Viral Load and Genotype Analysis of Hepatitis E Virus among HEV Infected Women in Bangladesh

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN BIOTECHNOLOGY

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Dedicated to

My beloved parents, sister and brother

DECLARATION BY THE RESEARCHER

This is to declare that the undersigned, Shagoofa Rakhshanda, have carried out the research work embodying the results reported in this thesis entitled **"Viral Load and Genotype Analysis of Hepatitis E Virus among HEV Infected Women in Bangladesh"** under the joint supervision of Professor Naiyyum Choudhury, Biotechnology Program, Department of Mathematics and Natural Sciences (MNS), BRAC University, and Professor Firdausi Qadri, Senior Scientist and Executive Director, Institute for developing Science and Health Initiatives (ideSHi). It is further declared that the research work presented here is original and submitted in the partial fulfillment for the degree of Masters of Science in Biotechnology, BRAC University, Dhaka and has not been submitted anywhere else for a degree or diploma.

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Shagoofa Rakhshanda

Abstract

With an increase in the number of hepatitits E viral infected patients since the identification of the virus in 1983, there is a rising concern in Bangladesh. A better understanding of the virus at the genetic level can help further in the treatment of the patients. Acute and chronic HEV infections can be much severe in pregnant women, people with an underlying liver disease and immunocompromised and/or immunosuppressed patients. The present study aimed at determining the viral load in HEV positive patients at Dhaka, analyzing bioinformatically the genetic composition of HEV to find any new substitution mutation and, with the aid of the level of liver damage biomarkers found in the samples, predicting the effect and functional change due to nucleic acid sequence variation on binding affinity of peptides to HLAs. Although there was no significant differences in viral copy number among pregnant, post-natal and non-pregnant women, the average copy numbers for the pregnant and post-natal women were higher than that of the non-pregnant women (3360.84 copies/mL). At the 1592nd position in the nucleic acid sequence of three of the HEV RNA positive patients, A and T substituted each other resulting in an amino acid change at the 531st position, which was unlike those observed in other countries. The substitution corresponded with the liver damage biomarkers and binding properties of antigenic epitopes to the host cell receptor. These results may mend ways for further analysis of such substitutions and can eventually help in understanding the virus, and developing an appropriate treatment therapy.

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LIST OF ABBREVIATIONS

°C	Degree celsius
µL	Microliter
A	Adenine
Ag	Antigen
ALP	Alkaline phosphatase
ALT	Alanine transaminase
ANN	Artificial Neutral Network
AST	Aspartate transaminase
BLAST	Basic Local Alignment Search Tool
BRAC	Bangladesh Rural Advancement Committee
BSL	Biosafety level
BSMMU	Bangabandhu Sheikh Mujib Medical University
C	Cytosine
cDNA	Complementary deoxyribonucleic acid
CMV	Cytomegalovirus
C_t	Threshold cycle
D	Aspartic acid
DMCH	Dhaka Medical College Hospital
dNTP	Deoxyribonucleotide triphosphate
dsDNA	Double stranded deoxyribonucleic acid
E	Glutamic acid
EDTA	Ethylenediaminetetraacetic acid
e.g.	For example
ELISA	Enzyme-linked immunosorbent assay
et al	And others
ExPASy	Expert Protein Analysis System
g	Gram
G	Guanine
GNI	Gross national income
HEV	Hepatitis E virus
HEVCP	Hepatitis E virus capsid protein

HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
IAV	Influenza A virus
icddr,b	International Centre for Diarrhoeal Disease Research, Bangladesh
ideSHi	Institute for Developing Science and Health Initiatives
IDT	Integrated DNA Technology
IEM	Immune electron microscopy
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IU	International unit
IU/L	International unit per liter
L	Leucine
LDH	Lactate dehydrogenase
MEGA6	Molecular Evolutionary Genetics Analysis version six
MHC	Major histocompatibility complex
mL	Milliliter
mM	Millimole
MNS	Mathematics and Natural Sciences
MSA	Multiple sequence alignment
N	Asparagine
NAD	Nicotinamide adenine dinucleotide
NAT	Nucleic acid amplification techniques
NCBI	National Center for Biotechnology Information
ng	Nanogram
NK	Natural killer
ORF	Open reading frame
P	Proline
PCR	Polymerase chain reaction
pH	Potential of hydrogen
Q	Glutamine
R	Arginine
RNA	Ribonucleic acid

rpm	Revolutions per minute
RT-PCR	Real time polymerase chain reaction
S	Serine
SD	Standard deviation
SMM	Stabilized Matrix Method
SOP	Standard operating procedures
SOT	Solid-organ transplant
ssDNA	Single stranded deoxyribonucleic acid
T	Thymine
TBE	Tris/Borate/EDTA
UTR	Untranslated regions
V	Valine
W	Tryptophan

