EDCD) attributed to rupture of a waste water pipe during excavations for a road widening project\textsuperscript{74,75}

In the wake of the 2014 outbreak, the Nepali government pledged millions of rupees (NR) to fund replacement of the water supply pipes in Biratnagar. Half a year later, however, Biratnagar residents’ concerns about the safety of the drinking water supply remained unallayed.\textsuperscript{76} The large March 2015 earthquakes in Nepal, along with political unrest, strikes, and months-long border blockades in the Terai following the drafting of Nepal’s new Constitution further delayed progress toward ensuring access to safe, clean drinking water in Biratnagar.

More recent samples collected from people in Biratnagar and Dharan may help establish how the population seroprevalence of antibodies to HEV has changed in the wake of Biratnagar’s hepatitis E outbreak. Human health surveillance, water-quality monitoring, and infrastructure improvement and maintenance are ongoing needs made more challenging by the same economic and political realities that also make them more urgent. We echo the calls made by EDCD head Dr. Baburam Marasini\textsuperscript{77} and others\textsuperscript{78,79} for improved cross-sector coordination of epidemiologic prevention activities, surveillance, and response in Nepal.

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We thank Manish Das (Nobel Medical College Teaching Hospital), contacts at Koshi Zonal Hospital and Biratnagar Aspataal Pvt., and Ram Vinod Mahato (Institute of Science and Technology,
Tribhuvan University / Nepalese Association for Clinical Chemistry) for providing access to serum specimens. This study was funded through a grant from the Bill and Melinda Gates Foundation.
Figure 15. Map of Nepal with inset showing locations of Biratnagar (Morang District) and Dharan (Sunsari District) along the Koshi Highway (H8) in southeastern Nepal.

Base imagery from NASA satellite image VE-IMG-4753.
Figure 16. Epidemiologic curve showing course of 2014 Biratnagar hepatitis E outbreak. Curve graphed from data published by Shrestha et al., *EID*, 2015.
### Table 8. Demographic and Serologic Characteristics of Pre-Outbreak (Healthy Adults) and Outbreak Population Sample.

<table>
<thead>
<tr>
<th></th>
<th>Pre-Outbreak Healthy Adults (January 2014)</th>
<th>Biratnagar Outbreak Patients (April 27 - May 4, 2014)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Biratnagar</td>
<td>Dharan</td>
</tr>
<tr>
<td><strong>Total n</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Male</strong></td>
<td>62 (57.9%)</td>
<td>41 (56.2%)</td>
</tr>
<tr>
<td><strong>Female</strong></td>
<td>45 (42.1%)</td>
<td>32 (43.8%)</td>
</tr>
<tr>
<td><strong>Age: Years mean (sd)</strong></td>
<td>35.8 (15.1)</td>
<td>35.1 (13.4)</td>
</tr>
<tr>
<td>0-10</td>
<td>25 (23.4%)</td>
<td>13 (17.8%)</td>
</tr>
<tr>
<td>11-20</td>
<td>23 (21.5%)</td>
<td>20 (27.4%)</td>
</tr>
<tr>
<td>21-30</td>
<td>15 (14.0%)</td>
<td>14 (19.2%)</td>
</tr>
<tr>
<td>31-40</td>
<td>23 (21.5%)</td>
<td>16 (21.9%)</td>
</tr>
<tr>
<td>41-50</td>
<td>14 (13.1%)</td>
<td>7 (9.6%)</td>
</tr>
<tr>
<td>&gt;60</td>
<td>7 (6.5%)</td>
<td>3 (4.1%)</td>
</tr>
<tr>
<td>IgG+</td>
<td>6 (5.6%)</td>
<td>9 (12.3%)</td>
</tr>
<tr>
<td>IgM+</td>
<td>0</td>
<td>2 (2.7%)</td>
</tr>
<tr>
<td>Ag+</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Abbreviations: Nobel MCT Hospital – Nobel Medical College Teaching Hospital, n – number, sd – standard deviation, IgG+ – anti-HEV immunoglobulin G positive, IgM+ – anti-HEV immunoglobulin M positive, Ag+ – HEV antigen positive. Wantai HEV IgG, IgM, and Ag assays were used (Beijing Wantai BP Enterprise Co., Beijing, China).
Figure 17. Boxplots showing age distribution of pre-epidemic healthy adults by city and anti-HEV IgG serostatus.

Box shows median and interquartile range; extended lines show full range. The mean ages of IgG+ healthy adults in Biratnagar (52.7 years) vs. IgG- adults in Biratnagar (34.8 years) differed statistically significantly by the Wilcoxon rank-sum test (p=0.01). The mean ages of IgG+ and IgG- healthy adults in Dharan (31.3 years and 35.6 years) did not statistically significantly differ (p=0.31).
Figure 18. Venn diagram of Biratnagar epidemic patients who were seropositive for at least one serologic marker of past or acute infection with hepatitis E virus (HEV). Abbreviations: anti-HEV immunoglobulin G (IgG), anti-HEV immunoglobulin M (IgM), and HEV antigen (Ag). Ellipses generated with eulerAPE software (http://www.eulerdiagrams.org/eulerAPE/).
Figure 19. Age distribution of Biratnagar outbreak patients and age-specific seroprevalence of hepatitis E virus (HEV) infection biomarkers.

Bar heights indicate absolute numbers of specimens; seroprevalence of each biomarker in each age group is indicated as a percent above the bar. Wantai HEV IgG, IgM, and Antigen Assay Kits (Wantai Biologic Pharmacy Enterprise, Beijing, China) were used for serologic testing. Anti-HEV IgG seroprevalence was highest in the 21-30 year old age group (53%) and lowest in patients above age 60 (33%), but the seroprevalence was not statistically-significantly different across age groups by Pearson's. There was no clear age-related pattern of anti-HEV IgM seroprevalence, which ranged from a low of 8% among 41-50 year olds to a high of 22% among 21-30 year olds. HEV antigen was detected in ≤6% of patients in each age group. Abbreviations: HEV = hepatitis E virus; IgG = anti-HEV immunoglobulin G; IgM = anti-HEV immunoglobulin M; Ag = HEV antigen.
Figure 20. Map of Biratnagar, Morang District, Nepal, showing geographic distribution and hepatitis E serostatus of 405 outbreak patients from three hospitals.

Dots represent individual participants and positive serologic test results (anti-HEV IgG, anti-HEV IgM, and/or HEV Ag), geocoded and randomly distributed within neighborhood boundaries to protect participant confidentiality. Individuals seropositive for multiple biomarkers are represented once for each positive test. Excluded were 45 participants who gave no residential address (staff and staff relatives from Koshi Zonal Hospital); seroprevalence of IgG was 35.6% in this group and all were IgM- and Ag-. Numbered divisions are city wards. Red stars represent hospitals in this study (NMCTH= Nobel Medical College Teaching Hospital; BA = Biratnagar Aspataal, Pvt.; KZH = Koshi Zonal Hospital). Blue rectangles represent major water supply tanks. Increasing population density is represented by darker shading [pop. density map layer courtesy of GENESIS Consultancy Ltd.].
<table>
<thead>
<tr>
<th>Serostatus</th>
<th>Healthy Adults</th>
<th>Outbreak Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>180</td>
<td>378</td>
</tr>
<tr>
<td>Male</td>
<td>103 (57.2%)</td>
<td>211 (55.8%)</td>
</tr>
<tr>
<td>Female</td>
<td>77 (42.8%)</td>
<td>167 (44.2%)</td>
</tr>
<tr>
<td>Age, Years</td>
<td>35.5 (14.4)</td>
<td>30.7 (15.8)</td>
</tr>
<tr>
<td>Symptom Duration, Days</td>
<td>n/a</td>
<td>4</td>
</tr>
<tr>
<td>Liver Function Tests</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bilirubin (Total)</td>
<td>0.6 (0.3)</td>
<td>1.8 (1.3)</td>
</tr>
<tr>
<td>Bilirubin (Direct)</td>
<td>0.3 (0.2)</td>
<td>0.7 (0.7)</td>
</tr>
<tr>
<td>Aspartate Transaminase</td>
<td>29.9 (17.0)</td>
<td>49.6 (27.4)</td>
</tr>
<tr>
<td>(AST / SGOT)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine Transaminase</td>
<td>20.7 (12.2)</td>
<td>53.0 (33.1)</td>
</tr>
<tr>
<td>(ALT / SGPT)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkaline Phosphatase</td>
<td>89.8 (30.6)</td>
<td>261.7 (126.1)</td>
</tr>
<tr>
<td>(ALP)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 9. Clinical and biochemical comparison of healthy adults with outbreak patients by hepatitis E virus (HEV) serostatus.
Values for age and liver function tests are given as mean (standard deviation). Symptom duration is given as median because durations of 9 days or more were not recorded separately.
<table>
<thead>
<tr>
<th>Specimen</th>
<th>Age</th>
<th>Sex</th>
<th>Anti-HEV IgG</th>
<th>Anti-HEV IgM</th>
<th>HEV Antigen</th>
<th>ORF1 RNA</th>
<th>ORF2 RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>26</td>
<td>F</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>B</td>
<td>20</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>C</td>
<td>53</td>
<td>F</td>
<td>+</td>
<td>weak+</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>F</td>
<td>+</td>
<td>+</td>
<td>weak+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>30</td>
<td>F</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E</td>
<td>16</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>M</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>16</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>G</td>
<td>39</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>H</td>
<td>17</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 10. Demographic, serologic, and molecular characteristics of outbreak specimens positive for HEV RNA by RT-PCR.

Key: + = positive result; – = negative result.

Specimens assigned letter identifiers were those with sufficient material for nested PCR.
Figure 21. Phylogenetic tree of 540-nt partial hepatitis E virus (HEV) open reading frame 1 (ORF1) sequences.

Genotype/subgenotypes following Lu et al., are indicated at right; see comments in text. Biratnagar outbreak strains cluster within genotype 1, along with (but outside of) subgenotypes 1b and 1c. Nucleotides used in this analysis correspond to nt80-609 of the Burma reference strain (GenBank accession number M73218), including portions of the 5' untranslated region and the methyltransferase gene. Label format is GenBank accession number | country where specimen was obtained | strain designation (if available) | original host species. Bootstrap values from 1000 replicates shown at nodes. Consensus tree generated using maximum likelihood method in MEGA v.6.06 software.
Figure 22. Phylogenetic tree of 191-nt partial hepatitis E virus (HEV) open reading frame 2 (ORF2) sequences.
Nucleotides used in this analysis correspond to nt6369-6559 of the Burma reference strain (GenBank accession number M73218). Genotype/subgenotypes following Lu et al. \(^5\) are indicated at right; see comments in text. Label format is GenBank accession number | country where specimen was obtained | strain designation (if available) | original host species. Bootstrap values from 1000 replicates shown at nodes. Consensus tree generated using maximum likelihood method in MEGA v.6.06 software. \(^5\)
REFERENCES


Akbar SMF. (2016) Comparing HEV outbreaks in Japan, Bangladesh, and Nepal in US – Japan Cooperative Medical Sciences Program 18th International Conference on Emerging Infectious Diseases (EID), Hepatitis Panel.)
DISCUSSION

SUMMARY OF ORIGINAL RESEARCH

The research presented in this dissertation has focused broadly on the problem of hepatitis E in the Terai region of Nepal.

Hepatitis E Virus in Pregnant Women in Sarlahi District, Nepal

This manuscript detailed a community-based, observational, prospective cohort study of pregnant and postpartum women in Sarlahi District, Nepal, conducted from October 2012 to May 2014. The study was situated within an ongoing randomized, clinical trial, taking advantage of established research infrastructure to carry out the work in communities relatively enthusiastic about and accustomed to participation in health research studies.

