Serological and Molecular Analyses of Icteric Hepatitis E in Bangladeshi Women

A THESIS SUBMITTED TO THE DEPARTMENT OF MATHEMATICS AND NATURAL SCIENCES, BRAC UNIVERSITY FOR THE PARTIAL FULFILLMENT FOR THE DEGREE OF MASTER OF SCIENCE IN BIOTECHNOLOGY

Submitted by:

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Dedication

This work is dedicated to my beautiful beloved family, who offered me unconditional love and support and has always been there for me.

DECLARATION BY THE RESEARCHER

This is to declare that the research work embodying the results reported in this thesis entitled **"Serological and Molecular Analyses of Icteric Hepatitis E in Bangladeshi Women"** has been carried out by the undersigned under the joint supervision of Prof. Dr. Firdausi Qadri, Senior Scientist and Executive Director of Institute for developing Science and Health initiatives (ideSHi), Director of Center for Vaccine Sciences and Senior Scientist and Head of Immunology Laboratory at icddr,b, Bangladesh, Dr. Kaiissar Mannoor, Head and Scientist of the Institute for developing Science and Health initiatives (ideSHi) and Professor Naiyyum Choudhury, former Coordinator of the Biotechnology and Microbiology programs, Department of Mathematics and Natural Sciences, BRAC University and now the Chairman of Bangladesh Atomic Energy Regulatory Authority (BAERA); It is further declared that the research work presented here is original and submitted in the partial fulfillment for the degree of Master of Science in Biotechnology, BRAC University, Dhaka and has not been submitted anywhere else for a degree or diploma.

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Abstract

Bangladesh is an endemic zone for Hepatitis E Virus (HEV) which is associated with both epidemic and sporadic infections. Since there have been few population-based studies of this country's HEV burden, its epidemiological characteristics and virological features remain ambiguous. The study included a total of 30 female patients (13 pregnant and 17 non-pregnant women) between the ages of 18 and 70 years, who were all representative of the urban community in Dhaka city. All the subjects had visible jaundice and visited the physicians within the study period (i.e. from August 2016 to May 2017). Clinical and epidemiological data were collected from these patients using questionnaires and the serology-based diagnoses of the Hepatitis viruses, as manifested by the levels of IgM and IgG in case of Hepatitis A, C and E viruses and the presence of HBsAg in case of Hepatitis B virus (HBV), were determined. Nucleic acids were isolated from the serologically positive HEV samples and amplified with primers specific for the Open Reading Frame 2 (ORF2) of the HEV genome in order to ascertain their HEV genotypes. Out of 30 patients, 29 were serologically positive for HEV and 1 was positive for Hepatitis C Virus (HCV). Co-infections of Hepatitis A Virus (HAV) and HEV and that of HBV and HEV were detected. Both pregnant and non pregnant subjects had very high levels of serum bilirubin, Aspartate transaminase (AST), Alanine transaminase (ALT) and Alkaline phosphatase (ALP), with no significant difference in these levels between the HEV mono-infection and co-infection states. Both anti-HEV IgM and IgG antibodies were detected in 51.7% of the HEV positive patients while 41.4% and 6.9% of the patients were positive for only anti-HEV IgG and anti-HEV IgM respectively. 15 out of 29 HEV samples were found positive for HEV RNA. Sanger sequencing-based phylogenetic tree analysis of the HEV RNA positive samples resulted in the formation of a single cluster within genotype 1. This is one of the very first studies to report the clinical, epidemiological, and molecular characterization of an outbreak of Hepatitis E in Bangladesh.

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LIST OF ABBREVIATIONS

ACLF	Acute Chronic Liver Failure
Ag	Antigen
ALP	Alkaline Phosphatase
ALT	Alanine Transaminase
AST	Aspartate Transaminase
BAN	Bangladesh
cDNA	complementary DNA
CHN	China
COV	Cut-off value
CP	Capsid Protein
CRE	cis-reactive element
DEU	Germany
dNTP	deoxynucleotide tri-phosphate
dsDNA	double stranded DNA
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
et al	And Others
FHF	Fulminant Hepatic Failure
Grp78	Glucose-Regulated Protein 78
HAV	Hepatitis A Virus
HBV	Hepatitis B Virus
HBsAg	Antigen of the Hepatitis B Virus
HCV	Hepatitis C Virus
Hel	RNA Helicase
HEV	Hepatitis E Virus
HRP	Horseradish Peroxidase
HVR	Hypervariable Region

HSC	Higher Secondary Certificate
HSC70	Heat Shock Cognate Protein 70
HSPG	Heparan Sulfate Proteoglycans
IgA	Immunoglobin A
IgG	Immunoglobin G
IgM	Immunoglobin M
icddr,b	International Centre for Diarrhoeal Disease Research, Bangladesh
IND	India
INSDC	International Nucleotide Sequence Database Collaboration
ITA	Italy
IU	International Unit
IU/L	International Unit per Liter
JPN	Japan
JSC	Junior School Certificate
KGZ	Kyrgyzstan
LPC	Low Positive Control
Met	Methyltransferase
MEX	Mexico
MFP	Multifunctional Protein
$MgCl_2$	Magnesium Chloride
mg/dl	Milligram per Deciliter
ml	Milliliter
mM	Milli-Molar
mm Hg	Millimeter Mercury
NC	Negative Control
NCR	Non-Coding Region
nt	Nucleotide
ORF	Open Reading Frame
PCP	Papain-like Cysteine Protease
PCR	Polymerase Chain Reaction
PSC	Primary School Certificate
RdRp	RNA-dependent RNA polymerase

RNA	Ribo-Nucleic Acid
RT-PCR	Reverse Transcriptase PCR
SD	Standard Deviation
SOD	Sample Optical Density
SSC	Secondary School Certificate
ssRNA	single-stranded RNA
TMB	3,3', 5,5;-tetramethylbenzidine
UN	United Nation

Chapter 1: Introduction

1. Introduction

1.1 Background

Hepatitis E virus (HEV) is the most common cause of acute viral hepatitis in the world especially in developing countries where it causes significant morbidity and mortality. This emerging infectious agent spread enterically via the fecal-oral route, predominantly through contaminated water and therefore is responsible for waterborne outbreaks and sporadic cases in developing countries with poor sanitation conditions.

HEV has one serotype and four reported genotypes out of which genotypes 3 and 4 are primarily zoonotic and genotypes 1 and 2 are associated with acute viral hepatitis in endemic countries that exclusively infect humans. Infections are primarily subclinical, with only 20–30% of cases experiencing symptoms [1]. Although the disease is self-limiting and no chronic sequelae or carrier state has been documented in the general population, it is observed to have a high attack rate in young adults and is particularly severe among pregnant women (especially in their second and third trimesters) where the mortality secondary to symptomatic infection was estimated to be ten-fold higher than that in men or non-pregnant women [1] [2].

Even though the highest incidence of epidemics of HEV infection was mostly recorded in South and Southeast Asia, where seasonal HEV outbreaks have been documented in India and Nepal, few studies have characterized the burden of HEV infections in Bangladesh. Despite the absence of recognized seasonal outbreaks, Bangladesh is suspected to be HEV endemic, with seroprevalence projections ranging from 27% to 60% [1].

This study represents the first urban community-based seroprevelance survey among Hepatitis E infected female patients (both pregnant and non-pregnant women) and the molecular characterization of HEV in Bangladesh. The availability of the resulting comprehensive molecular and serological data can be useful in informing disease prevention policy and in developing novel antiviral compounds.

1.2 The History of Hepatitis E Virus

Hepatitis E virus (HEV) is the most recently discovered of the five well-recognized hepatotropic viruses, named A to E. The earliest well-documented report of this infection was an epidemic of acute viral hepatitis, which had occurred in New Delhi, India in 1955–1956 which was initially thought to be related to Hepatitis A [3]. It was first recognized during an epidemic of hepatitis, which occurred in Kashmir valley in 1978 when the stored sera from the Kashmir outbreak and the epidemic in Delhi were tested [4]. The sera exhibited no specific serological markers for Hepatitis A and Hepatitis B, and therefore was provisionally named as enterically transmitted non-A, non-B Hepatitis virus. These viral-like particles were identified in the feces of a volunteer using immune electron microscopy after self-ingestion of acute phase stool suspensions from a water-borne epidemic of non-A hepatitis in Central Asia [3]. These viruses were subsequently named Hepatitis E virus (HEV), based on its enteric route of transmission and its tendency to cause epidemics. Soon thereafter, Hepatitis E virus (HEV) was cloned and sequenced in 1990 [3].

Hepatitis E infection is most prevalent in areas of poor sanitation and hygiene, which include large parts of Africa, Asia, the Mediterranean region, Mexico and South America [4]. HEV is a responsible medium to large-sized waterborne epidemics and is the commonest cause of acute sporadic viral hepatitis and fulminant hepatic failure (FHF) in the endemic regions. HEV infections occurring in developed countries may have zoonotic origin or is imported in travelers and tourists who have travelled to the disease-endemic regions.

In Europe when sanitary conditions were still very poor during the 19th century and early part of the 20th century numerous hepatitis outbreaks in that region had features similar to those of Hepatitis E which tend to suggest that the disease was once endemic in the regions where it is currently non-endemic [4].

1.3 Molecular virology of Hepatitis E virus

1.31 Morphology and genomic organization of Hepatitis E virus

HEV is the only member of the genus Hepevirus in the family of Hepeviridae [4]. It is a small, round non-enveloped viral particle of 27–34 nm with a genome of approximately 7.2 kb in length that consists of a polydenylated, single strand RNA molecule containing three discontinuous and partially overlapping open reading frames (ORFs), and 5′ and 3′ cis-acting elements which have important roles to play in HEV replication and transcription (Fig. 1.1) [5]. ORF1 extends 5 kb from the 5′ end, consists of 5,079 nt and encodes the nonstructural proteins required for replication and protein processing, including an RNA helicase, an RNA-dependent RNA polymerase, a methyltransferase, and a cysteine protease [5][6]. ORF2, which is of 1,980 nt in length, contains a broadly reactive, diagnostically useful epitope at its 3' end and a signal sequence at its extreme 3′ end and encodes a viral capsid protein [5]. The smallest ORF, ORF3, consists of 372 nt and overlaps ORF1 by one nucleotide at the 5′ end and extensively overlaps ORF2 by 328 nt at the 3′ end [5]. In a very recent study, it was found that the ORF3 encodes a protein that functions as an ion channel and has viroporin activity [7]. At present, there are approximately 1,600 HEV sequences available at the International Nucleotide Sequence Database Collaboration (INSDC, previously known as DDBJ/EMBL/GenBank) and the number of sequences is expanding at a great rate [5].

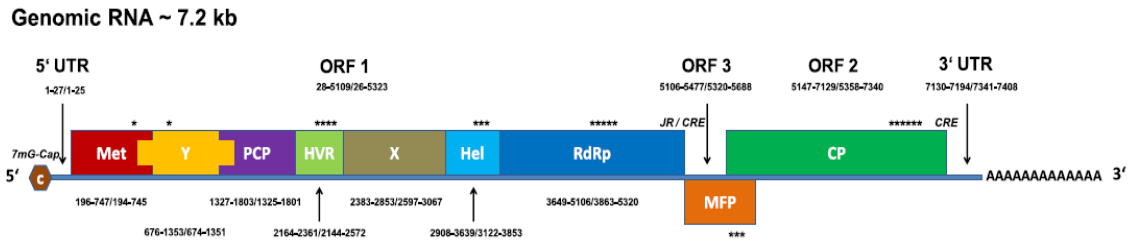

Figure 1.1 [18]: Schematic description of the HEV genome and viral proteins. The Figure shows linear, ssRNA (+) genome of ~7.2 kb HEV genome and corresponding viral proteins. The 5′-end is capped and the 3′-terminus is polyadenylated. ORF1

encodes the nonstructural polyprotein, including methyltransferase (Met), Y-domain (Y), papain-like cysteine protease (PCP), hypervariable region (HVR), macro-domain (X), RNA helicase (Hel) and RNA-dependent RNA polymerase (RdRp). The ORF2 encodes the capsid protein (CP). The ORF3 encodes a small multifunctional protein (MFP). CRE is cis-reactive element; Nucleotide positions are relative to the HEV-1 Burmese strain (Acc. No. M73218)/HEV-3 47832 strain (Acc. No. KC618402). Asterisk (*) indicates the hot spot region for clinical mutations.

1.32 Life Cycle of Hepatitis E Virus

Although the life cycle of Hepatitis E Virus (HEV) relates to other ssRNA viruses, it calls for further examination (Fig. 1.2). HEV attaches to the cells via the interaction of ORF2 (i.e. the capsid protein) with attachment receptors such as heparan sulfate proteoglycans (HSPGs) and heat shock cognate protein 70 (HSC70) and enters the cells through dynamin-2, clathrin,membrane cholesterol and actin dependent endocytosis [15]. After entry, the virion uncoats and releases the viral RNA into the cytoplasm where the 7-methylguanosine cap structure in the 5' non-coding region (NCR) of the HEV genome recruits the 40S ribosomal subunit to initiate cap-dependent translation of ORF1 polyproteins which include viral enzymes. The viral genomes are replicated by the viral RNA helicase and RdRp [16]. The ORF2 and ORF3 proteins are also translated from the viral subgenomic RNA [16]. The replication complex of HEV is likely positioned at the ER Golgi intermediate compartment, where the viral proteins and positive single-stranded RNA could be localized [17]. The assembly of RNA and ORF2 protein forms the progeny viral particles, which are then released from the host cells via the ORF3 protein which has viroporin activity that is critical for viral egress [7].