CHAPTER ONE

INTRODUCTION

Introduction

Human beings have been afflicted by disease and injury since the onset of human life on earth. Among the recorded communicable diseases so far, handful have been eradicated, some are eliminated, while most are kept under control by various vaccines, drugs and lifestyle changes.

Viral hepatitis is a type of disease that is caused by five distinct viruses named hepatitis A, B, C, D and E. Hepatitis E virus (HEV) and the disease associated with it is a global public health concern, especially in many developing countries in Asia, Africa, and Latin America. HEV infection is usually acute and self-limiting in developing countries, requiring no or minimal treatment. However, in the developed countries, it causes chronic enteric infection, which is treated by reducing immuno-suppression or by using antiviral therapy. Hepatitis E virus is the only hepatitis virus to infect non-primitive animals like swine, wild boars and deer. It can appear as sporadic (mostly in developed countries), and as endemic or epidemic (mostly in developing countries) cases as an outcome of poor hygiene and sanitation (Guu *et al.*, 2009; Kar *et al.*, 2008; Kamar *et al.*, 2014; Bouquet *et al.*, 2012; Smith, Purdy and Simmonds, 2013).

1.1 Background of the Study

The mechanism of transmission of the hepatitis E virus is not yet fully understood, but it is known to spread via fecal-oral route through contaminated water, undercooked meat, direct exposure to infected animals and contaminated environment. The presence of anti-HEV antibodies is mostly observed amongst veterinarians and farmers who work with pigs. The IgM class of antibodies is short lived and are detectable in the blood serum of the infected patients for the first couple of months. On the other hand, IgG antibodies are longer lasting and are detectable for several years after the onset of the infection. Many infected children do not have symptoms but they may nonetheless release virus into the environment (Legrand-Abravanel *et al.*, 2010; Teshale *et al.*, 2010; Vasickova *et al*., 2007). The last ten years have been revolutionizing in terms of understanding the background of this virus and the infection caused by it. The use of less sensitive assays has led to the under diagnosis

of the disease thus prompting development of better and improved diagnostic tools (Kamar *et al.*, 2014).

1.2 Hepatitis E Virus Genome

Hepatitis E is a small, non-enveloped, positive-sense, single stranded RNA virus containing a genome size of about 7.2 kb and diameter 32-34 nm. It has three open reading frames (ORFs) (Abravanel *et al.*, 2011; Khuroo and Khuroo, 2008; Ahmad, Holla and Jameel, 2011; Takahashi *et al.*, 2007). ORF1, situated at the 5'end of the HEV genome, consists of 5,079 bases. It codes for a 1693 amino acids long protein, with six conserved sequences of functional domains, like methyltransferase, protease, helicase and polymerase. ORF2, situated near the 3' end of the genome. It is 1,980 bases long and codes for a capsid protein (HEVCP) that comprises the outside shell of the Hepatitis E virus. The HEVCP has 660 amino acids. The N terminus contains a signal peptide along with a domain that is rich in arginine; which may play a role in the encapsidation process of viral RNA. The third ORF is 369 bases long that codes for 123 amino acids and overlaps with the first two ORFs (Guu *et al.*, 2009; Koonin *et al.,* 1992; Borkakoti *et al.,* 2013; Meng *et al.,* 2002; Surjit, Jameel and Lal, 2004). It codes for an immunogenic protein or viroprotein which serves as an ion channel protein whose function, much like that of influenza A virus (IAV) matrix-2 protein, is to release infectious particles (Ding *et al.*, 2016).