The study enrolled a total of 2363 pregnant women and followed them through three months postpartum or the end of the study period. These pregnant women collectively contributed 8716 symptom surveys at roughly monthly intervals over the course of the study. Of the 2363 participants, 1669 also provided baseline serologic samples at enrollment, 811 provided samples shortly after delivering, and 659 provided samples three months after the end of their pregnancies. These samples were tested for markers of infection with hepatitis viruses and used to identify seroconverters.

At baseline, 11.7% of the participants were seropositive for anti-HEV IgG. Greater age, lower caste and land ownership, and meat consumption were associated with seropositivity, while primary drinking water source and latrine access were not. We identified 20 seroconverters over the study period, 9 of whom seroconverted between enrollment and delivery, yielding an incidence of roughly 32.3/1000 person-years during pregnancy and 44.3/1000 person-years from enrollment
through 3 months postpartum. We also observed the phenomenon of seroreversion, described in other populations, in several participants, including all 5 of the women who seroconverted during pregnancy and provided a 3 month postpartum blood sample.

During follow-up, we identified 15 cases of hepatitis-like illness based on a symptom score, one of which (discussed in the following chapter) was confirmed to be hepatitis E. The remainder could not be definitively diagnosed as viral hepatitis. There were 2 maternal deaths, one of which was determined by retrospective verbal autopsy to involve jaundice, but we could not confirm hepatitis E and some features of the course of the woman’s illness suggest another cause is more likely.

We did not detect disproportionately poor birth outcomes among seroconverters or in the symptomatic case. This finding, however, is qualified by the fact that many women with early or mid-pregnancy losses were not formally enrolled into the parent influenza vaccine trial. Thus, women with adverse outcomes that could potentially have been associated with HEV infection were systematically excluded. This, and the relatively low number of infections we observed, prevents us from drawing robust and valid conclusions about broader associations of HEV infection with adverse pregnancy outcomes.

**Case Report: Hepatitis E Virus (HEV) in an Acutely-Ill Pregnant Woman in Nepal**

Although in most ways an unremarkable case of uncomplicated acute hepatitis E, in context, this case was nonetheless notable for several reasons:

- It was the *only* case of acute hepatitis E detected in our cohort of ~2000 Nepali women followed during pregnancy and postpartum.
• Blood samples provided by the woman two weeks apart were both seropositive for anti-HEV IgG and IgM and viremic, allowing for real-time hepatitis E diagnosis in the field by auxiliary nurse-midwives using a rapid IgM ELISA, and for later extraction of viral RNA.

• A collaborating laboratory provided a phylogenetic tree indicating that the virus isolated from this Nepali woman was infected with HEV of a subgenotype (1d) previous known only from North Africa. While this tree was independently replicable and the isolate does represent genotype 1 HEV, we found that reliance on a short sequence from a conserved segment of the genome likely yielded misleading subgenotype and phylogenetic information.

• The course of this woman's illness and its ultimately positive outcome provide an anecdotal counterpoint to the hospital-based, case-based literature, which is dominated by the most severe cases – especially in areas of South Asia where access to care is beyond the reach of much of the population. In communities across South Asia and the developing world, most hepatitis E goes undiagnosed, but the burden of these milder hepatitis E illnesses is not without impact on people's lives. Although many people who develop hepatitis E are not as fortunate as our study participant and her young son, this case serves as a reminder that the broader hepatitis E story is still evolving in often-unexpected ways.

The 2014 Biratnagar, Nepal Hepatitis E Outbreak

In April 2014, a hepatitis E epidemic affecting thousands of people struck the city of Biratnagar, along the Indian border in Morang District, Nepal. We collaborated with a Nepali virologist to obtain serum samples and medical record information for 450 patients presenting at three Biratnagar hospitals (one teaching hospital, one private hospital, and one government hospital) and referred for liver function tests during the outbreak. We also obtained 180 samples from healthy adults collected in January 2014 from Biratnagar and the nearby city of Dharan as part
of a separate study to establish reference values for clinical biomarkers. We tested the samples for anti-HEV IgM and IgG and for HEV Ag. Using RT-PCR, we attempted to extract viral RNA from samples positive for HEV Ag or high-titer IgM. We used primers covering two different segments of the hepatitis E virus genome to extract nucleic acids and performed phylogenetic analysis on sequences obtained from these samples.

We found a seroprevalence in healthy adults of 5.6% in Biratnagar and 12.3% in Dharan prior to the outbreak. Among outbreak patients, 2.2% (190/450) were IgG+, 14.7% (66/450) were IgM+, and 2.9% (13/450) were HEV Ag+. Seropositivity varied by hospital and by patient neighborhood of origin, but did not vary tremendously by age or by sex. However, the absolute number of patients mirrored frequently-reported patterns during hepatitis E outbreaks, with a greater number of cases being seen in young-to-middle-aged adults and relatively fewer in very young or very old populations. Liver function tests were generally elevated in outbreak patients relative to the healthy adult sample, but did not vary substantially with serostatus.

From among the viremic patients, we obtained 3 sequences using HEV ORF1 primers and 6 sequences using ORF2 primers. We determined the outbreak strains to share high % identity and to belong to genotype 1 as expected, though their relationship to other genotype 1 strains was obscured by variability in the trees produced using the two different genomic segments.

We observed significant differences in both serologic results and patient catchment area by hospital. Lacking detailed information on patients’ water sources, or, failing that, water tank service areas, we could not definitively link the outbreak to a single water source. Furthermore, the geographic distribution of patients for whom specimens were available from the three hospitals did not cover all areas of the city known to be affected by the outbreak.
This work adds dimension to accounts of the outbreak and reveals differences in populations seen at each of three Biratnagar hospitals during the epidemic. It suggests that one contributor to the large size of the outbreak may have been low seroprevalence of anti-HEV antibodies and corresponding high susceptibility of the population in Biratnagar.

**Public Health Implications**

The literature on hepatitis E virus reviewed in the early chapters of this work describes a pathogen responsible for a large and yet underappreciated share of illnesses and deaths in young adults in developing countries. It highlights the sometimes-dire consequences of hepatitis E observed in pregnant women both during and in the absence of broader outbreaks. It posits various pathways by which the virus, in concert with changes in hormones and immune function and the placental environment, may provoke severe or even fatal outcomes for both mother and infant, and laments the dearth of available primary and secondary prevention strategies in the settings where they are most desperately needed.

In pregnant women in Sarlahi – as in healthy adults from Dharan, Sunsari, and Biratnagar, Morang – we found a somewhat lower seroprevalence of antibodies to HEV than has typically been reported in the Kathmandu Valley and other rural and urban areas in South Asia. This relatively lower seroprevalence, however, does not imply an absence of infection or even of serious illness. We estimated, based on serology, an incidence of 32.3 infections / 1000 p*y in pregnant women, and 43.5 / 1000 p*y in pregnant and postpartum women in Sarlahi. As a further caveat, our surveillance period covered only a few seasons, we may have systematically missed women who developed HEV infections earlier in pregnancy, and the number of deaths was sufficiently small in our sample to preclude making firm statements about the proportion of maternal deaths attributable to hepatitis E. Although one pregnant woman who became the single, verified hepatitis E case did recover and give birth to a healthy, full-term baby boy, even this relatively benign course
of illness took a toll on the mother’s health, leading to symptoms lasting at least a month and a
decrease in body weight during the late second trimester.

Striking disparities in seroprevalence of HEV in Sarlahi by caste and land ownership suggest
that the burden of infection and illness falls disproportionately on certain communities, and in
those communities, the impact of hepatitis E may be felt more acutely and the need for prevention
and intervention may be far greater.

The outbreak that affected thousands of people in Biratnagar is another clear signal that
HEV poses a serious threat in the Terai. There, a large population with low prior immunity to HEV
was hit hard when the virus was introduced into a municipal water system serving densely-
populated neighborhoods. The Biratnagar outbreak was attributed, perhaps correctly, to the
accidental rupture of a pipe during road construction, which allowed sewage to contaminate the
water supply.¹

But to view the Biratnagar outbreak as the result of a singular, unfortunate accident is to
disregard the context that contributed to its occurrence. Nepal is undertaking a variety of
infrastructure development projects, often with the financial backing of other governments or
international development agencies. The pace of road widening in Kathmandu has been furious
over the past decade, the façades of historic brick buildings sheared clean off to make room for
asphalt and utility poles.

Although perhaps less dramatically, life in the Terai has also undergone substantial changes.
What was formerly jungle behind the NNIPS headquarters and clinic in Hariyon just a decade ago is
now a plain of plowed fields, and the traditional open bamboo-and-mud-walled homes that dot the
dirt roads are increasingly punctuated by imposing two- or three-story concrete structures built
with money sent back by family members working overseas. Hariyon, whose population now
exceeds 20,000, and Lalbandi, at ~15,000,² were both recently upgraded to “municipality” status as part of an urbanization initiative.¹ Neighborhoods in Biratnagar, like Jamungachhi (near the airport) have expanded rapidly, and this growth has challenged the ability of the municipal infrastructure to keep up. The major earthquake in April 2015 ⁴ and the chronic political unrest in the Terai (especially in border cities like Biratnagar)⁵ surrounding the drafting of Nepal’s first Constitution disrupted, but did not halt, these trends.

Much of the larger-scale infrastructure development is aimed, nobly, at increasing the access of poorly-connected communities to much-needed services and opportunities. Nonetheless, the circumstances surrounding the hepatitis E outbreak in Biratnagar underscore that the bodies tasked with overseeing the maintenance of even existing infrastructure – the leaking water pipes running through sewage channels adjacent to the newly-paved roads – and with monitoring and responding to infectious disease outbreaks, are already stretched perilously thin.⁶-⁸

Increasingly, young Nepalis from the countryside are seeking work in these expanding population centers or in cities abroad. Remittances are a large and growing source of income.⁹ Among the women in the Sarlahi cohort, nearly 40% (857/2250) reported that at least one member of the household was employed outside of the local area. Of these women reporting one or more migrant laborers, 23% had family working in Kathmandu, 23% had family working elsewhere in Nepal, 15% had family working in India, 38% reported family working in the Gulf States (often building other countries’ burgeoning infrastructure), ¹⁰,¹¹ and 13% had family in Southeast Asia.

This large-scale movement of the population presents a two-way risk for hepatitis E, for those who leave and for those who remain at home. Migrant laborers (mostly men, more often from lower socioeconomic classes) coming from areas with low HEV seroprevalence are likely to lack prior immunity to HEV, and they are therefore at risk of contracting hepatitis E when they move to
Kathmandu or Bhojpur or Doha. Many them, especially those in the Gulf States, work in risky professions and, unable to afford more comfortable accommodations, live in extremely crowded conditions with poor sanitation,\textsuperscript{10,11} further increasing their risk of exposure to enteric pathogens.

Meanwhile, the effects of family members’ migrant labor on the risk of HEV infection among women in the Terai are likely to be mixed. There is evidence that women whose husbands work abroad are less likely than other women to engage in paid employment outside the home,\textsuperscript{12} which could potentially reduce their risk of exposure to HEV. Conversely, the work of tending to the family’s own crops outdoors may fall more to women as the result of a family member’s absence, which could increase risk of exposure. Family members who return home to Sarlahi from larger cities may bring with them HEV infections. Intra-household transmission appears to be uncommon with HEV,\textsuperscript{13} in contrast to HAV, though it has been reported.\textsuperscript{14,15} Still, repeated reintroduction of HEV into the environment by migrants returning from larger cities may help boost its circulation in the Terai, increasing the risk to women who lack prior immunity.