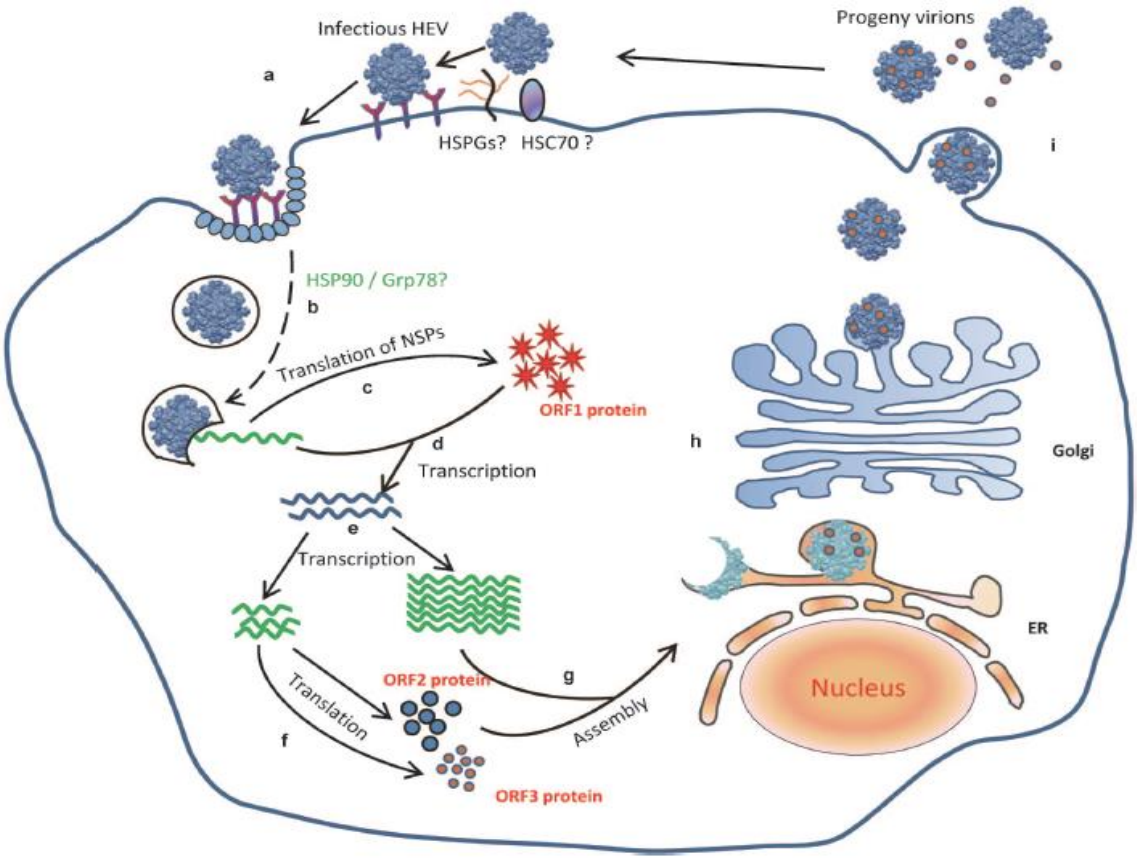

Figure 1.2 [15]: Proposed life cycle of HEV;

Step a: HEV attaches to the cell surface via HSPGs, HSC70 or other putative attachment receptor(s) and then enters the cell via a unknown specific cellular receptor.

Step b: The HEV virion penetrates the membrane and enters the cells. HSP90 and Grp78 may be involved in this transport. The virion then uncoats and releases the positive-sense genomic RNA into the cytoplasm of the cell.

Step c: The positive-sense genomic viral RNA serves as the template to translate the ORF1 nonstructural polyprotein in the cytoplasm.

Step d: The viral RdRp synthesizes an intermediate, replicative negative-sense RNA from the positive-sense genomic RNA that (**Step e**) serves as the template for the production of positive-sense, progeny viral genomes.

Step f: The ORF2 and ORF3 proteins are translated from the subgenomic, positive-stranded RNA, and (**Step g**) the ORF2 capsid protein packages the genomic viral RNA and assembles new virions.

Step h: The nascent virions are transported to the cell membrane; The ORF3 protein facilitates the trafficking of the virion.

Step i: The nascent virions are released from the infected cells;

1.33 Genotypes and Subtypes of Hepatitis E virus

To date, HEV is known to have one serotype and four reported genotypes namely, genotypes 1, 2, 3, and 4 according to analysis of either the complete genome sequence and/or the 371 base region, the largest base region from ORF1, spanning nucleotide 80–450, since it was demonstrated unequivocally that the use of this region in phylogenetic analysis generates a tree consistent with full length sequences [5][8]. The prevalence and distribution of HEV infection varies genotypically by global region (Fig. 1.3). Genotype 1 is epidemic in developing countries in Asia and North Africa while Genotype 2 is mostly found in Mexico and in central African countries and both these genotypes occur exclusively in humans [9]. Genotype 3 is widely distributed and has been isolated from humans in North and South America, Europe, Japan, and the Pacific region and in domestic pigs in many countries except in Africa. Genotype 4 has been isolated from humans in China, Japan, Taiwan, and Vietnam and from domestic pigs, boar, and deer in many countries [9]. Therefore genotypes 3 and 4 suggest a zoonotic nature of HEV. A new genotype or strain of HEV was isolated from chicken with hepatitis-splenomegaly syndrome and was proposed to belong to either a new genotype 5 or to a separate genus [5]. HEV genotype 1 is more conserved and is classified into five subtypes, 1a, 1b, 1c, 1d, and 1e and Genotype 2 sequences are classified into two subtypes, 2a and 2b. Genotype 3 and 4 are extremely diverse and are divided into ten subtypes, 3a, 3b, 3c, 3d, 3e, 3f, 3g, 3h, 3i, and 3j and seven subtypes 4a, 4b, 4c, 4d, 4e, 4f, and 4g, respectively [5]. These genotypes and subtypes are useful in understanding the disease epidemiology, its mode of transmission and the geographical distribution of the virus.

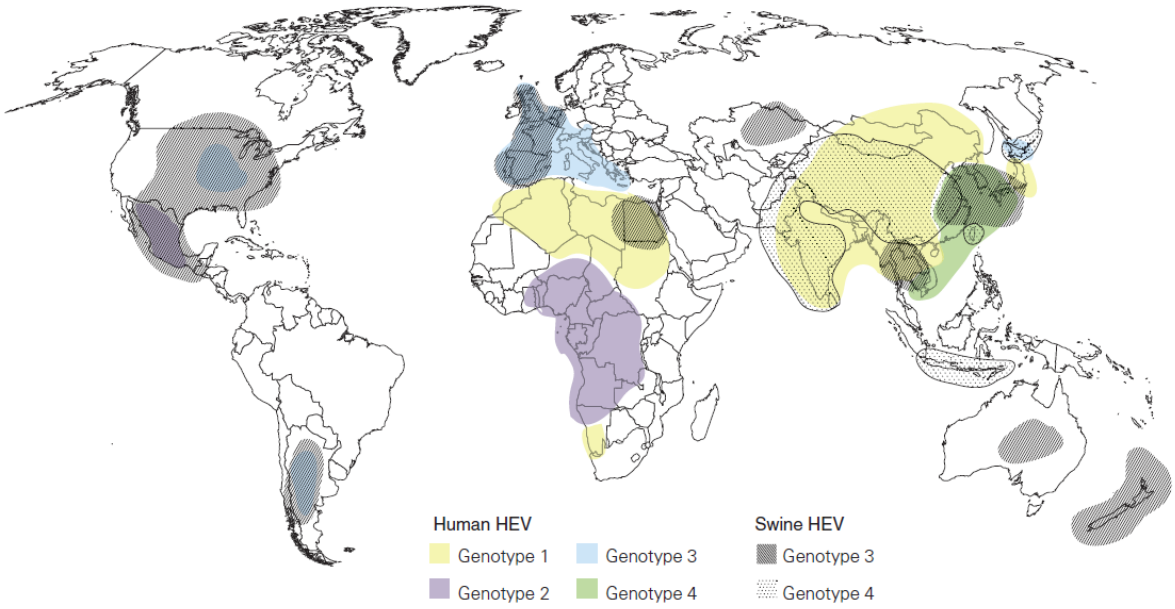

Figure 1.3 [4]: Map showing geographical distribution of hepatitis E virus genotypes among (a) human isolates, and (b) swine isolates

1.4 Epidemiology

Hepatitis E Virus has two distinct pattern of epidemiology in different geographical regions which correlate with the disease prevalence. Large outbreaks involving several hundred to several thousand persons have been reported in the developing countries which include Southeast and Central Asian countries, several parts of the Middle East, Africa and Mexico [4]. The predominant route of transmission of HEV infection in these regions is the fecal–oral transmission (usually through contaminated water). Other routes of transmission include food-borne transmission, transfusion of infected blood products and vertical (materno-fetal) transmission [10]. The peak incidence in sporadic cases of Hepatitis E in endemic regions occurs in adult within the age range of 15 to 40 years. Since HEV infection generally manifests as a self-limiting, acute, icteric hepatitis, the mortality rate is therefore quite low. However, the viral attack rate and the severity have increased among pregnant women with overall mortality rising to 20%-45% in pregnant women in the second and third trimester respectively [3]. The HEV infected women develop fulminant hepatic failure (FHF) more readily (55%) than non-HEV-infected women; maternal mortality was also found to be higher secondary to FHF in the HEV-infected group (41%) vs. 7% in the non-HEV group [10]. Also, the disease can progress to chronicity in immune-compromised patients, notably

organ transplant recipients and individuals co-infected with HIV [7]. Recently a high seroprevalence of anti-HEV antibodies have been reported in the epidemiological studies carried out in the developed countries suggesting a zoonotic transmission or exposure to the virus from travel to HEV endemic areas.

1.41 Hepatitis E Virus Infection in Bangladesh

Seasonal epidemics are known to occur in endemic populations of South and Southeast Asia, especially in India and Nepal. However, the epidemiologic profiling of HEV in Bangladesh has not yet been rightly done. In one case study conducted by icddr,b (International Centre for Diarrhoeal Disease Research, Bangladesh) in 2008, it was reported that a large outbreak of jaundice (>4000 suspected cases) in a densely populated, low-income, urban community in Bangladesh was likely due to HEV, with the men working outside home being at the highest risk of developing the illness and a high mortality rate recorded among women with confirmed pregnancies and their neonates, or women of reproductive age whose pregnancy status was unconfirmed [12]. In the early 1990s, the reports published of travelers to Bangladesh acquiring HEV infections, followed by a documented outbreak of Hepatitis E in a group of Bangladeshi UN peacekeepers suggested very strongly of the HEV endemicity in Bangladesh [1]. Also, in another study executed between 2003 and 2005, the baseline seroprevalence to HEV in an enteric-disease–prone population of rural Bangladesh was found to be 22.5% which was similar to that of the neighboring South Asian countries [13]. HEV in Bangladesh is known to spread mainly by fecal contamination of the municipal water system and since Bangladesh is networked with rivers and there is flood annually, the occurrence of HEV outbreaks is high. Like its neighboring country India and parts of Nepal, poor sanitary conditions and enteric risk factors exist in Bangladesh. Given the high infant and maternal mortality rates in Bangladesh, HEV may be an important contributor to poor pregnancy outcomes, deaths in late pregnancy, and infant mortality in early life. Better data on the true burden of HEV need to be assembled to provide evidence of HEV endemicity in Bangladesh.

1.5 Clinical Course and Pathogenesis of HEV Infection

HEV infections are mostly known to have a clinically silent course. The infections are rarely associated with clinical symptoms during childhood and frequent detection of anti-HEV antibodies among residents of endemic regions in the absence of a history of prior acute hepatitis indicates that asymptomatic HEV infection is common [4][6]. In symptomatic cases, the incubation period ranges from 2 to 8 weeks, with a mean of 40 days, although some patients have a somewhat prolonged illness with prominent cholestatic manifestations. In studies where experimental cynomolgus monkeys were infected with HEV, it was shown that on day 9 the virus was first detected in serum and reached its peak at higher titer by PCR between days 14 and 23, disappearing by day 28 and on day 27 serum anti-HEV IgG appeared at the height of ALT elevation (Fig 1.4) [5]. Initial symptoms of acute Hepatitis E are typically unspecific and include flulike myalgia, arthralgia, weakness, nausea, vomiting, jaundice, dark urine, anorexia, enlarged tender liver, abdominal pain, and tenderness accompanied by increased levels of liver transaminases, bilirubin, alkaline phosphatase, and γ-glutamyltransferase [6]. The disease may range in severity from sub-clinical to fulminant in patients with underlying chronic liver diseases and also during pregnancy where the death rate approaches 20%-45% [3]. The severe course of infection in pregnant women might result from the hormonal balance and immunologic complexity during pregnancy. Both the reduced expression of the progesterone receptor in HEV infected pregnant women and a high viral load due to weaker T cell responses are the factors associated with fatal outcomes of this disease during pregnancy [6]. The discovery of HEV quasispecies in serum and cerebrospinal fluid additionally suggest a possible role in neurological disorders and relate to the emergence of neurotropic HEV variants which infect extrahepatic tissues and cause local tissue damage and inflammation. However, the mechanism of extrahepatic manifestations by HEV still needs to be further elucidated [14].

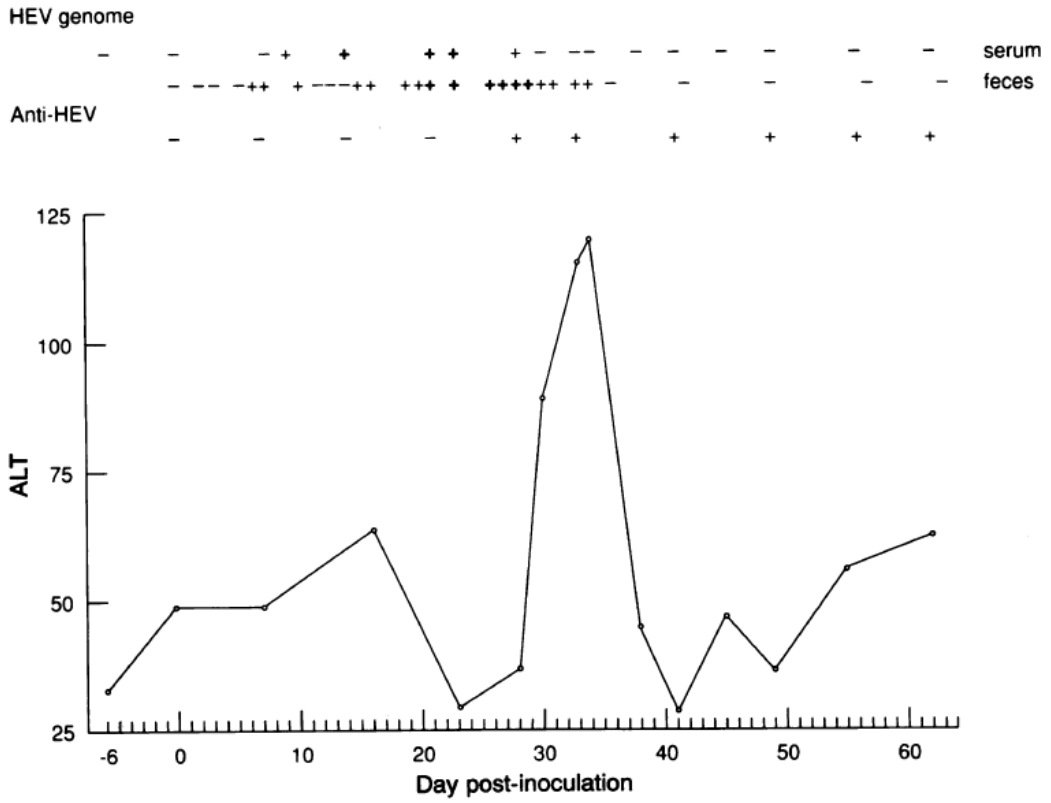

Figure 1.4 [34]: Evidence for SAR-55 infection of Cyno-376. Serum alanine aminotransferase (ALT) levels are plotted as units/liter and the presence (+) or absence (-) of viral genomes or antibody to HEV is indicated. Viral genomes in the feces and serum were detected by RT-PCR. The presence of total serum antibodies to HEV was monitored by ELISA using recombinant antigens from both the Mexican and Burmese strains of HEV