Hepatitis E viral genome

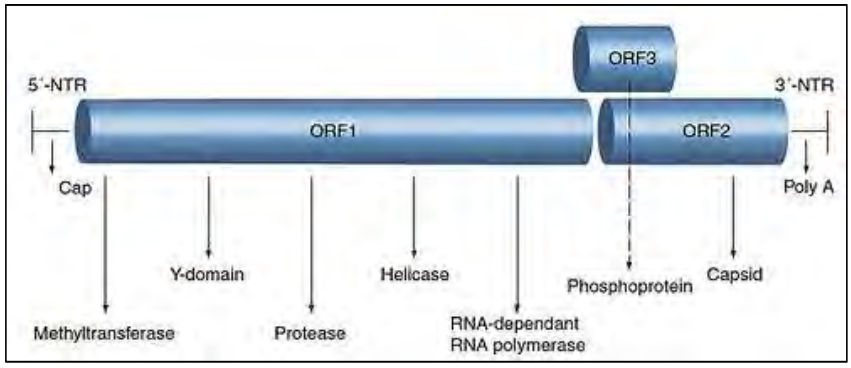


Figure 1.1: Detail of the hepatitis E viral genome

Source: An Online Resource for Students, Researchers and Policy Makers. <https://rickjohnson303.wordpress.com/2011/01/28/hello-world/>

Due to the complete genome sequencing of HEV, HEV isolates have been divided into at least four genotypes, and 24 subtypes. Genotype 1 is mostly found in isolates from Pakistan, Nepal, India and China. Genotype 2 was detected from an epidemic outbreak in Mexico. HEV genotype 1 and 2 are said to be the reason for the

waterborne hepatitis E in developing countries. These genotypes are mainly restricted to humans (Abravanel *et al.*, 2011; Bouquet *et al.*, 2012; Vollmer *et al*., 2012). The HEV genotype 3 and 4 causes the autochthonous cases in industrialized countries and is mostly found in animals like pigs and rats. In the United States and other countries, Genotype 3 was detected while genotype 4 was found in Taiwan, Japan and China. However, a new strain of HEV was recently detected in chickens with hepatitis–splenomegaly syndrome. This strain was named avian HEV, and was proposed to belong to either a new genotype 5 or a separate genus. Through recent researches, genotype 6 and 7 have also been identified. Considering the heterogeneity of the HEV strains circulating in humans and other animals, development of a broadly reactive assay is needed for detection of the various HEV strains that can infect humans (Jothikumar *et al.,* 2005; Legrand-Abravanel *et al.*, 2010; Rein *et al.,* 2012; Doceul *et al*., 2016).

This study aims to better understand the genetic profile and to identify the mutant genetic codes that are found associated with the occurrence of hepatitis E virus infection among cases reporting to the Institute for Developing Science and Health Initiatives (ideSHi), and International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b).

1.3 Literature Review

1.3.1 HEV history and classification

Hepatitis E virus (HEV) was first identified in 1983. It was then responsible for large-scale waterborne epidemics in developing countries (Panda and Varma, 2013). It was observed under the electron microscope from a patient who was a non-A and non-B hepatitis suspect. It was viewed as a spherical viral particle of 27 to 30 nm size. At first it was suggested to be classified into the family of *Picornaviridae* as hepatitis A type 2 virus. But later it was proven not to be from this family. Based on virion morphology, HEV was classified in the *Caliciviridae* family during the years 1988 to 1998. However, after the phylogenetic analysis of the HEV genome, this classification was also rejected (Kar *et al.*, 2008; Borkakoti *et al.,* 2013; Vasickova *et al.*, 2007). Finally, in the seventh Report of the International Committee on the Taxonomy of Viruses, it was classified as an independent family of 'HEV-like virus'. This was later

termed as the *Hepevirus* family from the *Hepeviridae* genus (Panda, Thakral and Rehman, 2006; Vollmer *et al*., 2012; Takahashi *et al*., 2007). During the early years, it was established that variants that were 20% different in the ORF 2 region was classified into different genotypes (Smith, Purdy and Simmonds, 2013).

During 1997, Hepatitis E virus was first found in swine in United States. Later it was found that the swine HEV was genetically closely related to human HEV. Since then zoonotic and cross species transmission of HEV have been demonstrated (Jothikumar *et al.,* 2005).