If development outpaces the ability to address the challenges that urbanization often presents for human health – particularly with regard to drinking water treatment and distribution, human waste disposal, and crowding – then more Biratnagar scenarios may be inevitable, particularly in Terai communities that have not historically had the massive outbreaks seen in the capital, and where most of the population may be susceptible. Addressing these challenges to forestall future epidemics of waterborne diseases like hepatitis E will require coordinated efforts on the part of the government, international aid and development organizations, health care providers at all levels, and local communities.
DIRECTIONS FOR FUTURE RESEARCH

The work presented herein provides preliminary answers to some of the questions we sought to address, but inevitably provokes others.

Detection of HEV in the Environment and in Animals in the Terai

In Sarlahi, we observed an association of meat-eating with HEV positivity, and a lack of association of drinking water source or latrine access with seropositivity, despite HEV in Nepal being considered a primarily waterborne infection. Our survey data were limited in scope for practical reasons, and as a result, we may have missed some relevant nuances that could help clarify the relationships of each of these variables with socioeconomic factors and with HEV.

Mapping latrines and well locations relative to cases could improve our understanding of the distribution of infections in this population. In the current study, we had the ability to look at individual-level associations and limited larger-scale associations; however, aggregating data to the administrative ward or VDC level, as we did to approximate community-level exposure, may be a poor representation of natural community configurations. Analysis that does not define communities based on administrative boundaries, but rather uses the spatial distribution of individual-level data to define communities, could provide useful insights.

Environmental testing of groundwater from local wells, as well as sampling of surface water and of fish or snails obtained from local ponds and streams, would complement spatial analyses by providing evidence of the presence, degree, and variability of fecal contamination (and perhaps of HEV). Determining whether wells of various designs or depths are more likely to be contaminated might help in shaping interventions.

In addition to testing fish and snails, it would be illuminating to test a variety of other local animals – both livestock and wild animals – for evidence of HEV. The presence of HEV in animals in
Nepal has previously been investigated among swine in the Kathmandu Valley, where antibodies were present but phylogenetic information was not published, and in sewage runoff from small swine farms where no HEV RNA was detected. [Kerry Morrison et al., unpublished data] A study that purported to show that HEV was prevalent in rats in Kathmandu was later retracted after the samples were found to have been contaminated.

Thus, there is a dearth of information on the presence, prevalence, and genotype(s) of HEV strains in Nepali livestock and wild animal species. It is unknown if these animals may represent a potential reservoir for human infection in Nepal. The conflicting results from studies in India make the situation all the more intriguing.

If zoonotic transmission did occur, would these infections be similar to genotype 1 infections in terms of disease outcomes in this population? This could help address several questions, including whether the milder and frequently asymptomatic infections with HEV genotypes 3 and 4 that occur in Europe, North America, and Japan, are primarily a function of general population health status or of reduced virulence in humans of these animal-adapted strains. Whether such infections would provoke similar antibody responses is also not entirely clear, but is also of interest in explaining the global epidemiology of HEV and the occurrence of outbreaks.

**Replication of HEV Findings from Bangladesh on Cytokines, Micronutrients, and Arsenic**

The seroprevalence of anti-HEV IgG among pregnant women in Sarlahi is similar to that among pregnant women in rural areas of Bangladesh. Although the incidence of infection in Sarlahi was estimated to be somewhat lower overall, the differences may be explained by statistical uncertainty in the estimates, year-to-year differences, or differences in methodology.

Assuming that the population risk of HEV is similar, are there also similar associations of HEV in Sarlahi with micronutrient levels, taking into account potential seasonal confounding?
Would we observe similar patterns of cytokine activity prior to infection in women who eventually became seroconverters vs. those who remained uninfected? 

Do toxins like arsenic or other environmental contaminants increase susceptibility to infection, as colleagues have recently posited? Bangladesh has historically dealt with major arsenic contamination of the water supply, but in Nepal the issue has received less attention. Studies from the Terai have shown that arsenic levels in groundwater may exceed safe levels, with perhaps a quarter of all tubewells in the Terai affected. In adults aged 15-49 from several Terai districts, the prevalence of clinically-apparent arsenicosis was generally low (<2%), and lower in women than in men. However, there are focal areas of higher arsenic levels where a greater proportion of the population is exposed, and health effects may occur at levels lower than those required to produce overt manifestations of toxicity. In the Terai, as in Bangladesh, might people with greater arsenic exposure also be more susceptible to HEV infection? If so, could widespread promotion of locally-suitable arsenic filters for water treatment have the welcome side effect of reducing the incidence of hepatitis E by a small amount, in addition to its other benefits?

On a related note, aflatoxin, produced by fungal contamination of grains with Aspergillus niger, has received little attention in relation to hepatitis E. Aflatoxin is ubiquitous in both Nepal and Bangladesh, and parallel studies conducted in Sarlahi and in Gaibandha show that lifetime exposure to aflatoxin and its metabolites begins before birth. Though perhaps best known for its association with liver cancer, aflatoxin has been linked to a wide range of adverse health effects, including immune dysregulation and stunted growth. Chronic hepatitis B viral infection and aflatoxin exposure together have a synergistic effect on the risk of hepatocellular carcinoma. While the pathogenesis and natural history of HEV differ significantly from HBV, it is not unreasonable to wonder whether aflatoxin’s deleterious effects on the liver, on immune function,
and on overall health might also increase the risk of infection, serious illness, or death in people exposed to HEV. Given the high levels of human exposure to aflatoxin via food across tropical and subtropical South Asia, where HEV is a perennial menace, it may be worthwhile to investigate whether there is any association of aflatoxin with HEV susceptibility or outcomes.

**Characterization of Antibody Responses to HEV During Pregnancy and Beyond**

In Sarlahi, we observed a total of 20 seroconversions: 9 during pregnancy (incidence ~33/1000 p*y), 8 during the first 3 months postpartum (incidence ~99/1000 p*y), and 3 from enrollment in pregnancy to 3 months postpartum (where no intermediate sample was available). Among the women who seroconverted during pregnancy and provided later samples (n=5), all had seroreverted by 3 months postpartum. In Bangladesh, 39/40 women who were identified as seroconverters based on seropositivity at 3 months postpartum had apparently seroconverted very late in pregnancy or postpartum. 18

Even allowing for seasonal coverage and the limited sample size in the Sarlahi study, it is curious that the postpartum incidence of seroconversion is so strikingly high relative to the incidence during pregnancy in both of these studies.

Do these observations reflect a pregnancy-independent pattern of very transient antibody production in response to mild HEV infection, which, on purely probabilistic grounds, would have had more opportunity to disappear over the longer periods of observation during pregnancy than during the 3 months of observation postpartum? Seroreversion has been reported in diverse settings, though with wildly inconsistent rates of occurrence. 30

If production of antibodies to HEV is simply very ephemeral, this suggests that we may be (perhaps vastly) underestimating the specific and cumulative incidence of infections in the population, particularly with longer intervals between samples. Such “missed” asymptomatic
infections may be of more than academic interest. Misclassification of infection status may obscure small-to-moderate effects of potentially-modifiable exposure-related variables, reducing opportunities for intervention to prevent infections. In addition, we have not yet adequately assessed the potential effects of mild infections on fetal development and viability, and ephemerality of maternal antibody response does not necessarily imply lack of effect on the fetus.

Shorter observation windows would provide more sensitive detection of infection events; however, balancing this goal with practical and ethical considerations in a community-based trial may prove difficult. Testing a subset of existing, banked late-pregnancy samples from Bangladesh (regardless of postpartum serostatus) could help to clarify whether the postpartum-skewed incidence of seroconversion observed in that cohort is an artifact of the retrospective seroconversion identification strategy or whether it is a real phenomenon. If corroborated in Bangladesh, and if replicated in longitudinal studies of pregnant women conducted in other settings, this would be a fascinating and heretofore unidentified feature of the epidemiology of HEV. Until and unless that occurs, it will remain a curiosity.
REFERENCES


Flow-Chart of Hepatitis E Substudy: Blood Sample Processing

5mL Blood Sample Collected in Field
- Transport to Field Office in cooler
  - Centrifuge
    - Aliquot ~2.5mL serum
      - X####A ~500ul to WARUN
      - X####B ~750ul to JHSPH via Cincinnati Children's Hospital
      - X####C ~750ul to Cincinnati/Seattle
      - X####D ~750ul to Cincinnati/Seattle
    - Cytokines
      - Micronutrients
  - X####E ~500ul to WARUN
  - HC####B,C,D ~2.0mL to JHSPH via Cincinnati Children's Hospital

Update Antibody & Hepatitis Substudy Form [Form 51]
- Hepatitis Cases: (Symptomatic, Women Only)
  - Anti-HEV IgM
  - HbsAg
  - Anti-HCV IgM
  - Anti-HAV IgM
- Routine Blood Draws:
  - (All Women @ Enrollment, Delivery, 3 Mos. Postpartum)
  - Anti-HEV IgG -> IgM
  - Anti-HAV Igs (enrollment only)
  - Anti-HBc -> HbsAg (PE & 3P)
  - Anti-HCV (PE & 3P)

Sub-Aliquot to AFRIMS: RNA Seq (HEV+ only)

Key to Specimen Labels:

<table>
<thead>
<tr>
<th>X</th>
<th>X</th>
<th>#</th>
<th>#</th>
<th>#</th>
<th>#</th>
</tr>
</thead>
<tbody>
<tr>
<td>*Specimen Type: PE = enrollment/baseline</td>
<td></td>
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<tr>
<td>1P = 1st postpartum (delivery)</td>
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<td>3P = 3 months postpartum</td>
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<td>HC = symptomatic case</td>
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<td></td>
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<tr>
<td>Number 0001-9999 (sequential)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Aliquot A-D (sequential)</td>
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</tbody>
</table>
# Appendix 2: Sarlahi Cohort Study Forms and Data Collection