1.6 Laboratory Diagnosis of HEV Infection

The routine laboratory diagnosis of Hepatitis E depends on serology and nucleic acid amplification techniques. The serological assays were designed for the detection of serum antibodies to HEV (IgA, IgM, and IgG). The diagnostic assays for anti-HEV antibodies, which are commercially available, are based on recombinant proteins or synthetic peptides derived from ORFs 2 and 3 [19][20]. HEV-specific IgM is usually detectable at the onset of symptoms or deranged liver function which is why IgM serology is useful for the identification of acute cases of HEV infection [23]. However, strong positive results are very rare after 3 months from the onset of infection and sometimes it is not detected at all. Also, there is always a chance of false positive result [21][22]. HEV antigen-specific IgA is sometimes used as an additional

confirmatory antibody for the diagnosis of acute HEV infection and anti-HEV IgA assays were utilized by many investigators who considered it to be highly specific [5]. Specific IgG on the other hand, reaches a peak shortly after the onset of symptoms and persist for years [23]. A very practical and useful ELISA has been developed which is highly specific for neutralizing antibodies against HEV, and is specifically useful for quantifying the humoral immune response for future Hepatitis E vaccine trials and the durability of the immune response [5]. Two of such commercially available HEV antibody assays are Abbott Immunoglobulin G Assay and Genelabs IgG Assay [24]. To detect HEV RNA in clinical specimens (mainly blood) conventional and real-time RT-PCR assays are used which tend to be more sensitive than serology for Hepatitis E diagnosis [23]. Assuming that there is no contamination, a positive result proves HEV infection and allows for further study including sequencing and genotyping of the infecting viruses. Unfortunately, the viral load of HEV in blood remains detectable for a range of 17–48 days (mean of 28 days) which is a very narrow window and therefore infected patients show a negative result for the presence of HEV RNA since they show up until sometime after the onset of illness [25]. Therefore, along with the demonstration of HEV-specific IgM and IgA during the diagnosis of acute Hepatitis E infection, rising levels of IgG, or detection of HEV RNA and a substantially raised serum transaminase concentrations can be useful for detecting an acute HEV infection [26].

1.7 Treatment and Prevention of Hepatitis E Virus

Hepatitis E is one of the major reasons behind high morbidity in the developing countries and therefore viable treatment and prevention measures need to be taken to lower the incidence of this viral infection. Since HEV is a self-limiting disease, the infected individuals who are immune-competent do not require any antiviral therapy in the acute cases of the infection as the immune system is able to clear the HEV infection spontaneously. Treatment options for patients with chronic Hepatitis E include reduction of immunosuppression and administration of pegylated interferon alfa or ribavirin [6]. Patients with pre-existing chronic liver disease who develop hepatic failure as a result of Hepatitis E infection should be considered for liver transplant, since such patients have a poor outcome [23]. Prevention of HEV in regions

where it is sporadic is based on the implementation of appropriate hygiene and sanitary measures to avoid fecal-oral transmission which is the predominant mode of transmission [4]. These include proper treatment and safe disposal of human excreta, provision of safe drinking water supply, implementation of sanitary food-handling practices, and avoiding consumption of undercooked or uncooked meat and vegetables as the virus was found to be heat-labile [4]. Passive immunoprophylaxis is another preventive measure which can be executed where anti-HEV antibodies have been shown to neutralize the virus and to prevent disease in laboratory animals experimentally inoculated with HEV. However, in human studies this measure failed to provide any protection against the disease. HEV infection can also be prevented with effective HEV vaccines and ORF2 is widely used as a target for vaccine development [27]. Since patients with chronic liver disease are most at risk if infected with HEV, vaccination should be targeted at these individuals. However, mutations in ORF2 may lead to a failure of an adaptive cellular immune response in the vaccinated individuals [18]. Therefore mutations taking place in ORF2 that is only sensitive in affecting the ORF2 protein structure is one of the many challenges for producing an efficient vaccine. On the contrary, a number of mutations resulting in HEV attenuation (e.g., F51L, T59A, S390L, N562Q/D/P/Y, L477T, L613T and HVR deletion) may provide a basis for the development of live-attenuated vaccines against HEV [28][29][30]. Other factors that need to be considered when developing an efficient vaacination program include the assessment of the long-term and duration of protection, cost effectiveness as well as tolerability in pregnant women and young children [5]. Therefore, the control and eventual elimination of this disease is very attainable if a highly effective vaccine is developed.

1.8 Aims and Objective

Hepatitis viruses constitute the mainstay of liver diseases in Bangladesh and poses huge burden to the health care delivery system as well as to the economy of Bangladesh [36]. Out of all the hepatitis viruses, HEV is known to be the leading cause of acute hepatitis, acute chronic liver failure (ACLF) and also fatal conditions like acute hepatitis in pregnancy with maternal mortality rate varying from 20%-45% [3][31][37][38]. Little is known about the prevalence of Hepatitis E and the genotype

distribution of the virus in Bangladesh. Therefore, the following study was undertaken to gain information about the sero-prevalence of HEV, to find the widespread distribution of Hepatitis E viral genotype, to find the presence of any Hepatitis co-infection and to assess the impact of these factors in HEV-infected female (both pregnant and non-pregnant) patients in Bangladesh. This project aims to:

- evaluate and study the seroprevalence of HEV and other Hepatitis virus specific immunoglobin molecules in Hepatitis infected patients
- ascertain whether HEV is the predominant virus to cause viral Hepatitis in Bangladesh
- analyze the co-infection rate of HEV with other Hepatitis viruses
- analyze and compare the disease impact of both co-infection and single infection of HEV
- analyze and distinguish the severity of Hepatitis E infection between pregnant and non-pregnant female patients
- analyze the genotype of HEV among the study participants (pregnant and non-pregnant women) who were serologically positive for HEV

Chapter 2:

Methods & Materials

2. Methods & Materials

2.1 Study Population

The study group comprised of patients attending the Department of Obstetrics and Gynecology, Department of Medicine and Department of Hepatology in Dhaka Medical College & Hospital and Bangabandhu Sheikh Mujib Medical University Hospital, Bangladesh during the period from August 1, 2016 to May 28, 2017. A total of 30 female patients (13 pregnant and 17 non-pregnant women) were categorized as acute viral hepatitis and acute liver failure. The patients of acute liver failure were diagnosed on the basis of development of encephalopathy within 8 weeks of the onset of jaundice without any past history of pre-existing chronic liver disease [32]. Acute viral hepatitis was diagnosed as a self-limiting disease and a serum aspartate aminotransferase elevation of at least fivefold or clinical jaundice or both [33]. The patients were evaluated on the basis of history, physical examination, and liver function tests. The pregnant women were examined for a palpable uterus and all were subjected to urine pregnancy test and ultra sonogram to confirm pregnancy. The pregnant patients were observed until delivery for the development of any obstetric complications and the final outcome was vigilantly assessed on the successful completion of pregnancy, maternal death, fetal loss, preterm delivery, and intrauterine death.

The study has been approved by the Ethical Committee of Dhaka Medical College and the Ethical Review Committee of Bangladesh University of Health Sciences, Bangladesh. All patients gave their informed consent prior to enrolment.

2.2 Sample Collection

Blood samples were collected in EDTA vacutainer tubes during the period of four weeks of the onset of clinical symptoms in the patients. Plasma was separated by centrifugation at 3000 rpm for 10 minutes at +4°C and aliquoted and preserved separately in 1.5 ml Eppendorf tubes at -70°C to prevent frequent thawing of the

samples and to keep the viral RNA intact. The first aliquot was used for serological screening and the rest were used to detect HEV RNA and genotyping.

2.3 Serological Analysis

Serological tests were performed using commercially available ELISA kits according to the instructions given in the manufacturer's manual. The various serological tests performed in all the study samples were: detection of anti HAV IgM using WANTAI HAV-IgM ELISA diagnostic kit (Beijing Wantai Biological Pharmacy Enterprise Co., Beijing, CN), detection of HBsAg using bioelisa HBsAg 3.0 (BIOKIT, S.A., Barcelona, Spain), detection of anti-HCV antibodies using bioelisa HCV 4.0 (BIOKIT, S.A., Barcelona, Spain), detection of anti-HEV IgM using WANTAI HEV-IgM ELISA diagnostic kit (Beijing Wantai Biological Pharmacy Enterprise Co., Beijing, CN) and detection of anti-HEV IgG using HEV-IgG ELISA diagnostic kit ((Beijing Wantai Biological Pharmacy Enterprise Co., Beijing, CN). The samples were further examined when they came out positive for both HEV IgM and IgG or HEV IgM or HEV IgG. HEV positive cases were correlated clinically and grouped as acute liver failure and acute viral hepatitis patients and these positive samples were further analyzed for presence of HEV RNA.

2.31 Anti-HEV IgG and IgM ELISA Procedure

WANTAI HEV-IgG ELISA employed solid phase, indirect ELISA method for detection of IgG-class antibodies to HEV (anti-HEV) in two-step incubation procedure. The wells were first prepared by marking three wells for Negative control, two wells for Positive control and one well for Blank. Then, 100µL of the Specimen Diluent was added into each well except the Blank. 10µL of each Positive control, Negative control and specimen was added to their respective wells. The plate was then covered and incubated for 30 minutes at 37^0C. At the end of the incubation, the plated cover was removed and the each of the wells was washed 5 times with Wash Buffer, allowing the microwells to soak for 30-60 seconds after every wash. After the final wash cycle the plate was turned down onto a paper towel and tapped to remove any remainders. Then, 100µL of HRP-Conjugate was added into each well except the

Blank. The plate was covered and incubated for 30 minutes at 37^0C. At the end of the incubation, the wells were again washed 5 times with Wash Buffer, allowing the microwells to soak for 30-60 seconds after every wash. At the end of the wash cycle the plate was turned down onto a paper towel and tapped to remove any remainders. For coloring, Chromagen A and Chromagen B solutions, 50µL each, was added into each well including the Blank. The plate was incubated at 37^0C for 15 minutes, making sure that light is avoided. The enzymatic reaction between the Chromagen solutions and the HRP- Conjugate produced blue color in positive control and in HEV IgG positive sample wells. The reaction was stopped by adding 50µL of Stop Solution into each well. The solution turned yellow in Positive control and HEV IgG positive sample wells. The plate reader was then calibrated with the Blank well and the absorbance was read at 450nm. The cut-off value was calculated using the following formula: Cut-off value (COV) =NC+ 0.26; where NC is the mean absorbance value for three negative controls. If the Sample Optical Density (SOD) was less than the COV then the sample was considered IgG negative but if the SOD was more than the COV the sample was considered to be IgG positive.

The WANTAI HEV-IgM ELISA employed the exact same procedure (as that of WANTAI HEV-IgG ELISA) for the detection of anti-HEV IgM from the serum samples of the patients in this study except anti-human IgM antibodies (anti-IgM) conjugated to horseradish peroxidase (HRP-conjugate) was used in this procedure. The cut-off value was calculated using the following formula: Cut-off value (COV) =NC+ 0.26; where NC is the mean absorbance value for three negative controls. If the Sample Optical Density (SOD) was less than the COV then the sample was considered IgM negative. However, if the SOD is more than the COV the sample was considered as IgM positive.

2.32 Anti-HAV IgM ELISA Procedure

WANTAI HAV-IgM ELISA is a solid-phase, two-step incubation antibody capture ELISA assay in which, polystyrene microwell strips are pre-coated with antibodies directed to human immunoglobin M proteins (anti-µ chain). At first, the wells were prepared by marking three wells for Negative control, two wells for Positive control and one well for Blank. The Specimen was diluted 1:1000 with normal saline. Then,

50µL of Positive and Negative Control and 100µL of the Specimen was added to their respective wells except the Blank and mixed by tapping the plate gently. The plate was covered and incubated for 20 minutes at 37^0C. At the end of the incubation, the plate cover was removed and the each of the wells was washed 5 times with diluted Wash Buffer, allowing the microwells to soak for 30-60 seconds after every wash. After the final wash cycle the plate was turned down onto a paper towel and tapped to remove any remainders. Then, 100µL of HRP-Conjugate was added into each well except the Blank. The plate was covered and incubated for 40 minutes at 37^0C. At the end of the incubation, the wells were again washed 5 times with Wash Buffer, allowing the microwells to soak for 30-60 seconds after every wash. At the end of the wash, the plate was turned down onto a paper towel and tapped to remove any remainders. For coloring, Chromagen A and Chromagen B solutions, 50µL each, was added into each well including the Blank. The plate was incubated at 37^0C for 15 minutes, making sure that light was avoided. The enzymatic reaction between the Chromagen solutions and the HRP- Conjugate produced blue color in the positive control and in the HAV IgM positive sample wells. The reaction was stopped by adding 50µL of Stop Solution into each well. The solution turned yellow in Positive control and HEV IgG positive sample wells. The plate reader was then calibrated with the Blank well and the absorbance was read at 450nm. The cut-off value was calculated using the following formula: Cut-off value (COV) =NC+ 0.16; where NC is the mean absorbance value for three negative controls. If the Sample Optical Density (SOD) was less than the COV then the sample was considered HAV IgM negative but if the SOD was more than the COV the sample was considered to be HAV IgM positive.