Earlier, it was considered that HEV is an infection that only occurred in developing countries, but this misconception was nullified after discovering a significant number of HEV patients in developed countries. Anti-HEV immunoglobulin G (IgG) was found in about 30% of the population in United States and Europe. The presence of IgG indicates the presence of a previous HEV infection (Behrendt *et al.*, 2016).

1.3.2 HEV in pregnant women

Acute HEV infections are generally self-limiting and symptomatic or asymptomatic in immunocompetent patients, but it can be much severe in pregnant women or people with an underlying liver disease. Until recently, it was thought that chronic HEV infection does not occur, but it is now seen that HEV may lead to chronic infection in immuno-suppressed patients like solid-organ transplant (SOT) recipients, patients who are given chemotherapy, and human immunodeficiency virus (HIV)–positive patients. In immuno-compromised patients, liver fibrosis too can develop very rapidly, leading to liver cirrhosis (Legrand-Abravanel *et al.*, 2010; Behrendt *et al.*, 2016). HEV is also responsible for non-hepatic problems such as neurological (e.g., Bell's palsy, neuralgic amyotrophy, acute transverse myelitis, and acute meningoencephalitis), renal (e.g., cryoglobulinemia), pancreatic (e.g., acute pancreatitis), and hematological disorders (e.g., thrombocytopenia and aplastic anemia) (Kamar *et al.*, 2014; Doceul *et al*., 2016).

Pregnant women with jaundice and acute viral hepatitis caused by HEV infection have worse fetal and obstetric outcomes and higher maternal mortality compared to other types of viral hepatitis (Borkakoti *et al.*, 2013; Jaiswal *et al*., 2001; Emerson and

Purcell, 2003). Among the pregnant females in India, the incidence of HEV infection is much higher, specially during the second and third trimesters of pregnancy (Kar *et al.*, 2008; Arankalle *et al*., 1993; Balayan, 1997). The mortality rate of pregnant women due to HEV infection is significantly higher in Asian women, with maximum severity during the third trimester. The rate ranges from 11.4% to 21%. Studies carried out in Iran, Africa, and Middle East also show similar results. Thus, hepatitis E infection during pregnancy is a serious health concern. Reports indicate that abortion, death of the fetus, premature delivery, or death of the baby soon after birth are seen in women with hepatitis, specially Hepatitis E virus infection. However, it is observed from studies conducted in Europe and United States that there is no difference in the severity of viral hepatitis in pregnant and non-pregnant women (Kar *et al.*, 2008; Cahill, 1962; Adams and Combes, 1965).

Generally, pregnancy is associated with altered status of sex steroid hormones and immunity. These hormones play crucial role in the course of viral infections including viral hepatitis during pregnancy. Steroid hormones, through their effects on viral regulatory elements, influence viral replication. It is postulated that malnutrition coupled with folate deficiency influences immune response which also contributes to greater risk of multiple viral infection and higher viral load in pregnant women (Kar *et al.*, 2008). Studies from various developing countries show that a significant proportion of pregnant women can progress to fulminant hepatitis with a mortality rate varying from 30% to 100% (Borkakoti *et al.*, 2013). The NF-kB signaling pathway regulating at the transcriptional level through p50 subunits have been suggested to correlate with the severe liver damage, leading to multiple organ failure and the death of both the mother and the fetus. In spite of such associations, the exact cause or mechanism(s) for its occurrence is yet not clear (Kar *et al.*, 2008).

The incidence of HBV related acute liver failure is known widely in comparison to HCV infection in which acute liver failure is rare. But the severe course of HEV infection causing acute liver failure during pregnancy is unique to this virus with chronicity occurring in recipients of solid organ transplants (Borkakoti *et al.*, 2013). Vertical transmission of the HEV infection has been reported (Khuroo, Kamili and Jameel, 1995).