## List of MaGIFT Forms and Scripts Used in the Hepatitis Substudy

<table>
<thead>
<tr>
<th>Form #</th>
<th>Form Name &amp; Purpose / Data Collected</th>
<th>Version</th>
<th>Date</th>
<th>Version</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Household Consent Script</td>
<td>5</td>
<td>12-Apr-11</td>
<td>2</td>
<td>12-Apr-11</td>
</tr>
<tr>
<td></td>
<td>Describes overall household participation in MaGIFT and seeks consent.</td>
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</tr>
<tr>
<td>2</td>
<td>Reproductive Age Women’s Consent Script</td>
<td>4</td>
<td>15-May-13</td>
<td>4</td>
<td>15-May-13</td>
</tr>
<tr>
<td></td>
<td>Describes pregnancy surveillance, immunization, and follow-up of women 15-40 and seeks consent.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5A</td>
<td>Household Women’s Census Form</td>
<td>6</td>
<td>14-Jan-11</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Records household size, members, ID numbers, ages, and women’s current pregnancy status.</td>
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</tr>
<tr>
<td>5B</td>
<td>New Household Women’s Census Form</td>
<td>5</td>
<td>14-Jan-11</td>
<td>—</td>
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</tr>
<tr>
<td></td>
<td>Records information as above for new households established after baseline census.</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>6</td>
<td>Household Characteristics Form (HCF)</td>
<td>5</td>
<td>10-Dec-10</td>
<td>1</td>
<td>28-Mar-11</td>
</tr>
<tr>
<td></td>
<td>Records household demographics, home construction, socioeconomic indicators.</td>
<td></td>
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</tr>
<tr>
<td>7</td>
<td>Pregnancy Surveillance Form (PSF)</td>
<td>3</td>
<td>02-May-11</td>
<td>2</td>
<td>02-May-11</td>
</tr>
<tr>
<td></td>
<td>Records dates of pregnancy surveillance and menstrual periods and results of pregnancy tests for women in a single sector of a ward of a village development committee.</td>
<td></td>
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<tr>
<td>8</td>
<td>Pregnancy Enrollment Form (PEF)</td>
<td>6</td>
<td>16-Dec-11</td>
<td>2</td>
<td>16-Dec-11</td>
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<tr>
<td></td>
<td>Records health and reproductive history, tobacco and alcohol use, anthropometry, and provision of antenatal care package.</td>
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<tr>
<td></td>
<td>Form Name</td>
<td>Start Date</td>
<td>End Date</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Maternal Weekly Morbidity Form</td>
<td>27-Aug-12</td>
<td>27-Aug-12</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Records symptoms of flu-like illness and care visits among pregnant women.</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>8</td>
<td>Infant Weekly Morbidity Form</td>
<td>30-Jul-12</td>
<td>30-Jul-12</td>
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<tr>
<td></td>
<td>Records symptoms of flu-like illness or pertussis and care visits among infants &lt;6 months of age.</td>
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</tr>
<tr>
<td>1</td>
<td>Vaccine Receipt Eligibility Form – Phase II</td>
<td>01-May-12</td>
<td>01-May-12</td>
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<tr>
<td></td>
<td>Records pregnant woman's eligibility to receive vaccine and history of tetanus toxoid injections.</td>
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</tr>
<tr>
<td>2</td>
<td>Monthly Pregnancy Follow-Up Form (PFF)</td>
<td>15-Oct-12</td>
<td>15-Oct-12</td>
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<tr>
<td></td>
<td>Records woman’s vital status, pregnancy status, 30-day history of illness symptoms, alcohol/tobacco use, anthropometry, and hepatitis score (calculated from symptom report).</td>
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<tr>
<td>3</td>
<td>Maternal Birth Assessment Form (MBAF)</td>
<td>10-Jan-13</td>
<td>10-Jan-13</td>
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</tr>
<tr>
<td></td>
<td>Records date and nature of pregnancy outcome, details of delivery, pre- and post-delivery care, maternal complications, and woman's vital signs.</td>
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<td>4</td>
<td>Infant Birth Assessment Form (IBAF)</td>
<td>23-Nov-11</td>
<td>23-Nov-11</td>
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</tr>
<tr>
<td></td>
<td>Records date and time of birth, vital status and sex of infant, infant health, cord care and breastfeeding practices, and infant anthropology.</td>
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<tr>
<td>5</td>
<td>Maternal Verbal Autopsy Form (MVAF)</td>
<td>25-Mar-13</td>
<td>15-Mar-13</td>
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<td>Documents death of enrolled woman and records information solicited from relatives or others pertaining to cause of death.</td>
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<td>6</td>
<td>Child Verbal Autopsy Form (CVAF)</td>
<td>31-Jan-11</td>
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<td>Date 2</td>
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<td>19</td>
<td>6 Month Child Anthropometry Follow-Up Form</td>
<td>15-Nov-11</td>
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<tr>
<td></td>
<td>Records vital status, height, weight, and head circumference of infants at 6 months of age.</td>
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</tr>
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<td>23</td>
<td>Pregnancy Report Form</td>
<td>03-Mar-11</td>
<td>28-Mar-11</td>
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<tr>
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<td>Documents pregnancies reported via pregnancy surveillance procedures or other means.</td>
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<tr>
<td>25</td>
<td>Pregnancy Tracking Log</td>
<td>19-Oct-12</td>
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</tr>
<tr>
<td></td>
<td>Used to schedule and track individual study milestones, follow-up visits, and pregnancy outcome.</td>
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<tr>
<td>26</td>
<td>Post-Partum Morbidity Form (MPPF)</td>
<td>31-Jan-11</td>
<td>28-Mar-11</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Records symptoms of illness or complications and chlorhexidine use shortly after delivery.</td>
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</tr>
<tr>
<td>27</td>
<td>Birth Report Form</td>
<td>14-Sep-12</td>
<td>14-Sep-12</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Records date of birth and parents’ names upon receipt of notification.</td>
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<tr>
<td>29</td>
<td>Married New Woman Form</td>
<td>02-May-11</td>
<td>—</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Records arrival / household change and consent of married woman aged 15-40 in study area.</td>
<td></td>
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<tr>
<td>30</td>
<td>Adverse Event Report Form</td>
<td>30-May-11</td>
<td>29-May-11</td>
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<tr>
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<td>Records occurrence, details, care-seeking, and outcome of adverse event.</td>
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<tr>
<td>34</td>
<td>Weekly Morbidity Script for Women and Other Adults</td>
<td>04-Dec-10</td>
<td>04-Dec-10</td>
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<tr>
<td></td>
<td>Script to solicit information on symptoms of flu-like illness among enrolled pregnant women.</td>
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<td>35</td>
<td>Weekly Morbidity Script for Infants and Other Children</td>
<td>4</td>
<td>27-Aug-12</td>
<td>4</td>
<td>27-Aug-12</td>
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</tr>
<tr>
<td></td>
<td>Script to solicit information on symptoms of flu-like illness or pertussis and care visits among infants &lt;6 months of age.</td>
<td></td>
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<tr>
<td>51</td>
<td>Antibody and Hepatitis E Substudy Form (AHSSF)</td>
<td>3</td>
<td>10-Oct-12</td>
<td>4</td>
<td>31-Jul-13</td>
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<tr>
<td></td>
<td>Documents dates and label numbers for specimens collected as part of hepatitis substudy.</td>
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<td>58</td>
<td>Post-PEF Tracking Form</td>
<td>1</td>
<td>23-Mar-12</td>
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</tr>
<tr>
<td></td>
<td>Documents home and maiti (parents’ home) addresses and visit/return dates.</td>
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<tr>
<td>62</td>
<td>Shipper Tank / Liquid Nitrogen Maintain Log</td>
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</tr>
<tr>
<td></td>
<td>Used to track depth of liquid nitrogen in tanks to ensure maintenance of cold chain.</td>
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<tr>
<td>65</td>
<td>Weekly Lab-Work Schedule (WLS)</td>
<td>3</td>
<td>05-Oct-12</td>
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</tr>
<tr>
<td></td>
<td>Used to schedule visits for specimen collection, document label numbers, and track met status.</td>
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<tr>
<td>66</td>
<td>Hepatitis E Substudy Pregnant Woman’s Consent Script</td>
<td>2</td>
<td>12-Mar-12</td>
<td>2</td>
<td>12-Mar-12</td>
</tr>
<tr>
<td></td>
<td>Describes substudy, follow-up procedures, and specimen collection to pregnant women enrolled in MaGIFT and seeks consent.</td>
<td></td>
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<tr>
<td>67</td>
<td>Specimen Shipment Log</td>
<td>4</td>
<td>04-Dec-12</td>
<td>—</td>
<td></td>
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<tr>
<td></td>
<td>Records specimen numbers and aliquot types being shipped.</td>
<td></td>
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<td>69</td>
<td>Laboratory Specimen Collection Log (Blood &amp; Breastmilk) (LSBL)</td>
<td>2</td>
<td>05-Oct-12</td>
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<tr>
<td></td>
<td>Records specimen and participant ID numbers for specimens received in field lab.</td>
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</tr>
</tbody>
</table>
Highlighted forms were specifically developed or significantly modified for the hepatitis substudy and are included in this appendix.

<table>
<thead>
<tr>
<th></th>
<th>Form Description</th>
<th>Date 1</th>
<th>Date 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>71</td>
<td>Specimen Management Log (Blood)</td>
<td>2</td>
<td>04-Dec-12</td>
</tr>
<tr>
<td></td>
<td>Records aliquots made from blood specimens and tracks storage and destination.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>78</td>
<td>Hepatitis E Symptomatic Pregnant Woman’s Consent Script</td>
<td>1</td>
<td>12-Apr-11</td>
</tr>
<tr>
<td></td>
<td>Describes, and seeks consent for, questionnaire and blood draw from women with symptoms of hepatitis-like illness.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>79</td>
<td>HEV Exposure Assessment Questionnaire (HEV-EAQ)</td>
<td>5</td>
<td>18-Oct-12</td>
</tr>
<tr>
<td></td>
<td>Records potential hepatitis E-related risk factors, including hygiene and sanitation practices, water and food sources and storage, occupation, travel history, and animal exposures.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>Hepatitis Case Visit Form (HEV-CVF)</td>
<td>5</td>
<td>18-Oct-12</td>
</tr>
<tr>
<td></td>
<td>Records care sought, treatments used and their perceived effectiveness, and beliefs about illness, as well as label number and results of rapid HEV test and referral status.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>81</td>
<td>Hepatitis E Report Card (HERC)</td>
<td>2</td>
<td>18-Oct-12</td>
</tr>
<tr>
<td></td>
<td>Records date of positive rapid HEV test and provides basic information about hepatitis E and when to seek medical care.</td>
<td></td>
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</tr>
</tbody>
</table>
FORM 14: MONTHLY PREGNANCY FOLLOW-UP FORM (PFF)

MATERNAL INFLUENZA VACCINE TRIAL
MONTHLY PREGNANCY FOLLOW UP FORM (PFF)

Week of Interview: __________ Date: __________ Worker ID: __________

VDC: ____________ Ward: __________ Sector: __________ HH: __________

NNIPS #: __________ Woman’s Name: __________________________

Husband: __________________________

Hev Status: □ 0 = not eligible for participation
□ 1 = eligible for participation

Vital Status: □ 1 = alive
□ 2 = died (STOP → MVAF)
□ 3 = don’t know (STOP)

Met status: □ 1 = Met
□ 2 = not met (STOP)
□ 7 = permanently moved (STOP)

Pregnancy status: □ 1 = still pregnant
□ 2 = live birth and/or stillbirths (STOP → MVAF/IBAF)
□ 3 = miscarriage/abortion (STOP → MVAF/IBAF)
□ 9 = don’t know (STOP)

Form Status: □ 1 = completed
□ 6 = refused (STOP)

SECTION A: CURRENT PREGNANCY

1. In the past 30 days, have you sought consultation or medical treatment regarding this pregnancy?
□ 0 = No
□ 1 = Yes (go to C1a)
□ 9 = Don’t know

1a. If yes, where did you go for care?

2. How many doses of tetanus toxoid vaccine have you had in the past 30 days?
□ 0 = None (go to Section B)
□ 1 - 5 = Number of Times
□ 9 = Don’t know
SECTION B: 30-DAY MORBIDITY

I am now going to ask you questions about your health.