2.33 Anti-HCV IgG and IgM ELISA Procedure

"bioelisa HCV 4.0" was an immunoenzymatic method in which the wells of a microplate were coated with recombinant antigens representing epitopes of HCV: Core, NS3, NS4 and NS5. The number of strips was chosen as required for the test (Table 2.1). At first, 8 wells were reserved for blank and controls. The sample diluents were then added at 200µl of volume in rest of the wells and 10µl of each sample was added to the designated wells. If the antibodies specific for HCV were present in the samples, they formed stable complexes with the HCV antigens on the wells. Then, 200µl of negative control were transferred into each of 2 wells, 200µl of low positive

control into each 3 wells and 200µl of positive control into each of 2 wells and one well was left empty for the substrate blank. The microplate was then covered with an adhesive seal and incubated for 1 hour at 37°C. After the incubation, the adhesive seal was removed and discarded. The contents of the wells were aspirated and were filled completely (approximately 350µl) with the washing solution. The process of aspiration and washing was repeated 5 more times allowing each column of wells to soak for at least 15 seconds before the next aspiration cycle. After the last washing, the microplate was blotted on a pad of absorbent tissue to remove any excess liquid from the wells. Then, 100µl of diluted conjugate (rabbit anti-human IgG conjugated with peroxidase) was transferred into each well except the one reserved for the substrate blank. The conjugate would bind to any antigen-antibody complexes formed. Any formation of bubbles was avoided upon addition of the diluted conjugate. The plate was again covered with an adhesive seal and incubated for 30 minutes at 37°C. During the last 5-10 minutes of this incubation the substrate-chromogen solution was prepared. If the entire plate was used 280µl of chromogen (TMB) was added to the bottle containing the substrate buffer (14ml) and were mixed well. If the entire plate was not used, then Table 2.1 was followed. The final solution was colourless. After the incubation, the adhesive seal was removed and discarded. The contents of the wells were aspirated and were filled completely (approximately 350µl) with the washing solution, repeating this process 5 more times, allowing each column of wells to soak for at least 15 seconds before the next aspiration cycle. Then, 100µl of the substrate-TMB solution was added to each well, including the blank. The plate was incubated for 30 minutes at room temperature (20-25°C). This solution would develop a blue colour if the sample was positive. The blue colour changed to yellow after blocking the reaction by adding 100µl of stop solution (sulphuric acid) in the same sequence and time intervals as for the substrate-TMB. The plate reader was then calibrated with the Blank well and the absorbance was read well within 30 minutes at 450nm. The intensity of colour was proportional to anti-HCV antibodies concentration in the sample. Wells containing negative samples remained colourless. The mean absorbance of the low positive control (LPCx) was calculated. The obtained value, multiplied by 0.9, was the cut-off value. Therefore, Cut-off = LPCx x 0.9; the absorbance of the sample was then divided by the cut-off value. If the samples were positive, the ratio absorbance/cut-off should be greater than or equal to1.0. If the samples were negative then the ratio

absorbance/cut-off should be less than 0.9. If equivocal, then the ratio absorbance/cut-off should be greater than or equal to 0.9 but less than 1.0.

Strips required	1	2	4	6	8	10	12
Conjugate diluent (ml)	1.0	2.0	4.0	6.0	8.0	10.0	12.0
Concentrate conjugate (µl)	20	40	80	120	160	200	240

Table 2.1: Usage of specific volumes of conjugate diluents and concentrate conjugate according to the number of strips

2.34 HBsAg ELISA Procedure

"bioelisa HBsAg 3.0" is a direct immune-enzymatic method of the "sandwich" type in which guinea pig anti-HBs antibodies are coated to microplate wells and act as the capture antibody. The numbers of strips were chosen as required for the test (Table 2.1). At first, three wells were marked for Negative control, one well for Positive control and one well for Blank which was left empty. Then, 100µl of each control, positive and negative, were transferred to their assigned wells. Then 100µl of each of the samples to be analyzed were transferred to the corresponding wells. The plate was then covered with an adhesive seal and incubated for 1 hour at 37°C. If the sample contained HBsAg, the antigen would bind to the antibody on the plate. After the incubation, the adhesive seal was removed and discarded. The contents of the wells were aspirated and were filled completely (approximately 350µl) with the washing solution. The process of aspiration and washing was repeated 3 more times allowing each column of wells to soak for at least 15 seconds before the next aspiration cycle. Then, 100µl of diluted conjugate (goat anti-HBs antibodies marked with peroxidase) was transferred into each well except the one reserved for the substrate blank. The plate was again covered with an adhesive seal and incubated for 30 minutes at 37°C. The conjugate would react with the antigen-antibody complex if any formed in the first incubation. During the last 5-10 minutes of this incubation the substrate-chromogen solution is prepared. If the entire plate was used 280µl of chromogen (TMB) was added to the bottle containing the substrate buffer (14ml) and mixed well. If the entire plate was not used, then Table 2.1 was followed. This working substrate solution was

pink in colour and if changed to blue at this step then it was discarded. After the incubation, the adhesive seal was removed and discarded. The contents of the wells were aspirated and were filled completely (approximately 350µl) with the washing solution, repeating this process 3 more times, allowing each column of wells to soak for at least 15 seconds before the next aspiration cycle. Then, 100µl of the substrate-TMB solution was added to each well, including the blank. The plate was incubated for 30 minutes at room temperature (20-25°C). This solution would develop a blue colour if the sample was positive for HBsAg. The blue colour changed to yellow after blocking the reaction by adding 100µl of stop solution (sulphuric acid) in the same sequence and time intervals as for the substrate-TMB. The plate reader was then calibrated with the Blank well and the absorbance was read well within 30 minutes at 450nm. The intensity of colour was proportional to the amount of HBsAg in the test specimens. The wells containing negative samples remained colourless. The mean absorbance of the negative control (NCx) was calculated. The obtained value must be less than 0.120, after subtracting the blank. The absorbance value obtained for the positive control (PC) should be equal to or higher than 0.700, after subtracting the blank. The presence or absence of HBsAg in the samples was then determined by relating the absorbance value of each sample to the cut-off value. The cut-off value was calculated by adding 0.040 to the mean absorbance of the negative control (i.e. Cut-off = NCx + 0.040). The absorbance of the sample was then divided by the cut-off value. If the samples were positive, the ratio absorbance/cut-off should be greater than or equal to1.0. If the samples were negative, then the ratio absorbance/cut-off should be less than 0.9. If equivocal, then the ratio absorbance/cut-off should be greater than or equal to 0.9 but less than 1.0.

2.4 Extraction and Detection of Viral RNA

Viral RNA was extracted from 140 µL serum using viral RNA extraction kit (QIAamp Viral RNA Mini Kit 52904) according to the manufacturer's instructions. The carrier RNA-buffer AVE mixture was prepared by adding 310 µL Buffer AVE to the tube containing 310 µg lyophilized carrier RNA which was then dissolved thoroughly. This prepared mixture was then divided into aliquots and stored at -30^0c to -15^0c until later use. For each sample, 560 µL of buffer AVL was mixed with 5.6 µL of the carrier

RNA-buffer AVE mixture in a 1.5mL eppendorf tube to which 140 µL of the serum sample was added and mixed thoroughly and incubated at room temperature (15-25^{0}C) for 10 minutes. Then, 560µL of ethanol (96-100%) was added to the sample, mixed by pulse vortexing for 15 seconds and then spun briefly. 630µL of the tube's content was transferred to the QIAamp Mini column without wetting the rim and the column was subjected to centrifugation at 6000×g for 1 minute. The QIAamp Mini column was then placed into a clean 2mL collection and the flow-through was discarded. This step was repeated until all the sample mixture was passed through the QIAamp Mini column. Then 500µL of Buffer AW1 was added to the QIAamp Mini column and subjected to centrifugation at 6000×g for 1minute. The QIAamp Mini column was then placed in a new 2mL collection tube and the flow-through discared. 500µL of Buffer AW2 was then added to the column and centrifuged at 20000×g for 3 minutes. The QIAamp Mini column was again placed in a new 2mL collection tube and the old collection tube was discarded. The column was again centrifuged at 20000×g for 1 minute. After centrifugation the QIAamp Mini column was placed in a new 1.5mL microcentrifuge tube and the old collection tube containing the filtrate was discarded. 60µL of nuclease free water was added to the column to elute the RNA and then incubated at room temperature for 1 minute. Finally the column was centrifuged at 6000×g for 1 minute and the flow through containing the extracted RNA was stored at -70°C in small aliquots.

For detection of the HEV RNA, real-time RT-PCR was carried out for each sample in the Bio-Rad's CFX96 Touch™ Real-Time PCR Detection System, with one set of primers using the SuperScript® III One-Step RT-PCR System with Platinum® Taq DNA Polymerase (cat no.: 11732-020). For each sample, 12.5 µL of specific master mix was prepared which contained 6.25 µL 2X reaction mix, 0.25 µL of each of the primers, 0.125 µL of the designed probe, 0.25 µL of RNase OUT, 0.25 µL of SuperScript® III/Platinum Taq mix, 150ng of the extracted RNA and finally nuclease free water was added to make a final volume of 12.5 µL. These reagents and enzymes present in the master mix were used for the specific amplification of HEV and for the direct detection of the specific amplicons in the fluorescence channels. The primers (sense and antisense) were designed to anneal to a nonvariable region of the ORF2 of all HEV genotypes. They consisted of the forward primer (nt 5237-5255): 5′ - GGTGGTTTCTGGGGTGAC - 3′ and the reverse primer (nt 5289- 5307): 5′-

AGGGGTTGGTTGGATGAA - 3′. Here, the sequence specific hybridization probe (5′ - Cy5/TGATTCTCAGCCCTTCGC/BHQ_2 - 3′) was designed which spanned nt 5260- 5278 of the ORF2 region of the HEV genome, with the flouroscent dye Cy5 included at the 5′ end and its optimal quencher BHQ_2 included at the 3′ end of the probe sequence. The positive and negative control samples and internal controls provided by the manufacturer were included in the amplification step as recommended.

2.5 cDNA synthesis and PCR amplification

cDNA was synthesized from the extracted viral RNA using the SuperScript® III First-Strand Synthesis System kit (Invitrogen: cat no. 18080051) according to the manufacturer's instructions, yielding 20 µL of purified cDNA. At first a total of 10 µL of RNA mixture was prepared in a 0.2 µL tube for each sample and the mixture included 1 µL of random hexamers, 1 µL of 10mM dNTPs (Invitrogen), 2.5 µL of the extracted RNA and finally nuclease free water was added to fill up the remaining volume. This RNA mixture was heated at 65^0C for 5 minutes and then immediately kept on ice for 2-3 minutes. Another mixture was prepared which included the following components: 2 µL of 10x RT buffer, 4 µL of 25mM $MgCl_2$, 2 µL of 0.1 mM DTT, 1 µL of SuperScript® III enzyme (Invitrogen) and 1 µL of RNase OUT. This mixture was then mixed with the prepared RNA mixture to make the final reverse transcription mixture. This reverse transcription mixture was incubated in the Thermal cycler (Bio-Rad) using the following thermal profile: 25^0C for 10 minutes, 50^0C for 50 minutes and 85^0C for 5 minutes. After this incubation step, the cDNA (20 µL) was cooled on ice and then treated with 1 µL of RNase H (Invitrogen) and finally incubated for 20 minutes at 37^0C.

The purified cDNA was then amplified by the Polymerase Chain Reaction (PCR). 5 sets of primers were designed which covered the ORF2 region of the HEV genome (Table 2.2). However, only the third set of primers was used in this step as it was the most efficient among all the sets of primers in detecting and amplifying the specified ORF2 region. The sequence of forward primer (nt 6554- 6573) was 5′-GCCGAGTATGACCAGTCCA- 3′ and the reverse primer (nt 7084- 7104) was 5′-GGGTAAAACTCGGGAGTTAT- 3′. PCR was performed in a final reaction volume

of 10.0 µL containing 1.0 µL of 10X PCR buffer (Qiagen; cat no. 203203), 0.4 µL of 25 mM $MgCl_2$ (Qiagen; cat no. 203203), 2.0 µL 5X Q-solution (Qiagen; cat no. 203203), 1.6 µL 2.5 mM dNTP mixture (Takara; cat no. 4030), 0.5 µL of forward and reverse primer each, 0.05 µL of HotStarTaq DNA polymerase (Qiagen: cat no. 203203), 1.0 µL of the cDNA preparation and finally the total volume was made to 10.0 µL with nuclease-free water. The thermal cycling profile was set as the following: pre-denaturation at 95°C for 15 minutes; 45 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C- 60°C temperature gradient for 50 seconds (the tubes are set at 58°C) and extension at 72°C for 40 seconds; and a final extension at 72°C for 10 minutes. After amplification the PCR products were detected by electrophoresis on a 1% agarose gel and visualized by GelRed staining.

Primer Set No.	Stage Polarity	Sequence (5'-3')
1	Sense	GGCCACCTCTGGTCTTGTTA
	Antisense	GCCGTAAGTGGACTGGTCAT
2	Sense	GTCTCCCGTTACTCCAGCAC
	Antisense	GGTGAGAGAAAGCCAAAGCA
3	Sense	GCCGAGTATGACCAGTCCA
	Antisense	ACAACTCCCGAGTTTTACCC
4	Sense	AATGTTGCGACCGGCGCGC
	Antisense	TAAGGCGCTGAAGCTCAGC
5	Sense	AGGACGGCACCAATACTCAT
	Antisense	AGCCGACGAAATCAATTCTGTC

Table 2.2: Hepatitis E Virus ORF2-Specific Oligonucleotide Primers Used in this Study

2.6 PCR Product Purification

The PCR products were purified on spin columns using the MinElute PCR Purification Kit (Qiagen, Cat.No. 28006). At first, the PCR tubes containing the PCR products were spun and its content was transferred to 1.5mL Eppendorf tubes. Then, 5 volumes of Buffer PB was added to 1 volume of the PCR product and mixed properly. The colour of the solution was checked whether it was yellow (similar to Buffer PB without the PCR sample). If the colour of the solution was orange/violet, then 10 µL 3 M sodium acetate (pH 5.0) was added and mixed by inverting The sample was then transferred to a a MinElute spin column that was placed in a provided 2ml collection tube and incubated for 5 minutes at room temperature. The column was then subjected to centrifugation at 17,900×g for 30-60 seconds. The flow-through was discarded and the MinElute column was placed back into the same collection tube. Then, 750µL of Buffer PE was added to the MinElute column. The column was incubated at room temperature for 5 minutes and was subjected to centrifugation at 17,900×g for 30-60 seconds. The flow-through was again discarded and the MinElute column was placed back into the same collection tube. The column was then centrifuged at 17,900×g for 1 minute. Each of the MinElute columns was then placed in a 1.5 ml micro-centrifuge tube. To elute the dsDNA, 15-20 µL nuclease-free water was carefully added to the center of the column membrane. The column was incubated at room temperature for 5 minutes and then centrifuged for 1 minute at 17,900×g. The column was then discarded and the resulting purified DNA was analyzed on a 1% agarose gel. The purified PCR products were then stored in the -70°C refrigerator.

2.7 Genotypic Characterization of HEV

The purified PCR products were further subjected to cycle sequencing using Big Dye Version 3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) following the manufacturer's instructions. Here, the PCR primers were used for the sequencing of both DNA strands. At first, a total of 10 µL of sequencing mixture was prepared (for each PCR product) that contained the following components: 2.1 µL of PCR buffer (Qiagen), 0.55 µL of Big Dye (Applied Biosystems, CA, USA), 0.2 µL of forward and reverse primer, 1 µL of the purified DNA and 6.3 µL of nuclease-free water. The

thermal profile of the cycle sequencing was as follows: Pre-denaturation at 95°C for 10 minutes; 25 cycles of denaturation at 94°C for 10 seconds, annealing at 57°C for 5 seconds and extension at 60°C for 4 minutes; and finally kept at 4°C until later use.

After cycle sequencing, the products were purified according to the manufacturer's protocol using BigDye® XTerminator™ purification kit (Applied Biosystems). At first the tubes that underwent cycle sequencing were centrifuged at 4000 rpm for 1 minute. Then 45 µL of SAM (Applied Biosystems) was added to each of the tubes followed by the addition of 10 µL X-terminator (Applied Biosystems). The tubes were then vortexed for 30 minutes followed by centrifugation at 4000rpm for 3 minutes. 15 µL of the supernatant was then separated out into PCR tubes. These purified cycle sequencing products were then subjected to capillary electrophoresis through POP-6 (Applied Biosystems) in an ABI PRISM 310 Automated Sequencer (Applied Biosystems). The genotype of each of the samples was determined by comparing its sequence with reference sequences for different HEV genotypes obtained from GenBank using the CLUSTAL W analysis software and the phylogenetic tree was constructed using the MEGA 5.2 software using the neighbor joining method based on the nucleotide sequence of the ORF2 region of the HEV genome.

2.8 Statistical Analysis

All quantitative variables were expressed as mean ± SD. The categorical variables were expressed as numbers. Quantitative variables were compared using unpaired t-test with Welch's correction. The P-values less than 0.05 were evaluated as statistically significant.

Chapter 3: Result

3.0 Result

3.1 Patient Characteristics

3.11 Demographic Profiles of the Patients

The descriptive data on age, level of education attained, the usage of multiple sources of drinking water and the pregnancy status of a total of 30 female patients are summarized in Table 3.1 to find if any correlation exists between their economic status and their maintenance of hygiene with the spread occurrence HEV infection. Out of the 30 patients, 13 were pregnant and the rest were not pregnant. All the patients were urban residents of Bangladesh with an average income of 246,000 bdt/annum. 40% of the patients completed education only till the primary level while 10% had no education at all. Also, the tubewell and municipal water supply were the major drinking water sources for about 73.3% of the patients. Only about 23.3% of the patients consumed boiled water and 3.3% of the patients consumed filtered water. These information indicate that the lack of education among these patients might link to the lack of knowledge regarding personal hygiene maintenance and also the lack of consumption of clean drinking water might be the main sources of Hepatitis infection.

Parameters		Data
Total Number of Patients (all women)	Pregnant	13
	Non-Pregnant	17
Age		30.6 (± 14.01133) (Range: 18- 70 years)
Edcation	No Education	3
	PSC	12
	JSC	6

	SSC	3
	HSC	1
	Graduate	2
	Post- Graduate	3
Sources of drinking water	Municipal supply	11
	Boiled	7
	Tubewell	11
	Filtered	1
Income/Annum	246,000 bdt (± 288814.98 bdt)	
Residence	Urban	

Table 3.1: Demographic Profiles of the Patients

3.12 Clinical and Laboratory Profiles of the Patients

The symptoms of the patients were recorded on a questionnaire by the attending physicians and nurses during the patient's first visit. All patients had visible jaundice. Only patients with bilirubin level greater than 1.2 were included in the study. The blood pressure and pulse rate of most of the patients of this study were normal. However, a high respiration rate (20.67 ± 11.63) was observed in most of the study participants. The clinical features of the patients are summarized in Table 3.2 (A). Biochemical analyses showed that the levels of bilirubin were above the upper normal limit (normal limit: 0.2–1.2 mg/dl) in all patients [Table 3.2 (B)]. The levels of ALT, AST and ALP varied from 24 to 4730 IU/L, 16 to7055 IU/L and 88 to 1803 IU/L respectively. Greater than 79% of the patients had levels of ALT, AST and ALP above the cut-off value (cut-off value of ALT, AST and ALP were 40 IU/L, 37 IU/L and 120 IU/L respectively). The abdominal ultrasonography detected no features of liver cirrhosis or hepatocellular carcinoma in the patients. One patient died of acute liver failure 3 days after presentation. This patient was a 19-year-old woman who had given

birth to her first child 3 days earlier. After the selection of the patients, their blood sample was used for serological analysis to detect the type of hepatitis infection that is present within these patients.

(A) Clinical Profile of the Patients (n=30)		
Parameters		**Data** (Mean ± Standard deviation)
Duration of Jaundice (days)		27 (± 16)
Respiration (per minute)		20.67 (± 11.63)
Pulse (beats/minute)		77.9 (± 11.01)
Blood Pressure (mmHg)	Systolic	110.5 (± 11.35245)
	Diastolic	73 (± 9.340309)

(B) Laboratory Profile of the Patients (n=30)		
Parameters	**Data** (Mean ± Standard deviation)	**Number of Patients** (with parameters above cut-off value)
Bilirubin (mg/dl)	11.53 (± 8.07) (range: 2- 37.4 mg/dl)	30
AST (IU/L)	481.23 (± 1301.08) (range: 16- 7055 IU/L)	25
ALT (IU/L)	650.86 (± 1088.7) (range: 24- 4730 IU/L)	24
ALP (IU/L)	308.6 (± 350.12) (range: 88-1803 IU/L)	25

Table 3.2 Clinical and Laboratory Profiles of the Patients

3.2 Serological Analysis

3.21 Types of Hepatitis Infections Detected

In order to find out the type and prevalence of the hepatitis infection in the study participants (n=30), their serum samples were subjected to serological analysis. The serological tests (using indirect and sandwich ELISA methods) were carried out to detect the presence of specific antibodies against Hepatitis A virus (HAV), Hepatitis B virus (HBV), Hepatitis C virus (HCV) and Hepatitis E virus (HEV). About 96.7% of the patients (i.e. 29 out of 30 patients) were serologically positive for HEV (IgM anti-HEV or IgG anti-HEV or both) infection while 4 (13.33%) patients expressed HBsAg, 3 (10%) patients were positive for IgM anti-HAV and only one (3.33%) patient was serologically positive for IgG anti-HCV(Fig 3.1). It can be clearly seen from figure below that HEV infection was the most common viral hepatitis among the patients and thus the data are consistent with the fact that HEV infection has more prevalence in Bangladesh than other types of Hepatitis infections (i.e. Hepatitis A, B and C). The Sample Optical Density (SOD) of HEV IgM and IgG calculated during the ELISA method are shown in Table 3.3. Both the immonoglobin molecules specific for HEV (anti-HEV IgM and IgG) were together detected in more than half (51.7%) of these HEV positive samples (i.e. 15 out of 29 samples) with an average sample optical density (SOD) of 2.32 for anti-HEV IgM and 2.16 for anti-HEV IgG. The patients who were serologically positive for Hepatitis A or B were also serologically positive for Hepatitis E. These co-infections resulted due to the co-presence of two different types of the hepatitis virus in the patients' system. This is more elaborately explained in the next section.

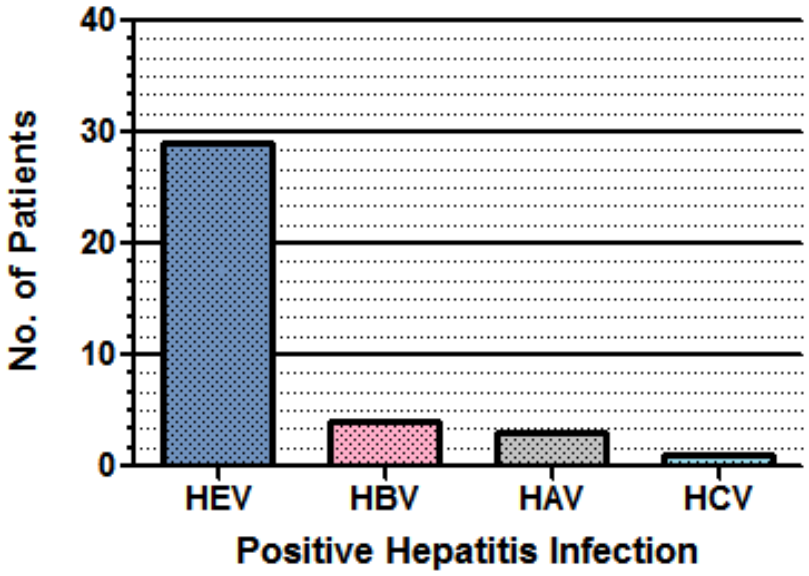

Figure 3.1: Types of Hepatitis Infection Detected in the Serum Samples (n=30)

Type of Hepatitis	Immunoglobin/ Antigen present	Number of Patients	SOD range	Mean SOD ± SD
Hepatitis E	anti-HEV IgM	2	0.92-2.6	1.76 ± 1.34
	anti-HEV IgG	12	0.47-2.6	1.39 ± 0.70
	anti-HEV IgM and IgG	15	IgM (1.8-2.7)	2.3173 ± 0.24
			IgG (0.33-2.7)	2.1647 ± 0.55
Hepatitis A	anti-HAV IgM	3	0.23- 2.7	1.196667 ± 1.077415
Hepatitis B	HBsAg	4	5.0-10.0	7.325 ± 1.92592
Hepatitis C	anti-HCV IgG	1	No range	25.8 ± 0

Table 3.3: HEV ELISA Result

3.22 Presence of Co-infection and its Significance

The viral hepatitis detected in the patients of this study was present as both mono-infection and co-infection. The co-infections of Hepatitis E virus (HEV) with either Hepatitis A virus (HAV), or Hepatitis B virus (HBV) were detected in a total of 7 (23.33%) patients and the highest co-infections were observed for HEV and HBV (Figure 3.2). This indicates that hepatitis co-infections do occur in Bangladesh and that co-infection prevalence is quite high. No co-infection with Hepatitis C virus and Hepatitis E virus was detected in the serum samples. This was very much expected since the occurrence of Hepatitis C mono-infection is generally very low and the chance of co-infection with this virus therefore would be highly unlikely.

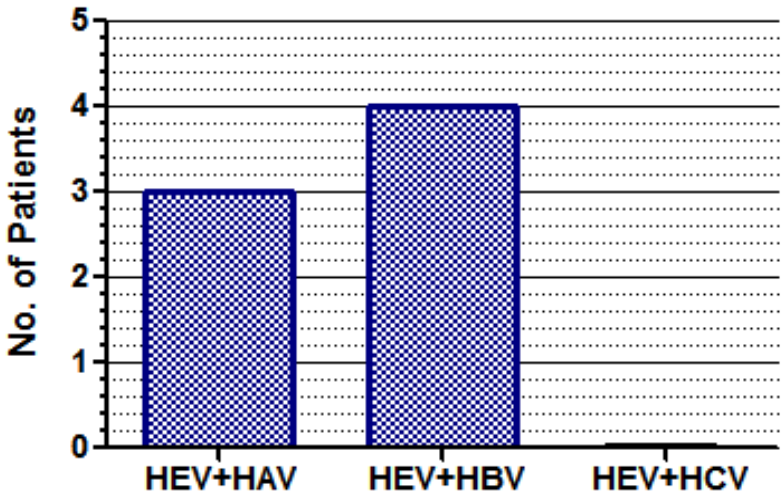

Figure 3.2: Presence of Hepatitis co-infection in the study patients

The Hepatitis E mono-infections and co-infections detected in the patients were then correlated with their respective levels of bilirubin and the liver enzymes i.e. Aspartate transaminase (AST), Alanine transaminase (ALT) and Alkaline phosphatase (ALP), to test the differences in the severity of Hepatitis infection in both the mono-infection and co-infection states [Fig. 3.3 (A-D)]. In Figure 3.3, the statistical analysis was performed using the unpaired t-test with Welch's correction. It is shown in Figure 3.3(A) that the average level of bilirubin in the patients having co-infection with HEV and HBV was 14.13±1.905 (Mean ± Standard Deviation) and that of patients co-infected with HEV and HAV was 8.133±1.915. Patients infected with only HEV had a mean bilirubin level of 11.19±1.920 and this was compared with the bilirubin levels of

the patients having the co-infection. The differences were not significant. Similarly, the average AST levels in patients having co-infection with HEV and HBV (405.3 ± 223.3) and the average AST levels in patients having co-infection with HEV and HAV (368.3 ± 307.0) were compared with the mean AST levels in patients infected with only HEV (525.0 ± 315.8) [Figure 3.3(B)]. There were no significant differences found. The average ALT levels in patients having co-infection with HEV and HBV (470.0 ± 222.0) and that in patients having co-infection with HEV and HAV (1982 ± 1394) were also compared with the mean ALT levels in patients with HEV mono-infection (528.5 ± 186.2) [Figure 3.3(C)]. The differences yet again turned out to be insignificant. On the other hand, Figure 3.3(D) revealed that the mean ALP level in patients having co-infection with HEV and HAV (129.0 ± 6.110) was found to be significantly higher (P value = 0.0148) than that of patients infected with only HEV (345.5 ± 81.62). Insignificant differences in the mean ALP levels were found between the co-infected patients with HEV and HBV (231.0 ± 52.36) and patients with HEV mono-infection (345.5 ± 81.62). One of the pregnant patients having co-infection with HEV and HBV died within three days after hospital admission. Although the patient did not have a significantly higher level of bilirubin and liver enzymes, the infection still turned out to be fatal and resulted in the death of the patients. This indicates the fact that the liver disease markers (i.e. elevated bilirubin and liver enzymes) cannot always be used to analyze the severity of hepatitis infection and that other factors may contribute to the fatality of the disease.