1. In the past 30 days, have you had a fever AND persistent cough on the same day?
   - Yes (go to 1a.)
   - No
   - Don’t know

   1a. IF YES, how many days did you have fever and persistent cough together on the same days?
   - 1 = Number of days
   - 0 = Don’t know
   - 99 = Don’t know

2. In the past 30 days, have you had a fever AND sore throat, on the same day?

   2a. IF YES, how many days did you have fever and sore throat together on the same days?
   - 1 = Number of days
   - 0 = No
   - 9 = Don’t know
   - 99 = Don’t know

<table>
<thead>
<tr>
<th>In the past 30 days, how many days have you had</th>
<th>A. Number of Days (00-30)</th>
<th>B. Treatment Sought, if any (Record highest number)</th>
<th>C. Hep E Score</th>
<th>D. How many days ago did the most recent episode begin?</th>
<th>E. Do you have this today?</th>
</tr>
</thead>
<tbody>
<tr>
<td>3. Persistent Cough?</td>
<td></td>
<td>XXXXXXXXXX</td>
<td>XXXXXXXXXX XXXXXXXXXX XXXXXXXXXX</td>
<td></td>
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<tr>
<td>4. Difficult or rapid breathing?</td>
<td></td>
<td>XXXXXXXXXX</td>
<td>XXXXXXXXXX XXXXXXXXXX XXXXXXXXXX</td>
<td></td>
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</tr>
<tr>
<td>5. Wheezing / Grunting?</td>
<td></td>
<td>XXXXXXXXXX</td>
<td>XXXXXXXXXX XXXXXXXXXX XXXXXXXXXX</td>
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<td></td>
</tr>
<tr>
<td>6. Shortness of breath?</td>
<td></td>
<td>XXXXXXXXXX</td>
<td>XXXXXXXXXX XXXXXXXXXX XXXXXXXXXX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8. Poor Appetite?</td>
<td></td>
<td>XXXXXXXXXX</td>
<td>XXXXXXXXXX XXXXXXXXXX XXXXXXXXXX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9. Nausea?</td>
<td></td>
<td>If either Q9 or Q10 &gt;00, Score = 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10. Vomiting?</td>
<td></td>
<td>XXXXXXXXXX</td>
<td>XXXXXXXXXX XXXXXXXXXX XXXXXXXXXX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11. Convulsions (excluding epilepsy)?</td>
<td></td>
<td>XXXXXXXXXX</td>
<td>XXXXXXXXXX XXXXXXXXXX XXXXXXXXXX</td>
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<tr>
<td>12. Swelling of Hands?</td>
<td></td>
<td>XXXXXXXXXX</td>
<td>XXXXXXXXXX XXXXXXXXXX XXXXXXXXXX</td>
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<tr>
<td>13. Swelling of Face?</td>
<td></td>
<td>XXXXXXXXXX</td>
<td>XXXXXXXXXX XXXXXXXXXX XXXXXXXXXX</td>
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<tr>
<td>14. Severe Headache (prevent normal activity)?</td>
<td></td>
<td>XXXXXXXXXX</td>
<td>XXXXXXXXXX XXXXXXXXXX XXXXXXXXXX</td>
<td></td>
<td></td>
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<tr>
<td>15. High fever/Very hot to touch?</td>
<td></td>
<td>If Q15 &gt;00 Score = 1</td>
<td></td>
<td></td>
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<tr>
<td>16. Watery Stool (4 or more times in a day)?</td>
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<td>XXXXXXXXXX</td>
<td>XXXXXXXXXX XXXXXXXXXX XXXXXXXXXX</td>
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<td></td>
</tr>
<tr>
<td>Question</td>
<td>Column A</td>
<td>Column B</td>
<td>Column C</td>
<td>Column D</td>
<td>Column E</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>---------</td>
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<td>---------</td>
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<tr>
<td>Blood or mucus in stool?</td>
<td></td>
<td></td>
<td>xxxxxxxx</td>
<td>xxxxxxxx</td>
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<tr>
<td>Painful or burning urination?</td>
<td></td>
<td></td>
<td>xxxxxxxx</td>
<td>xxxxxxxx</td>
<td></td>
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<tr>
<td>Foul smelling vaginal discharge?</td>
<td></td>
<td></td>
<td>xxxxxxxx</td>
<td>xxxxxxxx</td>
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<tr>
<td>Spots of blood from vagina?</td>
<td></td>
<td></td>
<td>xxxxxxxx</td>
<td>xxxxxxxx</td>
<td></td>
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<tr>
<td>Vaginal bleeding?</td>
<td></td>
<td></td>
<td>xxxxxxxx</td>
<td>xxxxxxxx</td>
<td></td>
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<tr>
<td>Night blindness?</td>
<td></td>
<td></td>
<td>xxxxxxxx</td>
<td>xxxxxxxx</td>
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<tr>
<td>Jaundice? (Yellow Eyes)</td>
<td></td>
<td></td>
<td>xxxxxxxx</td>
<td></td>
<td></td>
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<tr>
<td>Dark colored urine?</td>
<td></td>
<td></td>
<td>xxxxxxxx</td>
<td></td>
<td></td>
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<tr>
<td>Light colored stool (whitish)?</td>
<td></td>
<td></td>
<td>xxxxxxxx</td>
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<tr>
<td>Continuously dripping urine?</td>
<td></td>
<td></td>
<td>xxxxxxxx</td>
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<tr>
<td>Faces passing through the birth canal?</td>
<td></td>
<td></td>
<td>xxxxxxxx</td>
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<tr>
<td>Severe lower abdominal pain?</td>
<td></td>
<td></td>
<td>xxxxxxxx</td>
<td></td>
<td></td>
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<tr>
<td>Blurred vision?</td>
<td></td>
<td></td>
<td>xxxxxxxx</td>
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<tr>
<td>Itching of hands, feet or skin not from a rash?</td>
<td></td>
<td></td>
<td>xxxxxxxx</td>
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<tr>
<td>Feeling extremely weak?</td>
<td></td>
<td></td>
<td>xxxxxxxx</td>
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<td></td>
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<tr>
<td>Pain in upper right abdomen?</td>
<td></td>
<td></td>
<td>xxxxxxxx</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smell of food strange or unappealing?</td>
<td></td>
<td></td>
<td>xxxxxxxx</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**TOTAL HEP E SCORE (add scores in column C)**

- If Hep E score is 4 or more, inform ANM and enter check mark in the box. (cross out if informed previously or HEV status=0)

**B. Treatment Sought**

- 0 = Not Treated
- 1 = Dhami Jankhi/TBA
- 2 = Medicine shop/local (non MBBS) doctor
- 3 = Subhealth post/CMV auxiliary health worker/ANM
- 4 = Health post/health assistant private practitioner/Staff nurse
- 5 = Primary health center/MBBS doctor/nursing home
- 6 = Hospital
- 9 = Don’t Know

**D. Days ago last episode began**

- 00 = Within the past day
- 01-30 = # days previously episode began
- 90 = Refused
- 99 = DK

**E. Have it today?**

- 0 = No
- 1 = Yes
- 9 = DK
SECTION C: TOBACCO, ALCOHOL USE

In the past 30 days, have you...

1. Smoked bidi, cigarettes, hooka, or chimul?  
   - 0 = No  
   - 1 = Yes  
   - 2 = Don't know  
   - # of Days (00-30 days, 99 = don't know)  
   - If ≥1 day, how many times per day? (01-99 times, 99 = don't know)

2. Chewed tobacco?

3. Drunk jaard / tard?

4. Drunk rakshi?

SECTION D: ANTHROPOMETRY

1. Weight: _____ _____ kg

2. Temperature: _____ _____ °F

3. Blood Pressure: _____ _____ / _____ _____

4. Pulse: _____ _____

SECTION E: PREGNANCY CHECKLIST

** For each item in the checklist [0 = Not Given, 1 = Given by NNIPS, 2 = Received from Hith. Post, 3 = Not given (received from NNIPS), 4 = Not given (<13 weeks) 5 = Refused, 6 = Don't know]

- If more than or equals 13 weeks pregnant, give only iron-folate.
- If month of pregnancy is 5 or larger, give all items listed.

1. Deworming tablet offered/given:

2. Kawach (Chlorhexidine) for cord given

3. Instruction regarding notification of labor and delivery given:

4. Instruction regarding umbilical cord care during first 14 days provided:

5. Advice regarding antenatal / obstetric care provided:

6. Safe birthing kit offered/given:

7. Iron folate offered/given:
**FORM 51 v.3: ANTIBODY AND HEPATITIS SUBSTUDY FORM (AHSSF)**

<table>
<thead>
<tr>
<th>Specimen Status</th>
<th>Date</th>
<th>Specimen</th>
<th>Wk IO #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specimen Status</td>
<td></td>
<td>PE</td>
<td></td>
</tr>
<tr>
<td>Specimen Status</td>
<td></td>
<td>CB</td>
<td></td>
</tr>
<tr>
<td>Specimen Status</td>
<td></td>
<td>1P</td>
<td></td>
</tr>
<tr>
<td>Specimen Status</td>
<td></td>
<td>3P</td>
<td></td>
</tr>
<tr>
<td>Specimen Status</td>
<td></td>
<td>1B</td>
<td></td>
</tr>
<tr>
<td>Specimen Status</td>
<td></td>
<td>3B</td>
<td></td>
</tr>
<tr>
<td>Specimen Status</td>
<td></td>
<td>6B</td>
<td></td>
</tr>
<tr>
<td>Specimen Status</td>
<td></td>
<td>HC</td>
<td></td>
</tr>
</tbody>
</table>
**Form 51 v.4: Antibody and Hepatitis Substudy Form (AHSSF)**

### Maternal Influenza Vaccine Trial

**Antibody and Hepatitis E Sub-Study Form**

**Home Address:**  
- **VDC:**  
- **Ward:**  
- **Sector:**  
- **HH:**

**Mailing Address:**  
- **VDC:**  
- **Ward:**  
- **Sector:**  
- **HH:**

**Other Household Information**  
- **Tote:**  
- **Father’s Name:**

#### NNIPSNum  
- **First Names:**  
- **Last Name:**  
- **Mother’s Vaccination Code:**  
  - A, B, C, D, V

**Week of Birth Outcome**

### Specimen Status

<table>
<thead>
<tr>
<th>Specimen Status</th>
<th>Date</th>
<th>Specimen #</th>
<th>Wk ID #</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mother’s Enrollment Blood Collection:</strong></td>
<td></td>
<td>PE</td>
<td></td>
</tr>
<tr>
<td><strong>Baby’s Cord Blood Collection:</strong></td>
<td></td>
<td>CB</td>
<td></td>
</tr>
<tr>
<td><strong>Mother’s Post-Delivery Blood Collection:</strong></td>
<td>IF 8, STOP</td>
<td>1P</td>
<td></td>
</tr>
<tr>
<td><strong>Mother’s 3M PP Blood Collection:</strong></td>
<td>NA</td>
<td>3P</td>
<td></td>
</tr>
<tr>
<td><strong>Breastmilk 1 Month:</strong></td>
<td>NA</td>
<td>1B</td>
<td></td>
</tr>
<tr>
<td><strong>Breastmilk 3 Months:</strong></td>
<td>NA</td>
<td>3B</td>
<td></td>
</tr>
<tr>
<td><strong>Breastmilk 6 Months:</strong></td>
<td>NA</td>
<td>6B</td>
<td></td>
</tr>
<tr>
<td><strong>Hepatitis Case Follow-Up Blood Collection:</strong></td>
<td>IF 8, STOP</td>
<td>HC</td>
<td></td>
</tr>
</tbody>
</table>

**Specimen Status**  
1=Consented, collected  
2=Not Met  
3=Met, not collected  
6=Refused  
8=Died
JOHNS HOPKINS BLOOMBERG SCHOOL OF PUBLIC HEALTH

ORAL CONSENT SCRIPT

Pregnant Woman’s Consent Script

Study Title: Hepatitis E in Pregnancy Substudy (Mother’s Gift - Nepal Field Trial)

Principal Investigator: James M. Tielsch

IRB No. 00002458


Introduction

Namaste. We are grateful that you have agreed to participate in the Mothers Gift project. We would like to ask you if you would also like to participate in a study about an illness called hepatitis. Every year in Nepal and many parts of south Asia, people get an illness which sometimes causes yellow eyes and skin. This can be caused by several things, some of which we might be able to prevent in the future through improvements in water cleanliness or a vaccine.

Purpose:

The purpose of this part of the project is to find out how many women become infected and ill from hepatitis during their pregnancy, and to understand what is causing this illness in our community in Sarlahi. We will use what we learn from this part of the project to inform doctors and the government about the importance of hepatitis illness in Sarlahi.

Procedures:

For this part of the project, we would like to take a few teaspoons of blood from your arm three times: once when you first join the Mothers Gift study, once when you deliver your baby, and 3 months after you deliver your baby. The blood will be tested to see if you have ever been infected with a hepatitis virus, even if you did not feel ill or notice any signs and to see if you are protected from certain respiratory infections. We will share the results of the hepatitis test, when the laboratory test is completed.

When we visit you each week to ask about your health, we will also ask about signs of hepatitis illness. If you have signs of hepatitis illness, we will send study staff to visit you and we may ask you additional questions. If you or baby need treatment, we will refer you to a place for care.
We will also ask to take blood from your baby’s umbilical cord soon after they are born, to take a vaginal swab from you in late pregnancy, and a few teaspoons of breastmilk at 1, 3, and 6 months following the birth of your baby.