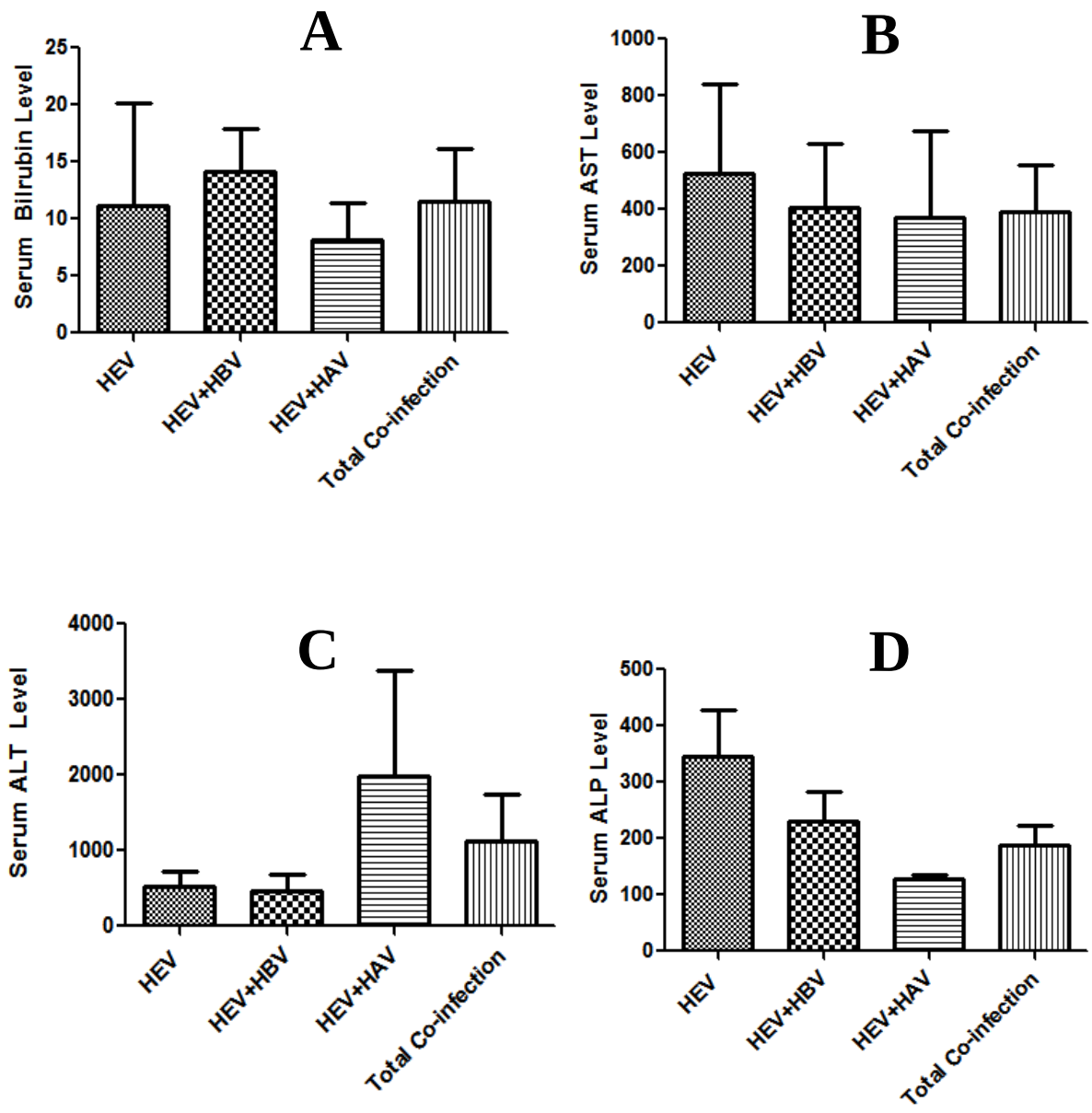

Figure 3.3: Comparison of the level of Bilirubin and Liver Enzymes in patients with Co-infection (HEV+HBV or HEV+HAV) and HEV monoinfection;

(A) Bilirubin Level Analysis

(B) AST Level Analysis

(C) ALT Level Analysis

(D) ALP Level Analysis

3.3 Severity of Hepatitis Infection between Pregnant and Non-pregnant patients

The elevated serum bilirubin and the liver enzymes in the patients of this study were analyzed to find out if any difference in the disease severity exist between the pregnant patients (n=13) and non-pregnant patients (n= 16) [Fig. 3.4 (A-D)]. No significant difference was found between the mean levels of bilirubin in the pregnant patients (9.623 ± 1.487) and that of the non-pregnant patients (13.11 ± 2.461 N=16). Insignificant differences were also found in the mean level of the liver enzymes between the pregnant (serum AST level: 187.6 ± 68.13; serum ALT: 258.4 ± 98.40; serum ALP: 222.5 ± 19.81) and the non-pregnant patients (serum AST level: 688.6 ± 431.4; serum ALT: 947.3 ± 349.6; serum ALP: 389.3 ± 116.6).

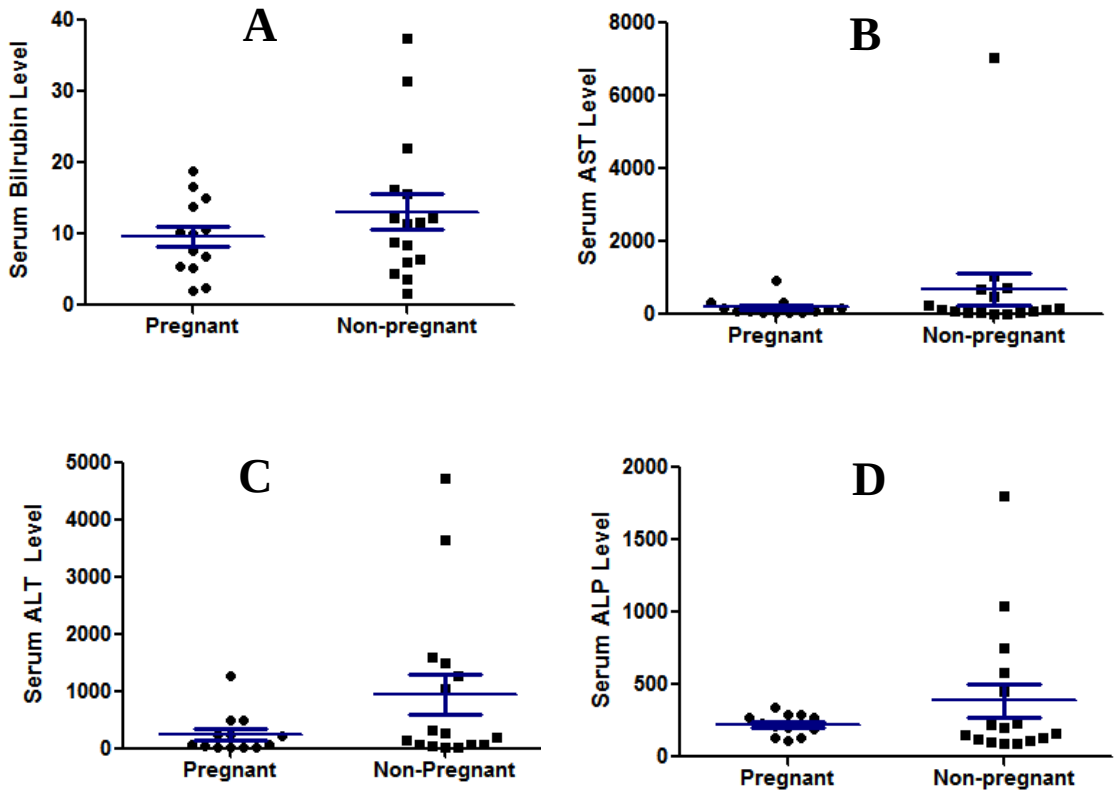

Figure 3.4: Severity of Hepatitis Infection between Pregnant and Non-pregnant patients by

(A) analysis of the level of serum bilirubin **(B)** analysis of the level of serum AST

(C) analysis of the level of serum ALT **(D)** analysis of the level of serum ALP

3.4 Hepatitis E Viral RNA Detection

The study participants that were serologically positive for HEV might not necessarily still have the active virus within their system. Therefore, to detect the presence of the viral RNA, a single-step real-time RT-PCR was carried out. Out of 29 serologically positive HEV infection cases, 15 (51.7%) were found positive for HEV RNA (Fig. 3.5). Out of 2 HEV-IgM positive cases, 1 was HEV RNA-positive. Among the 15 serum samples that were serologically positive for both HEV IgM and IgG, 14 were positive for HEV-RNA while only one came out to be HEV-RNA negative. HEV-RNA was not detected in any of the 12 HEV-IgG positive samples. This indicates that when IgG is the predominant immunoglobin within the patient's circulation, after its class-switching from IgM, the existence of any HEV particles is eliminated.

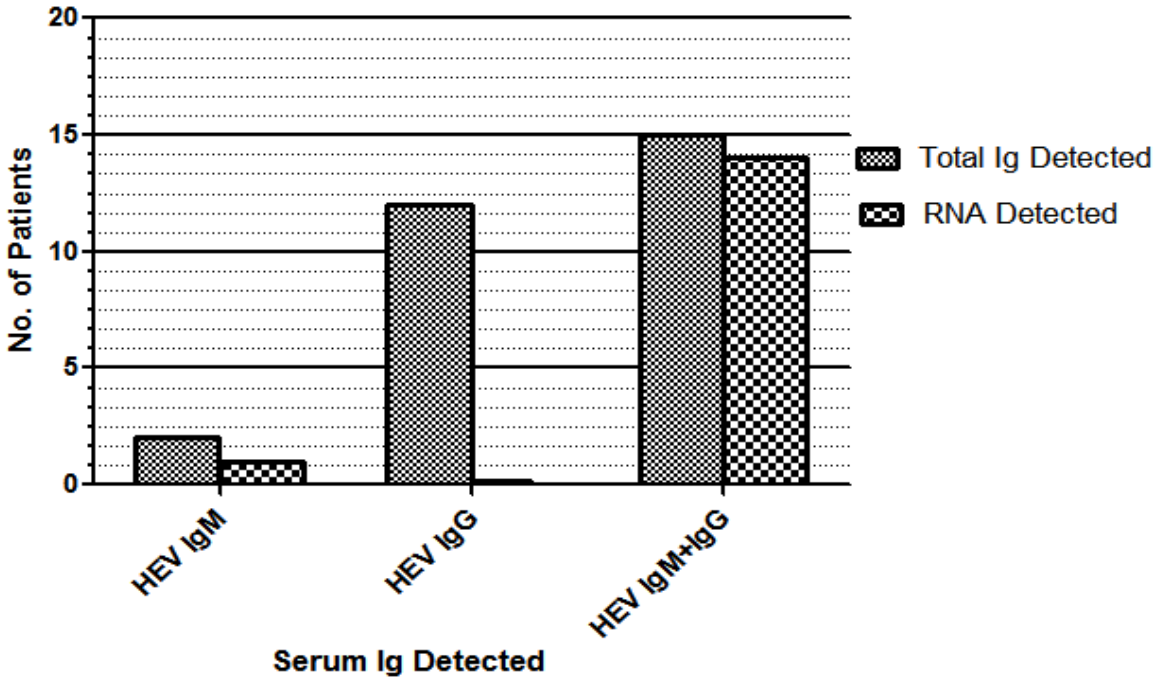

Figure 3.5: Hepatitis E Viral RNA detection in serologically positive HEV samples

3.5 Primer Selection

In order to amplify the required ORF2 region of the HEV genome, five sets of primers were designed. The primers' sequences are mentioned in Table 2.2. In order to analyze which primers produced the desired PCR products, agarose gel electrophoresis was performed on the PCR products on a 1% agarose gel which was visualized by GelRed staining. Out of these five sets, only one set (i.e. Primer Set 3) very efficiently amplified the required region as shown in the gel image in Fig 3.6. The product size of this primer set was 550bp. The rest of the primers did not produce any PCR products.

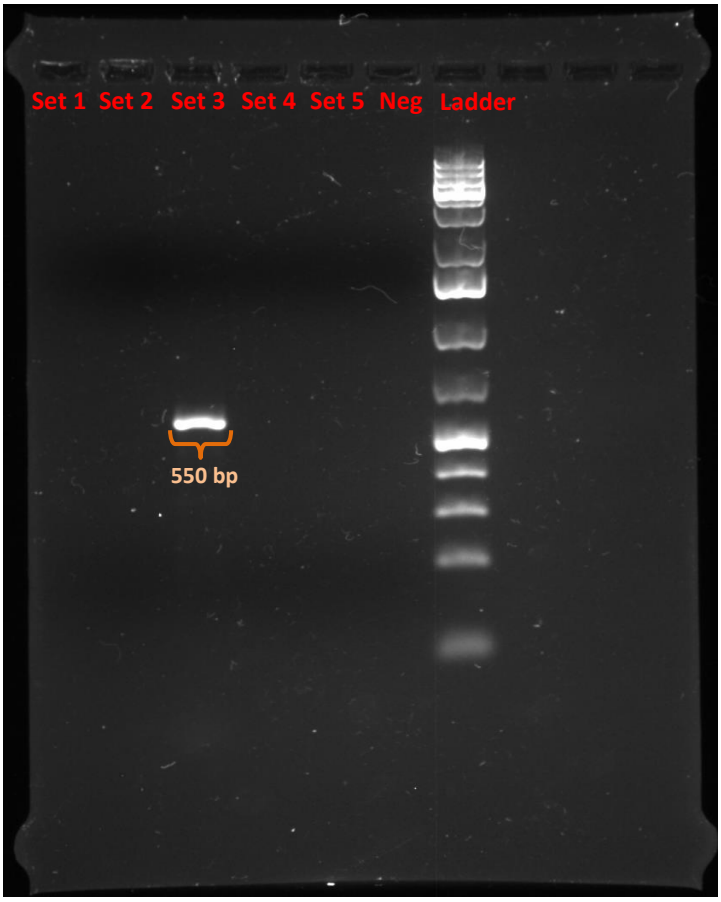

Figure 3.6: GelRed stained agarose gel electrophoresis of RT-PCR product of HEV-RNA showing desired amplicon of 550 bp produced by only primer set 3 after amplification by conventional PCR; Here, each of the primer sets is denoted by its respective number i.e. Set 1, Set 2, Set 3, Set 4 and Set 5; Neg: Negative control;

3.6 Phylogenetic Analysis

The HEV RNA-positive samples were amplified in the ORF2 region and then sequenced. Comparison with published reference sequences from GenBank showed that these samples clustered with genotype 1 in the phylogenetic tree of HEV (indicated by the orange dotted line in Fig. 3.7) that was constructed by neighbor-joining method based on the partial nucleotide sequence (550-nt) of the open reading frame (ORF) 2 region of the HEV isolates collected. In Fig. 3.7 the isolates from the current study are denoted by a prefix "S" followed by a number which together represents the sample number, followed by the three-letter country code (i.e. BAN for Bangladesh). The reference sequences are denoted by a prefix which represent the HEV genotype (G1, G2, G3, G4, G5) followed by its Accession No. and then a 3 letter country code (BAN: Bangladesh, IND: India, CHN: China, MEX: Mexico, JPN: Japan, DEU: Germany, KGZ: Kyrgyzstan, ITA: Italy) to indicate the country from which it has been isolated. Bootstrap values for the all the branches are given as percentages of trees obtained from 1,000 re-samplings of the data. The genome of Rubella virus (NC_001545.2) was used as an out-group because it is known to somewhat resemble the genome of HEV [35].