Risks/Discomforts:
When we take blood from your arm, there can be mild pain, but this lasts only a few seconds. Collecting the blood from the umbilical cord, the vaginal swab, the placenta, and the breastmilk do not cause any pain. The blood samples will be used to see how your body has responded to previous respiratory infections. The breastmilk will be used to see whether you can pass protection from certain respiratory infections to your baby. The vaginal swabs will be used to see if certain vaginal infections are related to the outcome of your pregnancy.

Anticipated Benefits:
There is not likely to be a direct benefit to you from participating in this part of the project. There are no costs to you for participating and you will not receive any compensation. We will use what we learn from this part of the project to inform doctors and the government about the importance of hepatitis illness in Sarpali.

Confidentiality:
All information you give us will be kept confidential and only our project staff will be allowed to see this information.

Voluntariness:
Your participation in the research project is completely voluntary. You can withdraw from the study at any time. Even if you do not want to join this part of the study, or if you withdraw from the study, you can still participate in future NNIPS projects. You can decide not to provide any or all of the specimens and you can still participate in the project.

Do you have any questions about this research study? If you have questions later, you can ask Dr. Subarna Khatri (telephone 046-530135) in Harihara or Dr. Laxman Subrasti (telephone 98510-36550) at the IOM in Kathmandu. You may also ask any of our study staff.

RECORD WOMEN’S CONSENT STATUS ON THE HEPATITIS SUBSTUDY ENROLLMENT FORM
## FORM 69: LABORATORY SPECIMEN COLLECTION LOG (BLOOD & BREASTMILK) (LSBL)

### Maternal Influenza Vaccine Trial
Laboratory Specimen Collection Log (Blood and Breastmilk)

<table>
<thead>
<tr>
<th>Specimen No.</th>
<th>VDC</th>
<th>Ward</th>
<th>Sector</th>
<th>III</th>
<th>NNIPS No.</th>
<th>First Name</th>
<th>Last Name</th>
<th>Collection Date</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
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</table>

**Specimen Types:**
1 = 1 month breast milk (1M), 2 = 3 month breast milk (3M), 3 = 6 month breast milk (6M), 4 = Post-EP blood (PE), 5 = Postpartum blood (PP), 6 = 3 month blood (3P), 7 = Hepatitis blood (HC), 8 = Cord Blood (CB)
### Maternal Influenza Vaccine Trial Specimen Management Log (Blood)

<table>
<thead>
<tr>
<th>Specimen Label</th>
<th>NNIPS No. / Address</th>
<th>Aliquot Date (DD MM YY)</th>
<th>Aliquot Status</th>
<th>Sock #</th>
<th>Route</th>
<th>Shipment Date (DD MM YY)</th>
</tr>
</thead>
<tbody>
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</tbody>
</table>

Aliquot:  
- N=No
- Y=Yes

Route (ship to):  
- 1 = WARUN  
- 2 = JHU  
- 3 = Seattle  
- 4 = Jesvan’s Lab KTM  
- 5 = Other  
- 6 = Cincinnati
Symptomatic Pregnant Woman’s Consent Script

Study Title: Hepatitis E Infection in Pregnancy Substudy (Mother’s Gift - Nepal Field Trial)

Principal Investigator: James M. Tisch

IRB No. 00002458

PI Version Date: Version 1, April 12, 2011

Introduction
   Namaste. We thank you for your participation in the Mothers Gift project and the hepatitis study. You had some signs of illness, so we wanted to check your health.

Purpose:
   We are trying to learn why some pregnant women suffer more illness from hepatitis type “E” than other people. We would like to learn if your symptoms are caused by hepatitis type E, and check your blood to see if any special treatment is needed.

Procedures:
   We would like to ask you some questions about your health and activities. Study staff will also examine you for signs of hepatitis.
   We would also like to take some blood from your arm, about 1.5 teaspoons (7mL). We will test this blood to check the health of your liver and to see if hepatitis E virus may be causing your illness.
   If you or baby need treatment, we will refer you for care.

Risks/Discomforts:
   When we take blood from your arm, there can be mild pain, but this lasts only a moment. About 1 in 10 women may see a bruise on the skin around where the needle was inserted. There is a very small risk of infection, but we will try to prevent any infection by cleaning the skin on your arm and by using only a new, sterile needle.

Anticipated Benefits:
   We will tell you the results of your blood test if you would like to hear about it and if we find you have hepatitis E infection. There are currently no medicines that can cure hepatitis E, but most people will recover even without medicine. There are no costs to you for participating and you will not receive any compensation. We hope that learning why many pregnant women become ill from hepatitis E will help us improve the health of pregnant women and their infants in the future.
Confidentiality:

All information you give us will be kept confidential and only our project staff will be allowed to see this information. We will not write your name on the blood sample, only a study identification number. We will not share the blood test results with anyone except you and with the health clinic or doctor if you need treatment.

Voluntariness:

Your participation in the research project is completely voluntary. You can withdraw from the study at any time. Even if you do not want to join this part of the study, or if you withdraw from the study, you can still participate in future NNIPS projects.

Do you have any questions about this research study? If you have questions later, you can ask Dr. Subarna Khatry (telephone 046-530135) in Hariana or Dr. Laxman Shrestha (telephone 98510-36550) at the IOM in Kathmandu. You may also ask any of our study staff.

RECORD WOMEN'S CONSENT STATUS ON THE HEPATITIS E SUBSTUDY BLOOD DRAW FORM
**Section A. Occupation and Travel**

<table>
<thead>
<tr>
<th>Question</th>
<th>During the winter season</th>
<th>During the summer season</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Housework in your own home or another home (such as preparing food, cleaning, washing clothing, caring for children)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Working with crops (such as planting, weeding, harvesting)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Collecting firewood or animal fodder</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. Working indoors, but not in a home (for example, in an office, shop, factory, or school)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

5. Do you usually stay inside the house compound all day?  
   - 0 = No  
   - 9 = Don’t know

6. Over the past 30 days, how many times have you visited a haat bazaar?  
   - 0 = None  
   - 01-60 = Number of times  
   - 99 = Don’t know

7. Other than going to a haat bazaar, over the past 30 days, how many trips have you made to the town centers of Hariyon, Lalbandi, Karmiya, or Nawalpur (to visit family or friends, take care of some business, go to a bank, or attend some festival or celebration, for example)?  
   - 0 = None  
   - 01-60 = Number of trips  
   - 99 = Don’t know
8. Over the past 30 days, how many days have you visited in any larger city (such as Janakpur, Birgunj, Hetauda, or Kathmandu)?

- 00= None
- 01-30= Number of days
- 99= Don’t know

**Section B. Food and Meals**

1. In the past 14 days, how many meals did you eat away from your home?

- 00= None
- 01-14= Number of meals
- 15= 15 or more meals
- 99= Don’t know

2. When you eat away from home, do you usually eat food that was prepared...
   *(Record up to 3 responses.)*

   - 1= Inside your own home and carried with you
   - 2= In the homes of family or friends
   - 3= By a hotel, restaurant, tea shop, or lodge
   - 4= By a haat bazaar vendor or other street vendor
   - 5= Other, specify: ____________________________
   - 9= Don’t know

3. In the past 14 days, how many meals did you eat in your home that were brought home from a haat bazaar, hotel, tea shop, or street vendor?

- 00= None
- 01-14= Number of meals
- 15= 15 or more meals
- 99= Don’t know

4. Are you a strict vegetarian who avoids every kind of meat and fish?

- 0 = not vegetarian *(Continue to Q5.)*
- 1 = vegetarian *(Skip to Q17.)*
- 9 = don’t know *(Continue to Q5.)*

<table>
<thead>
<tr>
<th>Do you ever eat.................................?</th>
<th>If yes, in the past 30 days, on how many days did you eat....?</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 = No</td>
<td>00= None</td>
</tr>
<tr>
<td>1 = Yes</td>
<td>1 = Yes</td>
</tr>
<tr>
<td>9 = Don’t know</td>
<td>9 = Don’t know</td>
</tr>
</tbody>
</table>

5. Pig or wild pig meat

6. Goat, lamb, or sheep meat

7. Water buffalo meat

8. Fish, crab, or snail

9. Chicken, duck, or other birds

10. Other meat *(specify)*:

11. Do you ever eat any part of an animal without cooking (raw)?

- 0 = No *(Go to 12.)*
- 1 = Yes *(Go to 11a.)*
- 9 = Don’t know *(Go to 12.)*
11a. What kind(s) of meat do you eat without cooking (raw)?

(Record up to 4 responses.)

☐ 1 = Pig or wild pig
☐ 2 = Goat, lamb, or sheep
☐ 3 = Water buffalo
☐ 4 = Fish, crab, or snail
☐ 5 = Chicken, duck or other birds
☐ 6 = Other, specify: ___________________________
☐ 7 = Don’t know

12. In the past year, have you eaten any food or soup prepared with the blood of an animal?

☐ 0 = No (Go to 13.)
☐ 1 = Yes (Go to 12a.)
☐ 9 = Don’t know (Go to 13.)

12a. From what animal(s) do you eat the blood?

(Record up to 3 responses.)

☐ 1 = Pig or wild pig
☐ 2 = Goat, lamb, or sheep
☐ 3 = Water buffalo
☐ 4 = Fish, crab, or snail
☐ 5 = Chicken, duck or other birds
☐ 6 = Other, specify: ___________________________
☐ 9 = Don’t know

13. In the past year, have you eaten brains, or food or soup made with brains (“gidi”)?

☐ 0 = No (Go to 14.)
☐ 1 = Yes (Go to 13a.)
☐ 9 = Don’t know (Go to 14.)

13a. From what animals do you eat the brains?

(Record up to 3 responses.)

☐ 1 = Pig or wild pig
☐ 2 = Goat, lamb, or sheep
☐ 3 = Water buffalo
☐ 4 = Fish, crab, or snail
☐ 5 = Chicken, duck or other birds
☐ 6 = Other, specify: ___________________________
☐ 9 = Don’t know

14. In the past year, have you eaten any food or soup made with stomach, intestines, or gizzard?

☐ 0 = No (Go to 15.)
☐ 1 = Yes (Go to 14a.)
☐ 9 = Don’t know (Go to 15.)

14a. From what animals do you eat the stomach, intestines, or gizzard?

(Record up to 4 responses.)

☐ 1 = Pig or wild pig
☐ 2 = Goat, lamb, or sheep
☐ 3 = Water buffalo
☐ 4 = Fish, crab, or snail
☐ 5 = Chicken, duck or other birds
☐ 6 = Other, specify: ___________________________
☐ 9 = Don’t know

15. In the past year, have you eaten any food or soup made with liver or kidney?

☐ 0 = No (Go to 16.)
☐ 1 = Yes (Go to 15a.)
☐ 9 = Don’t know (Go to 15a.)

15a. From what animals do you eat the livers or kidneys?

(Record up to 4 responses.)

☐ 1 = Pig or wild pig
☐ 2 = Goat, lamb, or sheep
☐ 3 = Water buffalo
☐ 4 = Fish, crab, or snail
☐ 5 = Chicken, duck or other birds
☐ 6 = Other, specify: ___________________________
☐ 9 = Don’t know
16. In the past year, have you eaten any food or soup made with an animal’s lung?
   0 = No (Go to 17.)
   1 = Yes (Go to 16a.)
   9 = Don’t know (Go to 17.)