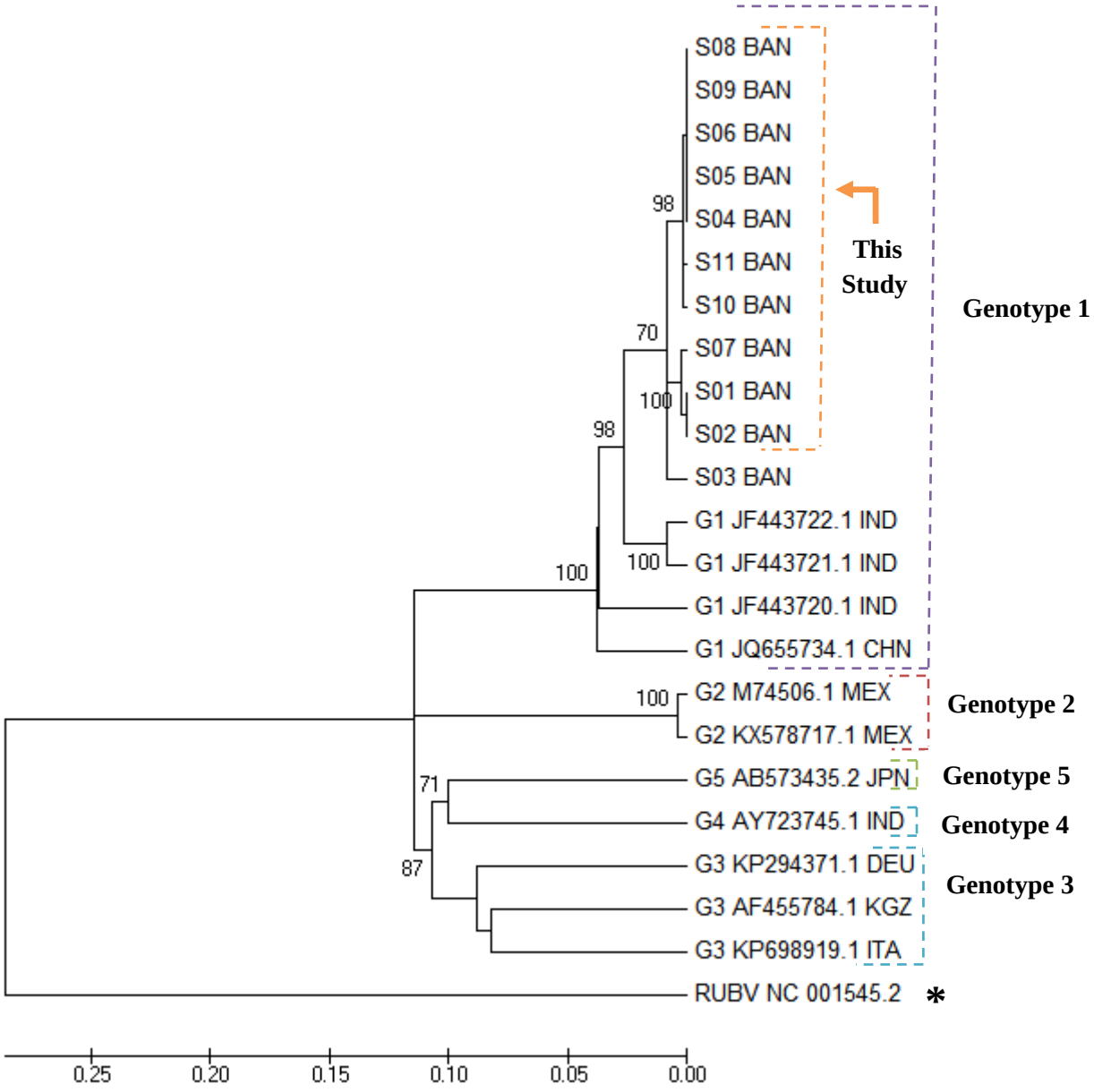

Figure 3.7: Phylogenetic tree showing the relationship of Hepatitis E virus (HEV) isolates from Bangladesh. The trees were constructed by the neighbor-joining method based on the partial nucleotide sequence of the open reading frame (ORF) 2 region of HEV samples. The genotypes are indicated by numbers (i.e G1: Genotype 1, G2: Genotype 2, G3: Genotype 3 and G4: Genotype 4). 15 isolates from the current study are denoted by a prefix "S" followed by a number which together represents the sample number, followed by the three-letter country code (i.e. BAN for Bangladesh). The branch lengths are proportional to the genetic distance. The scale bars indicate 0.05 nt substitutions per position. The bootstrap values for various branches are shown as the percentages of trees obtained from 1,000 re-samplings of the data. The

sequences used for the phylogenetic analysis were isolates from Genotype 1: India (GenBank accession nos. JF443722.1), India (JF443721.1), India (JF443720.1), and China (JQ655734.1); Genotype 2: Mexico (M74506.1), Mexico (KX578717.1); Genotype 3: Germany (KP294371.1), Kyrgyzstan (AF455784.1), Italy (KP698919.1); and Genotype 4: India (AY723745.1); Genotype 5: Japan (AB573435.2); an out-group (shown by an asterisk) was included for comparison. All isolates from the current study and the reference sequences start with a prefix to denote the HEV genotype (G1, G2, G3, G4, G5) followed by the Accession No. and then a 3 letter country code (BAN: Bangladesh, IND: India, CHN: China, MEX: Mexico, JPN: Japan, DEU: Germany, KGZ: Kyrgyzstan, ITA: Italy);

Chapter 4: Discussion

4.0 Discussion

Bangladesh is one of the least developed countries, with considerable poverty and poor hygienic conditions, thereby providing all the favorable conditions for nurturing a wide range of infections, among which Hepatitis E virus (HEV) infection is of major concern. Not only seasonal, but also sporadic, non-seasonal HEV outbreaks with significant mortality have been reported from Bangladesh [39]. Greater attention was focused on HEV in Bangladesh when the United Nations peace-keepers from Bangladesh developed acute icteric Hepatitis E in rapid succession prior to their deployment to Haiti in 1995 and also when people from Japan, Spain, and Italy developed acute Hepatitis E after traveling to Bangladesh [40][41][42][43]. However, the epidemiology of Hepatitis E, its seroprevelance and the genomic features of HEV in Bangladesh have not been investigated adequately.

In the present study, out of a cohort of 30 patients with suspected hepatitis, 96.7% of the study participants were found to be infected with Hepatitis E, thus indicating that Hepatitis E is more prevalent than the other Hepatitis infections (i.e. Hepatitis A, Hepatitis B and Hepatitis C) in Bangladesh. This outbreak of Hepatitis E can be traced to contamination of drinking water supplies with human fecal matter since the virus is known to spread enterically via the fecal-oral route. The contaminated drinking water may include water that has not been boiled or filtered, and it has been observed from our study that only 26.7% of the study participants consumed boiled or filtered water while the rest consumed the municipal-supplied water or water from the tubewell which are known to be sources of hepatitis infection, also confirmed in a recent study where HEV disease was significantly associated with drinking water from the above-mentioned drinking water sources [12]. The contamination of these drinking water sources was either due to poorer water treatment or placement of the tubewells at insufficient distance from latrines or sewage-contaminated ponds or tanks [44]. The contaminated water mainly relates to the practice of poor domestic or personal hygiene which tends to be one of the major issues in Bangladesh. Hygiene awareness and knowledge of the links between poor hygiene and disease are lowest among the typically poorly educated people. In this study 50% of the patients had either education till the primary level or no education at all.

An interesting finding of the study was the presence of co-infections which was confirmed during serological analysis of the serum samples of the study participants. A prevalence of 10% cases with co-infection of HAV and HEV and 13.3% cases with co-infection of HBV and HEV was noted in this study. These findings are similar to various other studies [46][47][48], indicating that association of HEV with other hepatotropic viruses such as HAV and HBV is not very unusual in Bangladesh. In order to test the difference in disease severity between hepatitis monoinfection and coinfections, the elevated level of bilirubin and the liver enzymes were compared. Only the ALP level was found to be significantly higher in patients with HEV monoinfection compared to that in patients having co-infections with HEV and HAV. The ALP level along with the level of other liver enzymes is supposed to be higher in the co-infection state rather than in the HEV monoinfection state as confirmed in a study conducted in 2010 [49]. However, the result obtained in this study was contradictory may be due to a small sample size. One pregnant patient with co-infection of HBV and HEV died within three days after hospital admission. This dual infection could be related to the reactivation of the latent HBV as a result of clinical HEV infection which proved fatal for this particular patient.

Also, there were no significant difference in the elevated levels of bilirubin and the liver enzymes when compared between pregnant and non-pregnant patients in order to test the severity of the liver injury. Similar findings were also reported in an animal study conducted in 1993 where the course of liver injury among pregnant primates experimentally infected with HEV was not very different from that among non pregnant animals [50]. Therefore, the increase in the level of the liver enzymes and bilirubin are not always reliable markers when testing the severity of liver injury because several other possible host factors may play a role in pathogenesis, such as differences in immune responses or hormonal factors between pregnant and non pregnant women. For instance, some studies looked at the immune parameters in pregnant women with HEV infection and compared the responses with that of HEV-infected non-pregnant women and found depressed levels of cytokines, suppressed T-cell-mediated immunity and a clear shift in the T-helper type 1 (Th1):Th2 cell paradigm during pregnancy [51-56]. These immunological changes during pregnancy are

known to occur to promote the maintenance of the antigenic fetus in the maternal environment. Also, hormones such as progesterone, estrogen and human chorionic gonadotropin (HCG) start to rise during pregnancy. In a number of animal studies, these hormones have been shown to have a clear suppressive effect on the cell-mediated immunity where oestrogen has been shown to cause shrinkage of thymus and depletion of the CD4 and CD8 population in mice, while HCG has been shown to inhibit the cell-mediated immunity in guinea-pigs and progesterone was found to cause a shrinkage of the thymus and block T-cell development and inhibit Th1 cell and promote Th2 cell development [57-59].

The comparison of the magnitudes of anti-HEV IgM and IgG responses were also made in this study. A higher percent positivity of anti-HEV IgG than IgM was reported which could have been due to two possible reasons. First, delayed sampling might account for negative anti-HEV IgM results in some patients as serum anti-HEV IgM is detectable only for about 4 to 6 months from the onset of symptoms since HEV viremia is short-lived, whereas anti-HEV IgG appears soon after the IgM response and can persist for up to 12 years after infection [60-63]. Secondly, poor host immune responses to HEV infection might account for the undetectable anti-HEV IgM in patients with acute hepatitis. Therefore, anti-HEV IgM alone may not be informative for the diagnosis of acute HEV infection. In the present study, 51.7 % of the patients were positive for both anti-HEV IgM and IgG, which represents a transition phase during the course of the infection.

As previously mentioned, the viremia in acute HEV infection lasts for a short period and disappears soon after the onset of symptoms. Thus, in acute cases, if the samples are taken at a later date after the development of symptoms, only antibodies might be detected, but RNA PCR may turn out to be negative [64]. In other cases, HEV RNA might be detected in patients negative for all HEV serological markers [65]. In the current study, out of 29 patients who were positive for HEV infection by serology, 15 (i.e. 51.7% of the patients) showed positivity for HEV RNA detected by a single-step real-time RT-PCR. About 93.3% of the patients positive for HEV RNA were in the transition phase of the infection (i.e. positive for both anti-HEV IgM and IgG). No viral RNA was detected in patients positive for only anti-HEV IgG since all the

Hepatitis E viruses have been cleared out from the system, as the presence of only anti-HEV IgG in the serum indicates a past Hepatitis E infection.

The genomic analysis showed that all the HEV isolates obtained from Bangladesh belonged to HEV genotype 1, which formed a single cluster in the phylogenetic tree (Fig. 3.7). This result for a few isolates confirms previous studies done on HEV genotypes in Bangladesh [39][66]. According to the phylogenetic tree analysis of the HEV isolates from this study, the isolates resembled that of India (JF443722.1, JF443721.1, and JF443720.1) and China (JQ655734.1) in genotype 1.

5.0 Conclusion

In conclusion, this study showed that there existed a high prevalence of Hepatitis E infection in Bangladesh compared to the incidence of other hepatitis infections caused by HAV, HBV or HCV. The presence of dual infections caused by HEV and HAV or HEV and HBV poses a challenging issue in Bangladesh. In addition to this, the HEV seroprevalence and its genotype were assessed in this study. This appears to be one of the first few studies to evaluate the almost complete gene sequences of HEV from Bangladeshi patients. With the improvement of socio-economic, general living, public education and hygienic conditions, occurrence of HEV infection in Bangladesh is expected to be on the decline within the next couple of decades. It is important to better understand the epidemiology of this pathogen to develop public health strategies that target the most vulnerable and high-risk groups in endemic countries like Bangladesh.

References

1. Labrique, A., Zaman, K., Hossain, Z., Saha, P., Yunus, M., Hossain, A., Ticehurst, J. and Nelson, K. (2009). Population Seroprevalence of Hepatitis E Virus Antibodies in Rural Bangladesh. *The American Society of Tropical Medicine and Hygiene*, 81(5), pp.875–881.
2. Renou, C., Gobert, V., Locher, C., Moumen, A., Timbely, O., Savary, J. and Roque-Afonso, A. (2014). Prospective study of Hepatitis E Virus infection among pregnant women in France. *Virology Journal*, 11(1), p.68.
3. Khuroo, M., Kamili, S. and Khuroo, M. (2009). Clinical course and duration of viremia in vertically transmitted hepatitis E virus (HEV) infection in babies born to HEV-infected mothers. *Journal of Viral Hepatitis*, 16(7), pp.519-523.
4. Aggarwal, R. and Naik, S. (2009). Epidemiology of hepatitis E: Current status. *Journal of Gastroenterology and Hepatology*, 24(9), pp.1484-1493.
5. Mushahwar, I. (2008). Hepatitis E Virus: Molecular Virology, Clinical Features, Diagnosis, Transmission, Epidemiology, and Prevention. *Journal of Medical Virology*, 80, pp.646–658.
6. Wedemeyer, H., Pischke, S. and Manns, M. (2012). Pathogenesis and Treatment of Hepatitis E Virus Infection. *Gastroenterology*, 142(6), pp.1388-1397.e1.
7. Ding, Q., Heller, B., Capuccino, J., Song, B., Nimgaonkar, I., Hrebikova, G., Contreras, J. and Ploss, A. (2017). Hepatitis E virus ORF3 is a functional ion channel required for release of infectious particles. *Proceedings of the National Academy of Sciences*, 114(5), pp.1147-1152.
8. Rein, D., Stevens, G., Theaker, J., Wittenborn, J. and Wiersma, S. (2012). The global burden of hepatitis E virus genotypes 1 and 2 in 2005. *Hepatology*, 55(4), pp.988-997.
9. Lam, W., Chan, R., Sung, J. and Chan, P. (2009). Genotype Distribution and Sequence Variation of Hepatitis E Virus, Hong Kong. *Emerging Infectious Diseases*, 15(5), pp.792-794.
10. Navaneethan, U., Al Mohajer, M. and Shata, M. (2008). Hepatitis E and pregnancy: understanding the pathogenesis. *Liver International*, 28(9), pp.1190-1199.

11. Mast, E. and Krawczynski, K. (1996). Hepatitis E: an overview. *Annual Review of Medicine*, 47, pp.257–66.

12. Gurley, E., Hossain, M., Paul, R., Sazzad, H., Islam, M., Parveen, S., Faruque, L., Husain, M., Ara, K., Jahan, Y., Rahman, M. and Luby, S. (2014). Outbreak of Hepatitis E in Urban Bangladesh Resulting in Maternal and Perinatal Mortality. *Clinical Infectious Diseases*, 59(5), pp.658-665.

13. Labrique, A., Zaman, K., Hossain, Z., Saha, P., Yunus, M., Hossain, A., Ticehurst, J. and Nelson, K. (2010). Epidemiology and Risk Factors of Incident Hepatitis E Virus Infections in Rural Bangladesh. *American Journal of Epidemiology*, 172(8), pp.952-961.