16a. From what animal(s) do you eat the lung?
(Record up to 4 responses.)
   1 = Pig or wild pig
   2 = Goat, lamb, or sheep
   3 = Water buffalo
   4 = Fish, crab, or snail
   5 = Chicken, duck or other birds
   6 = Other, specify: ______________________
   9 = Don’t know

17. Do you drink unboiled cow or water buffalo milk?
   0 = No
   1 = Yes
   9 = Don’t know

Section C. Live Animal Exposures

1. How many of the following animals are owned by your household?
   a. Water buffalo or cattle ............................. 00 = None
   b. Goats or sheep ......................................... 01-97 = Number of animals
   c. Chickens or ducks .................................... 98 = 98 or more
   d. Pigs ......................................................... 99 = Don’t know
   e. Pigeons ................................................... 
   f. Other, Specify: ______________________

2. Do ........................................... regularly come inside your house?
   a. Water buffalo or cattle .............................
   b. Goats or sheep .........................................
   c. Chickens or ducks ....................................
   d. Pigs or wild pigs ......................................
   e. Pigeons ................................................... 0 = No
   f. Other animal, specify: ______________________
   g. Other animal, specify: ______________________
3. In the past 7 days, how many times have you milked cows or water buffalo?

00 = None
01-30 = Number of times
31 = > 30 times
99 = Don’t know
4. Do you clean the living quarters of any animals on a regular basis?
   0 = No (Go to 5.)
   1 = Yes (Go to 4a.)
   9 = Don’t know (Go to 5.)

   4a. Which animals’ living quarters do you clean?  
   (Record up to 4 responses.)
   1 = Pig
   2 = Goat, lamb, or sheep
   3 = Water buffalo or cattle
   4 = Fish, crab, or snail
   5 = Chicken, duck or other birds
   6 = Other, specify: ______________
   9 = Don’t know

5. Have you slaughtered or butchered any animals in the past 1 year?
   0 = No (Go to 7.)
   1 = Yes (Go to 6a.)
   9 = Don’t know (Go to 7.)

   5a. Which animals have you slaughtered or butchered?  
   (Record up to 4 responses.)
   1 = Pig or wild pig
   2 = Goat, lamb, or sheep
   3 = Water buffalo
   4 = Fish, crab, or snail
   5 = Chicken, duck or other birds
   6 = Other, specify: ______________
   9 = Don’t know

6. Does your household have any pet?
   a. Monkey? ...........................................
   b. Bird? ...........................................
   c. Cat? ...........................................
   d. Dog? ...........................................
   e. Other, specify: ____________________

   0 = No
   1 = Yes
   9 = Don’t know

7. Do you use cow dung (“gobar”) for
   a. Fertilizer? ...........................................
   b. Fuel/fire? ...........................................
   c. Cleaning? ...........................................
   d. Pooja? ...........................................
   e. Other, specify: ____________________
Section D. Household Water Use

1. What is your household’s usual source of water for.................? (Record up to 2 responses.)
   a. Drinking ☐ ☐
   b. Cooking ☐ ☐
   c. Bathing ☐ ☐
   d. Washing dishes ☐ ☐
   e. Washing clothes ☐ ☐

   Water sources:
   1 = Tube well or tap
   2 = Protected ring well
   3 = Other (including pond, river, canal, unprotected well, stone tap)
   9 = Don’t know

2. From what vessel(s)/utensil(s) do you most often drink water when you are at home? (Record up to 2 responses.)
   1 = Hands ☐ ☐
   2 = Glass or metal cup ☐ ☐
   3 = Amkora ☐ ☐
   4 = Lhola ☐ ☐
   5 = Plastic bottle ☐ ☐
   6 = Other, specify______________
   9 = Don’t know

3. Do you drink water when you go out?
   0 = No ☐
   1 = Yes ☐
   9 = Don’t know

4. Does your household store drinking water?
   0 = No (Go to 5.) ☐ ☐
   1 = Yes (Go to 4a.) ☐ ☐
   9 = Don’t know (Go to 5.) ☐ ☐

   4a. Is the water stored in a covered or uncovered container?
   0 = Uncovered container ☐ ☐
   1 = Covered container ☐ ☐
   9 = Don’t know ☐

   4b. Is the primary stored drinking water shared with any other families or households?
   0 = No ☐
   1 = Yes ☐
   9 = Don’t know ☐

5. Does your family use some method to purify water before drinking?
   0 = No (Go to Section E.) ☐
   1 = Yes (Go to 5a.) ☐
   9 = Don’t know (Go to Section E.) ☐

5a. If yes, what method or methods do you usually use? (Record up to 3 responses.)
   1 = San or cloth filter ☐ ☐
   2 = Arsenic filter ☐
   3 = Tablets ☐
   4 = Pyush or drops ☐
   5 = Ceramic filter ☐
   6 = Boiling ☐
   7 = Other, please specify______________ ☐
   9 = Don’t know ☐
Section E. Sanitation Practices

1. What kind of latrine/toilet facility do you most often use when you are at home?
   
   0 = None/field/bush (Go to 2.)
   1 = Pit latrine
   2 = Water sealed/slab
   3 = Flush toilet
   6 = Other, specify: __________________________
   9 = Don’t know

   (Go to 1a.)

1a. How close is this latrine to your residence?
   
   0 = Within residence
   1 = Detached, nearby (<30m away)
   2 = Far (>30m away)

1b. Do you share this latrine with any other families or households?

   0 = No
   1 = Yes
   9 = Don’t know

1c. How often is your latrine usually cleaned?

   0 = Never (Go to 2.)
   1 = Less than once per month
   2 = 1-3 times per month
   3 = 4 or more times per month
   9 = Don’t know

   (Go to 1d.)

1d. Who cleans your latrine most often?

   1 = Self
   2 = Family member
   3 = Non-family member
   9 = Don’t know

2. Do you usually wash your hands before eating food?

   0 = No (Go to 3.)
   1 = Yes (Go to 2a.)
   9 = Don’t know (Go to 3.)

2a. If yes, what do you use to wash?

   1 = Water only
   2 = Water and Ash
   3 = Water and Earth
   4 = Water and Soap
   6 = Other, specify: __________________________
   9 = Don’t know

2b. How many hands do you wash?

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

3. Do you usually wash your hands after defecating?

   0 = No (Go to 4.)
   1 = Yes (Go to 3a.)
   9 = Don’t know (Go to 4.)

3a. If yes, what do you use to wash?

   1 = Water only
   2 = Water and Ash
   3 = Water and Earth
   4 = Water and Soap
   6 = Other, specify: __________________________
   9 = Don’t know

3b. How many hands do you wash?

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

4. Do you usually wash your hands after handling diapers or cleaning children’s bottoms?

   0 = No (Go to 5.)
   1 = Yes (Go to 4a.)
   9 = Not applicable (Go to 5.)

4a. If yes, what do you use to wash?

   1 = Water only
   2 = Water and Ash
   3 = Water and Earth
   4 = Water and Soap
   6 = Other, specify: __________________________
   9 = Don’t know

4b. How many hands do you wash?

   1 = One hand
   2 = Both hands
   9 = Don’t know
5. Do you currently have any type of soap in your household for washing hands?  

Section F. Health Conditions and Treatments

1. Over the past 3 months have you been around any person suffering from yellow eyes or yellow skin?  
   □ 0= No  
   □ 1= Yes  
   □ 9= Don't know

2. 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

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

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

END OF HEV-EAQ
FORM 80: HEPATITIS CASE VISIT FORM (HEV-CVF)

Maternal Influenza Vaccine Trial
HEPATITIS CASE VISIT FORM (HEV-CVF)

Week of Interview: Date: Worker ID: 

VDC: Ward: Sector: HH: 

NNIPS #: First Names Last Name 

Woman: 

Husband: 

Met status: 1=Met 2=Not met. (STOP) 

Case Follow-Up Consent Status: 

Case Report Date: 

SECTION A: TREATMENT

1. Have you (or someone on your behalf) sought any advice or treatment for the signs of illness you told us about yesterday? 0 = No (Go to 2.) 1 = Yes (Go to 1a.) 9 = Don't know (Go to 1a.)

1a. Where, or from whom, did you first seek advice or treatment for these signs of illness?

1b. Where else did you seek advice or treatment, if anywhere?

(Record up to 3 additional responses.)
<table>
<thead>
<tr>
<th></th>
<th>Have you used................ to treat this illness?</th>
<th>If yes, please describe:</th>
<th>If yes, did this treatment help?</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 = No</td>
<td>1 = Yes</td>
<td>9 = DK</td>
</tr>
<tr>
<td>a</td>
<td>Pills</td>
<td></td>
<td></td>
</tr>
<tr>
<td>b</td>
<td>An injection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c</td>
<td>Liquid medicine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>d</td>
<td>A special tea</td>
<td></td>
<td></td>
</tr>
<tr>
<td>e</td>
<td>Eating specific foods or plants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>f</td>
<td>Avoiding specific foods or plants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g</td>
<td>Applying a powder or paste</td>
<td></td>
<td></td>
</tr>
<tr>
<td>h</td>
<td>Wearing a special amulet, bracelet, necklace, or ring</td>
<td></td>
<td></td>
</tr>
<tr>
<td>i</td>
<td>A hand-washing ritual</td>
<td></td>
<td></td>
</tr>
<tr>
<td>j</td>
<td>A bathing ritual</td>
<td></td>
<td></td>
</tr>
<tr>
<td>k</td>
<td>Some other treatment</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3. What do you believe is the source or cause of your illness? (Write the details the participant provides.)


SECTION B: CASE NOTIFICATION AND REFERRAL

1. Rapid HEV test result:  
   0 = negative (-)  
   1 = positive (+) → Provide Hepatitis E Report Card (HERC)  
   2 = error, unclear result, or could not complete  

2. Provided verbal referral to nearby medical facility. (Check box to confirm.)  

3. Case Blood Draw:  
   0 = Did not complete (reason: ______________________)  
   1 = Completed (Go to 3a.)  
   6 = Refused  

3a. Blood Label #: H C □ □ □ □  

END OF HEV-CF
FORM 81: Hepatitis E Report Card (HERC)

Form 81 v.2 / Field v.2.0 18-October-2012
Maternal Influenza Vaccine Trial

Hepatitis E Report Card

Participant’s Name

Date of Positive HEV Test: 

We have tested your blood today. The test showed an illness called Hepatitis E. This is probably the main cause of the symptoms you reported.

People who become ill with Hepatitis E usually recover completely within a few weeks.

Hepatitis E is spread through water and food that has become contaminated with stool from people who are ill. You can help prevent others from becoming ill by washing your hands well after defecation and before preparing meals.

There are not currently any medical treatments to cure Hepatitis E or to make the symptoms go away more quickly. Still, a medical doctor or clinic can provide help and treatment if there are any complications.

Please seek medical care if your symptoms become worse or if you are concerned about your pregnancy.

Thank you again for your participation in our study. We will use what you have told us to try to help pregnant women avoid becoming ill in the future.

We wish you a quick recovery.

Namaste.
Appendix 3: Field Laboratory Safety & Informational Signs

Laboratory Biohazard & Emergency Contact Information

(Name and phone numbers of responsible personnel are blurred here for privacy.)
A safety problem was identified, wherein staff would occasionally handle socks of cryovials without insulated gloves. A primary reason for this was that the insulated gloves were typically kept in a different room from the liquid nitrogen dewars due to minimal storage space inside the room (which had previously been a restroom). Solution: Fabric loops were sewn onto the gloves, and a hook was affixed to the wall next to the dewars so that the gloves would be both visible and easily accessible. Safe handling of liquid nitrogen was discussed during a safety training presentation (given by K. Charron and A. Andrada) attended by all NNIPS staff who might encounter it, including vehicle drivers and the office sweeper.
LAB SAFETY

No food, drink, or smoking in lab.

Inside the lab, wear shoes that cover your feet (no sandals).

Wear lab coat and gloves when handling samples.

Wash your hands upon entering and leaving lab.

Disinfect work surfaces after samples are processed.

Keep the lab door closed.