14. Kamar, N., Izopet, J., Cintas, P., Garrouste, C., Uro-Coste, E., Cointault, O., Rostaing, L., et al. (2010). Hepatitis E Virus-Induced Neurological Symptoms in a Kidney-Transplant Patient with Chronic Hepatitis. *American Journal of Transplantation*, 10(5), pp.1321-1324.

15. Cao, D. and Meng, X. (2012). Molecular biology and replication of hepatitis E virus. *Emerging Microbes & Infections*, 1(8), p.e17.

16. Debing, Y., Moradpour, D., Neyts, J. and Gouttenoire, J. (2016). Update on hepatitis E virology: Implications for clinical practice. *Journal of Hepatology*, 65(1), pp.200-212.

17. Perttila, J., Spuul, P. and Ahola, T. (2012). Early secretory pathway localization and lack of processing for hepatitis E virus replication protein pORF1. *Journal of General Virology*, 94(Pt_4), pp.807-816.

18. van Tong, H., Hoan, N., Wang, B., Wedemeyer, H., Bock, C. and Velavan, T. (2016). Hepatitis E Virus Mutations: Functional and Clinical Relevance. *EBioMedicine*, 11, pp.31-42.

19. Anderson, D., Li, F., Riddell, M., Howard, T., Seow, H., Torresi, J., Perry, G., Sumarsidi, D., Shrestha, S. and Shrestha, I. (1999). ELISA for IgG-class antibody to hepatitis E virus based on a highly conserved, conformational epitope expressed in Eschericia coli. *Journal of Virological Methods*, 81(1-2), pp.131-142.

20. Dawson, G., Chau, K., Cabal, C., Yarbough, P., Reyes, G. and Mushahwar, I. (1992). Solid-phase enzyme-linked immunosorbent assay for hepatitis E virus IgG and IgM antibodies utilizing recombinant antigens and synthetic peptides. *Journal of Virological Methods*, 38(1), pp.175-186.

21. Lin, C., Wu, J. and Chang, T. (2000). Diagnostic value of immunoglobulin G (IgG) and IgM anti-hepatitis E virus (HEV) tests based on HEV RNA in an area where hepatitis E is not endemic. *J Clin Microbiol*, 38, pp.3915–18.

22. Takahashi, M., Kusakai, S., Mizuo, H., Suzuki, K., Fujimura, K., Masuko, K., Sugai, Y., Aikawa, T., Nishizawa, T. and Okamoto, H. (2005). Simultaneous Detection of Immunoglobulin A (IgA) and IgM Antibodies against Hepatitis E Virus (HEV) Is Highly Specific for Diagnosis of Acute HEV Infection. *Journal of Clinical Microbiology*, 43(1), pp.49-56.

23. Dalton, H., Bendall, R., Ijaz, S. and Banks, M. (2008). Hepatitis E: an emerging infection in developed countries. *The Lancet Infectious Diseases*, 8(11), pp.698-709.

24. Myint, K., Endy, T., Gibbons, R., Laras, K., Mammen, M., Sedyaningsih, E., Seriwatana, J., Glass, J., Narupiti, S. and Corwin, A. (2006). Evaluation of Diagnostic Assays for Hepatitis E Virus in Outbreak Settings. *Journal of Clinical Microbiology*, 44(4), pp.1581-1583.

25. Takahashi, M., Tanaka, T., Azuma, M., Kusano, E., Aikawa, T., Shibayama, T., Yazaki, Y., Mizuo, H., Inoue, J. and Okamoto, H. (2007). Prolonged Fecal Shedding of Hepatitis E Virus (HEV) during Sporadic Acute Hepatitis E: Evaluation of Infectivity of HEV in Fecal Specimens in a Cell Culture System. *Journal of Clinical Microbiology*, 45(11), pp.3671-3679.

26. Dalton, H., Thurairajah, P., Fellows, H., Hussaini, H., Mitchell, J., Bendall, R., Banks, M., Ijaz, S., Teo, C. and Levine, D. (2007). Autochthonous hepatitis E in southwest England. *Journal of Viral Hepatitis*, 14(5), pp.304-309.

27. Zhang, J., Zhang, X., Huang, S., Wu, T., Hu, Y., Wang, Z., et al. (2015). Long-Term Efficacy of a Hepatitis E Vaccine. *New England Journal of Medicine*, 372(23), pp.2265-2266.

28. Cordoba, L., Huang, Y., Opriessnig, T., Harral, K., Beach, N., Finkielstein, C., Emerson, S. and Meng, X. (2011). Three Amino Acid Mutations (F51L, T59A, and S390L) in the Capsid Protein of the Hepatitis E Virus Collectively Contribute to Virus Attenuation. *Journal of Virology*, 85(11), pp.5338-5349.

29. Pudupakam, R., Huang, Y., Opriessnig, T., Halbur, P., Pierson, F. and Meng, X. (2008). Deletions of the Hypervariable Region (HVR) in Open Reading Frame 1 of Hepatitis E Virus Do Not Abolish Virus Infectivity: Evidence for

Attenuation of HVR Deletion Mutants In Vivo. *Journal of Virology*, 83(1), pp.384-395.

30. Zhang, H., Dai, X., Shan, X. and Meng, J. (2008). The Leu477 and Leu613 of ORF2-Encoded Protein Are Critical in Forming Neutralization Antigenic Epitope of Hepatitis E Virus Genotype 4. *Cellular and Molecular Immunology*, 5(6), pp.447-456.

31. Borkakoti, J., Hazam, R., Mohammad, A., Kumar, A. and Kar, P. (2012). Does high viral load of hepatitis E virus influence the severity and prognosis of acute liver failure during pregnancy?. *Journal of Medical Virology*, 85(4), pp.620-626.

32. Trey, C. and Davidson, C. (1970). The management of fulminant hepatic failure. . *Prog Liver Dis*, 3, pp.282–298.

33. Smedile, A., Farci, P., Verme, G., Caredda, F., Cargnel, A., Caporaso, N., Dentico, P., Trepo, C., Opolon, P., Gimson, A., Vergani, D., Williams, R. and Rizzetto, M. (1982). Influence of delta infection on severity of hepatitis B. *Lancet*, 2, pp.945–947.

34. Tsarev, S., Emerson, S., Reyes, G., Tsareva, T., Legters, L., Malik, I., Iqbal, M. and Purcell, R. (1992). Characterization of a prototype strain of hepatitis E virus. *Proceedings of the National Academy of Sciences*, 89(2), pp.559-563.

35. Fagan, E. and Harrison, T. (2000). *Viral Hepatitis*. 1st ed. Garland Science, p.74.

36. Al-Mahtab, M. (2016). Past, Present, and Future of Viral Hepatitis in Bangladesh. *Euroasian J Hepato-Gastroenterol*, 6(1), pp.43-44.

37. Al-Mahtab, M., Rahman, S., Khan, M. and Karim, F. (2009). HEV Infection as an Aetiologic Factor for Acute Hepatitis: Experience from a Tertiary Hospital in Bangladesh. *Journal of Health, Population and Nutrition*, 27(1).

38. Mahtab, M., Rahman, S., Khan, M. and MF, K. (2009). Hepatitis E virus is the leading cause of acute-on-chronic liver disease: Experience from a tertiary centre in Bangladesh. *Hepato Biliar Pancreat Dis Int*, 8(1), pp.50-52.

39. Harun-Or-Rashid, M., Akbar, S., Takahashi, K., Al-Mahtab, M., Khan, M., Alim, M., Ekram, A., Khan, M., Arai, M. and Mishiro, S. (2013). Epidemiological and molecular analyses of a non-seasonal outbreak of acute icteric hepatitis E in Bangladesh. *Journal of Medical Virology*, 85(8), pp.1369-1376.

40. Drabick, J., Caudill, J., Hoke, C., Gouvea, V., Sun, W., Innis, B. and Gambel, J. (1997). A Cluster of Acute Hepatitis E Infection in United Nations Bangladeshi Peacekeepers in Haiti. *The American Journal of Tropical Medicine and Hygiene*, 57(4), pp.449-454.

41. Sanayama, Y., Ishiwada, N., Fukasawa, C., Kanazawa, M., Kohno, Y., Tamano, Y. and Yano, K. (2008). A pediatric patient with acute hepatitis E in Japan. *Journal of Infection and Chemotherapy*, 14(5), pp.374-376.

42. Fogeda, M., Avellón, A., Cilla, C. and Echevarría, J. (2009). Imported and autochthonous hepatitis E virus strains in Spain. *Journal of Medical Virology*, 81(10), pp.1743-1749.

43. La Rosa, G., Muscillo, M., Vennarucci, V., Garbuglia, A., La Scala, P. and Capobianchi, M. (2011). Hepatitis E virus in Italy: molecular analysis of travel-related and autochthonous cases. *Journal of General Virology*, 92(7), pp.1617-1626.

44. Islam, M., Siddika, A., Khan, M., Goldar, M., Sadique, M., Kabir, A., Huq, A. and Colwell, R. (2001). Microbiological Analysis of Tube-Well Water in a Rural Area of Bangladesh. *Applied and Environmental Microbiology*, 67(7), pp.3328-3330.

45. UNICEF Bangladesh (2010). *Sanitation, Hygiene Education and Water Supply in Bangladesh (SHEWA,B)*. [online] Bangladesh. Available at: https://www.unicef.org/bangladesh/SHEWAB_factsheet_-_FINAL-21April12.pdf [Accessed 11 Jun. 2010].

46. Jain, A., Jain, P., Prakash, S., Gupta, S., Singh, K., Shrivastava, S., Singh, D. and Singh, J. (2013). Prevalence of hepatitis A virus, hepatitis B virus, hepatitis C virus, hepatitis D virus and hepatitis E virus as causes of acute viral hepatitis in North India: A hospital based study. *Indian Journal of Medical Microbiology*, 31(3), p.261.

47. Singh, N., Kumari, A., Catanzaro, R. and Marotta, F. (2012). Prevalence of hepatitis E and hepatitis B dual infection in North India (Delhi). *Acta bio-medica : Atenei Parmensis*, 83(3), pp.197-201.

48. Arora, D., Jindal, N., Shukla, RK., Bansal, R. (2013). Water Borne Hepatitis A and Hepatitis E in Malwa Region of Punjab, India. *JOURNAL OF CLINICAL AND DIAGNOSTIC RESEARCH*.

49. Zhang, X., Ke, W., Xie, J., Zhao, Z., Xie, D. and Gao, Z. (2010). Comparison of effects of hepatitis E or A viral superinfection in patients with chronic hepatitis B. *Hepatology International*, 4(3), pp.615-620.

50. Arankalle, V., Chadha, M., Banerjee, K., Srinivasan, M. and Chobe, L. (1993). Hepatitis E virus infection in pregnant rhesus monkeys. *The Indian Journal of Medical Research*, 97, pp.4-8.

51. Orsi, N., Gopichandran, N., Ekbote, U. and Walker, J. (2006). Murine serum cytokines throughout the estrous cycle, pregnancy and post partum period. *Animal Reproduction Science*, 96(1-2), pp.54-65.

52. Mellor AL, R., Sivakumar, J., Chandler, P., et al. (2001). Prevention of T cell-driven complement activation and inflammation by tryptophan catabolism during pregnancy. *Nat Immunol*, 2, p.64.

53. PAL, R., AGGARWAL, R., NAIK, S., DAS, V., DAS, S. and NAIK, S. (2005). Immunological alterations in pregnant women with acute hepatitis E. *Journal of Gastroenterology and Hepatology*, 20(7), pp.1094-1101.

54. Cahill, K. (1962). Hepatitis in pregnancy. *Surg Gynaecol Obstet*, 114, pp.545–52.

55. Purtilo, D., Hallgren, H. and Yunis, E. (1972). Depressed maternal response to phytohaemagglutinin in human pregnancy. *Lancet*, 1, pp.769–71.

56. Lewis, J., Whang, J., Nagel, B., Oppenheim, J. and Perry, S. (1966). Lymphocyte transformation in mixed leukocyte cultures in women with normal pregnancy or tumors of placental origin. *American Journal of Obstetrics and Gynecology*, 96(2), pp.287-290.

57. BOLL, G. and REIMANN, J. (1996). Oestrogen Treatment Depletes Extrathymic T Cells from Intestinal Lymphoid Tissues. *Scandinavian Journal of Immunology*, 43(3), pp.345-350.

58. Rijhsinghani, A., Thompson, K., Bhatia, S. and Waldschmidt, T. (1996). Estrogen Blocks Early T Cell Development in the Thymus. *American Journal of Reproductive Immunology*, 36(5), pp.269-277.

59. Tibbetts, T., DeMayo, F., Rich, S., Conneely, O. and O'Malley, B. (1999). Progesterone receptors in the thymus are required for thymic involution during pregnancy and for normal fertility. *Proceedings of the National Academy of Sciences*, 96(21), pp.12021-12026.

60. Chauhan, A., Dilawari, J., Chawla, Y., Jameel, S., Kaur, U. and Ganguly, N. (1993). Hepatitis E virus transmission to a volunteer. *The Lancet*, 341(8838), pp.149-150.

61. Favorov, M., Fields, H., Purdy, M., Yashina, T., Aleksandrov, A., Alter, M., Yarasheva, D., Bradley, D. and Margolis, H. (1992). Serologic identification of hepatitis E virus infections in epidemic and endemic settings. *Journal of Medical Virology*, 36(4), pp.246-250.

62. Singh, N., Rai, R., Udawat, H. and Sharma, A. (2008). Evaluation of persistence of IgM anti HEV in patients with sporadic hepatitis E virus infection. *Asian J Exp Sci*, 22, pp.299–302.

63. Chadha, M., Walimbe, A. and Arankalle, V. (1999). Retrospective serological analysis of hepatitis E patients: a long-term follow-up study. *Journal of Viral Hepatitis*, 6(6), pp.457-461.

64. Kar, P., Jilani, N., Husain, S., Pasha, S., Anand, R., Rai, A. and Das, B. (2008). Does Hepatitis E Viral Load and Genotypes Influence the Final Outcome of Acute Liver Failure During Pregnancy?. *The American Journal of Gastroenterology*, 103(10), pp.2495-2501.

65. Chandra, N., Ojha, D., Chatterjee, S. and Chattopadhyay, D. (2014). Prevalence of hepatitis E virus infection in West Bengal, India: a hospital-based study. *Journal of Medical Microbiology*, 63(Pt_7), pp.975-980.

66. Sugitani, M., Tamura, A., Shimizu, Y., Sheikh, A., Kinukawa, N., Shimizu, K., Moriyama, M., Komiyama, K., Li, T., Takeda, N., Arakawa, Y., Suzuki, K., Ishaque, S., Roy, P., Raihan, A. and Hasan, M. (2009). Detection of hepatitis E virus RNA and genotype in Bangladesh. *Journal of Gastroenterology and Hepatology*, 24(4), pp.599-604.