ल्याब सुरक्षा

ल्याबमा खान-पीन वा धुम्पान गर्न निषेध छ

ल्याबमा जुत्ता लगाइ आफि खुटाको सुरक्षित राख्नुहोस्
(ल्याबमा चप्पल प्रयोग गर्न निषेध छ)

नमुनाहरूको परिक्षण गर्दा ल्याब कोट र पन्ना लगाउनुस

ल्याबमा प्रवेश गर्दा र निस्कंदा साखुन पानीले हात धुन्नुहोस्

ल्याबमा परिक्षण गरि कसैले नुसा ल्याब टेबल धुन्नुहोस्

सर्दी ठोका बन्द राख्नुहोस्
Wear Closed-Toe Shoes in the Lab

Don't Expose Your Toes!

Wear Lab Shoes

ल्याबको जुत्ता लाउनुस
### SPECIMEN CHART FOR ONGOING NNIPS STUDIES

**FLU**

<table>
<thead>
<tr>
<th>Form 69 (WLS) Code</th>
<th>Specimen Label Prefix</th>
<th>Specimen Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1B</td>
<td>1 month PP breast milk</td>
</tr>
<tr>
<td>2</td>
<td>3B</td>
<td>3 month PP breast milk</td>
</tr>
<tr>
<td>3</td>
<td>6B</td>
<td>6 month PP breast milk</td>
</tr>
<tr>
<td>4</td>
<td>PE</td>
<td>Post-PEF blood</td>
</tr>
<tr>
<td>5</td>
<td>1P</td>
<td>1 week PP blood</td>
</tr>
<tr>
<td>6</td>
<td>3P</td>
<td>3 month PP blood</td>
</tr>
<tr>
<td>7</td>
<td>HC</td>
<td>Hepatitis case blood</td>
</tr>
</tbody>
</table>

- **ILI infant nasal swab**

**NOMS**

Skin sample

**COOKSTOVE**

This wall chart was created because NNIPS was simultaneously conducting three major studies, each with sub-studies, and staff needed a handy reference for the different types of samples that were being processed and their associated codes.

Abbreviations:

- **FLU**= Maternal Global Influenza Immunization Field Trial (MaGIFT), in which the hepatitis study was nested.
- **WLS** = Weekly Lab Schedule; **PP** = postpartum; **PEF** = Pregnancy Enrollment Form; **ILI** = influenza-like illness.
- **NOMS** = Nepal Oil Massage Study, evaluating the effects of using sunflower vs. mustard oil on the health and skin flora of infants, who are traditionally rubbed with oil by their mothers.
- **Cookstove** = Cookstove Study, testing three types of improved cookstoves for acceptability and effects on indoor air pollution and respiratory health.
CURRICULUM VITAE

LISA J. KRAIN

born October 31, 1980, Evanston, Illinois, USA

EDUCATION

PhD Candidate, Epidemiology, Johns Hopkins Bloomberg School of Public Health to be completed 7/2016
Dissertation topic: Hepatitis E Virus (HEV) in the Terai of Nepal
Advisors: Kenrad Nelson and Alain Labrique

Master of Science (ScM), Epidemiology, Johns Hopkins Bloomberg School of Public Health 05/2008
Thesis topic: Treponema pallidum (yaws and syphilis) in Baka and Bantu populations, rural Cameroon
Advisors: Nathan Wolfe and Taha Taha

Bachelor of Arts (BA), Anthropology, University of California at Berkeley 05/2003
Graduated with high distinction in general scholarship, Phi Beta Kappa
Earth & Planetary Science minor

RESEARCH POSITIONS

Graduate Assistant (Researcher), Johns Hopkins School of Public Health, Baltimore, Maryland 04/2011 – present
Collaborated on design and implementation of longitudinal study of viral hepatitis among pregnant women in Nepal, funded by the Bill & Melinda Gates Foundation, and several related projects and manuscripts.

Predoctoral Fellow, Johns Hopkins Center for a Livable Future, Baltimore, Maryland 07/2009 – 06/2010
Assessed scope, components, and geographic coverage of methicillin-resistant Staphylococcus aureus (MRSA) surveillance programs under state and federal auspices. Evaluated availability and viability of surveillance data for epidemiologic study of sources, risk factors and transmission patterns of MRSA in community settings.

Graduate Assistant (Repository Manager), Wolfe Lab/Cameroon Program, Baltimore, Maryland 07/2006 – 02/2008
Maintained repository of human and animal blood and tissue samples, shipped specimens, prepared proposals for funding and IRB, developed data and repository management strategies for Zoonotic Emergence Network (now Global Viral Forecasting Initiative), ordered supplies for Baltimore & Yaoundé labs, supervised work-study students.

Graduate Assistant (Researcher), Johns Hopkins School of Public Health, Baltimore, Maryland 10/2005 – 12/2007
Reviewed and summarized literature on phylogeny, hosts, and emergence of viral, bacterial, and protozoan human pathogens. Contributed to "Characteristics of some major human infectious diseases (Table S1)", and "Details on each of our 25 major infectious diseases (Note S9)", supplements to Wolfe, ND, Panosian Dunavan C, and Diamond JM, Origins of major human infectious diseases, Nature 447, 279-283, 2007.

Intern, South Coast Air Quality Management District (AQMD), Diamond Bar, California 08/2004 - 10/2004
Integrated several existing data sets to produce airborne pollution estimates for ocean-going vessels at the Port of Long Beach; performed literature search on bioavailability of Cr(VI) in paints and primers; assembled information on rail shipping routes and facilities in Southern California from Internet sources.

Laboratory Assistant, Berkeley Seismological Laboratory, Berkeley, California 10/1999 - 12/2002
Produced digital maps using a variety of geographic information sources and earthquake databases; designed and maintained websites for education and research initiatives; prepared graphics; participated in public earthquake response.
**PEER-REVIEWED PUBLICATIONS**


**PRESENTATIONS AND SCIENTIFIC POSTERS**


* indicates presenter(s) for posters and talks

**HONORS AND AWARDS**

Center for a Livable Future Predoctoral Fellowship 07/2009 – 06/2010

Baltimore Albert Schweitzer Fellowship (for development and implementation of service project) 05/2007 – 05/2008

JHSPH Master’s Tuition Scholarship (for outstanding work in first two terms) 03/2006 – 05/2006

Superior Service Award, Natural Science Division, Pasadena City College (for work as TA) 06/2005

Elected to Phi Beta Kappa at UC Berkeley (Alpha of California Chapter) 05/2003

California Alumni Association Jonathan D. Sauer Scholarship (for natural science student) 08/2001 - 05/2002

California Alumni Association Emerging Leader Scholarship 08/2000 - 05/2001
**TEACHING EXPERIENCE**

**Teaching Assistant, Johns Hopkins University**, Baltimore, Maryland 06/2006-05/2011
- Epi 340.651 Emerging Infections (grad) – 4th term 2010-2011
- Epi 340.627 Epidemiology of Infectious Diseases (grad, online) – 3rd term 2010-2011
- Epi 280.350 Fundamentals of Epidemiology (undergrad) – spring 2009-2010
- Epi 340.601 Principles of Epidemiology (grad) – summer 2010
- Epi 340.608 Observational Epidemiology (grad, online) – 3rd term 2008-2009

**Teaching Assistant, Pasadena City College**, Pasadena, California 01/2004 – 06/2005
Designed discipline-specific critical-thinking activities in undergraduate courses Phys 2B: “Physiology and Anatomy” & Geol 1: “Physical Geology”; assisted students with quantitative, science reasoning, and writing skills.

Graded exams, gave feedback to undergraduates, and created website for Earth & Planetary Sci. 20: “Earthquakes”.

**SERVICE**

**Peer Review for Journals**
- *Clinical and Vaccine Immunology*, *Clinical Microbiology Reviews*, *Emerging Infectious Diseases*, *Journal of Clinical Microbiology*, *Journal of Viral Hepatitis*, *Pathogens and Global Health* since 2014

**Earthquake Engineering Research Institute (EERI) Nepal Earthquake Reconnaissance Team** 5/2015-6/2015
Served as “Virtual Team Collaborator”, compiling information and images from news, social media, and non-governmental organizations on physical and operational impact of the April 2015 earthquake on healthcare facilities in the Kathmandu Valley and in Gorkha District in preparation for recon team’s field survey.

**Dyslexia Tutoring Program** 11/2007-8/2011
Completed 20 hrs. training in Orton-Gillingham instruction methods. Worked one-on-one with dyslexic student to improve academic skills and to increase engagement in learning.

**Baltimore-Area Resources for Adults with Learning Disabilities and/or ADHD (Schweitzer Project)** 09/2006-12/2008
Initiated booklet/website project to facilitate access to information about free and low-cost diagnostic, treatment, support, and educational services, and to informally assess barriers to access.


**Epidemiology Comprehensive Exam Review Co-Coordinator** 02/2006 – 06/2006

**Errand and Escort Service, Huntington Memorial Hospital**, Pasadena, California 01/2004 – 05/2005
Logged 220+ hours transporting patients, ferrying charts and blood units, and delivering meals and flowers.

**Dean’s Student Advisory Committee, College of Letters and Science**, Berkeley, California 01/2002 - 5/2002

**Education and Outreach Coordinator, Cal Disabled Students’ Union**, Berkeley, California 08/1999 - 05/2001
Organized presentations about common disabilities for students and staff, put together campus-wide disability awareness day featuring speakers and approx. 50 disability organizations, designed website.

**Tutor/Teacher’s Aide, Washington Elementary School**, Berkeley, California 08/1999 - 05/2001
Spent 100+ classroom hours assisting K, 2, 5, and LD resource room students in language and math.

**PREVIOUS RESEARCH AND FIELDWORK EXPERIENCE**

Conducted **museum-based descriptive/stratigraphic study** of Neogene Caribbean gastropods (snails) 08/2002 - 12/2002  
Assisted with **tomographic study of Earth’s upper mantle** using cross-correlation of seismic waveforms 05/2000 - 08/2000  
Participated in 8-week **archaeological excavation at Tel Dor** 06/1999 - 08/1999

**OTHER EMPLOYMENT**

**Personal Care Attendant, Alameda County**, Berkeley, California 10/1999-12/1999  
Assisted college student with cerebral palsy with activities of daily living.

**PROFESSIONAL AFFILIATIONS**

American Society of Tropical Medicine and Hygiene (ASTMH)  
American Public Health Association (APHA), Epidemiology Section  
Geological Society of America (GSA), Geology and Human Health Division  
American Geophysical Union (AGU), Biogeosciences Division

**ADDITIONAL SKILLS AND CERTIFICATIONS**

**Computing Experience**  
UNIX, Windows, and Mac platforms  
HTML, CSS, and website design  
Statistical software: STATA, R  
Geographic information systems and digital cartography: ArcGIS, GMT  
Graphics software: Photoshop, PaintShop Pro  
Bioinformatics tools: NCBI (BLAST, PubMed, etc.), MEGA6 (sequence alignment/phylogenetics)  
Reference management: EndNote, RefWorks  
FreezerWorks repository management software

**Certifications**  
JHU Animal Care and Use (2006)  
JHMI Biosafety Laboratory Training (2006, 2013)  
JHMI Fire Safety and Hazard Communication (2009, 2013)  
National Weather Service Skywarn Spotter – Advanced Course (2013)  

**Languages**  
English – native language  
French – reading, writing, and conversational abilities (once fluent, now rusty)  
Nepali – beginning reading, writing, and conversational skills  
Spanish – intermediate reading knowledge, limited conversation and writing  
Hebrew – basic conversational, reading, and writing knowledge  
German, Yiddish – limited reading knowledge

**Research interests**: Epidemiology at the intersection of environmental health and infectious diseases; disease emergence; spatial and molecular epidemiology; natural disasters; ethics.