1.3.3 Diagnosis of HEV

A combination of serological tests and nucleic acid amplification techniques (NATs) are used for the diagnosis of HEV infections. Quantitative assay that involves real-time polymerase chain reaction (PCR) is a direct method that can be used to detect the presence and amount of HEV RNA in the patients' blood or other body fluids. Anti-HEV antigen (Ag)–specific enzyme-linked immunosorbent assay (ELISA) is an indirect method that is used to detect the presence of the serum anti-HEV antibody (i.e., IgM and IgG antibodies) against the HEV capsid protein, coded by the ORF 2. The presence of anti-HEV IgM is a marker of acute infection and the presence of anti-HEV IgG alone is a marker of past infection. Both the methods are quite efficient at diagnosing the infection and at determining the viral load in chronically infected patients. They are also good for monitoring the effect of any antiviral therapy given to affected patients, though the sensitivities and specificities of these assays differ greatly. There is no genotype-specific serologic testing (Abravanel *et al.*, 2011; Behrendt *et al.*, 2016; Kamar *et al*, 2014; Teshale, Hu and Holmberg, 2010). Other assays include reverse transcriptase polymerase chain reaction (RT-PCR), reverse transcription-loop-mediated isothermal amplification, Immune electron microscopy (IEM), fluorescent antibody blocking assay and immunochromatographic assay (Panda, Thakral and Rehman, 2006; Baylis *et al.*, 2011). Many serological tests are so sensitive and specific that they can detect anti-HEV antigen in individuals who did not have a history of hepatitis. This suggests that there can be the subclinical cases that are not recognized (Emerson *et al.*, 2003). After performing reverse transcriptase-PCR the PCR products are sequenced to identify the HEV genotype. This can help to identify the origin of infection (Rosa *et al*., 2014).

1.3.4 Viral load

There are two major viral markers for any hepatitis viral disease, the genotype and the viral load. Both of these help in determining viral proliferation, monitoring the response to treatments, evaluating the disease prognosis and differentiating between latent and active infection. High viral loads correlate with increased risk for the disease and thus require longer time to be treated. Quantitative PCR methods, using commercially available kits, are known to be used to determine viral load. It provides a high sensitivity and reproducibility and are used for quantitative analysis of a

number of clinically important viruses such as human immunodeficiency virus, hepatitis B and C viruses and cytomegalovirus (CMV) (Komurian-Pradel *et al.*, 2001; Piiparinen *et al.*, 2002; Watzinger *et al.*, 2004; Iloeje *et al*., 2006). PCR inhibition and the cell content of samples are two factors that can influence viral load (Schlecht *et al.*, 2003).

1.4 Liver Damage Biomarkers

Hepatitis is characterized by varying degrees of hepatocellular necrosis, damage and inflammation. Several liver diseases are associated with hepatitis E viral infection. The liver regulates many important metabolic functions. In general, only four serum biochemical tests are necessary to assess hepatic abnormalities. These include the levels of bilirubin, alanine aminotransferase (ALT) (EC 2.6.1.2), aspartate aminotransferase (AST), (EC 2.6.1.1), and alkaline phosphatase (ALP) (EC 3.1.3.1) (Desmet *et al*, 1994; Wolf, 1999).

Bilirubin is a brownish yellow substance found in the bile, which is produced by conjugating the unconjugated bilirubin formed on breakdown of red blood cells. Bile is secreted into the intestine from where it is removed by stool (feces). The kidneys also excrete part of this. It is this bilirubin that gives stool its normal color. It is assayed using tests and high levels of bilirubin may indicate the presence of hepatitis.

The enzyme, alanine aminotransferase (ALT), catalyzes the transfer of amino groups to form the hepatic metabolite oxaloacetate. Aspartate aminotransferase (AST) catalyzes the reversible transfer of an α-amino group between aspartate and glutamate. ALT and AST is found abundantly in hepatocytes where the activity of the enzymes are about 3000 times more than that in serum. During liver damage, these aminotransferase enzymes are released from damaged hepatic cells into the serum where the detection of unusually high level of ALT and AST indicates a high chance of liver damage. ALT and AST can also be found in much smaller quantities in the kidney, heart and skeletal muscle cells. The serum level of the enzyme can be detected using certain tests.

Alkaline phosphatase (ALP) is involved in metabolite transport across cell membranes. It is produced mostly in the liver, and in the placenta of a pregnant

woman. Alongside, it is also found in ileal mucosa, kidney and bone. The serum level of the enzyme is determined using certain biochemical tests. High level of ALP in the serum indicates hepatic damage (Dufour *et al*., 2000; Kim *et al*., 2008).

Beside these biomarkers, there is another way to find out whether liver damage due to hepatitis E viral infection has occurred or not. This is done by determining the level of binding affinity of human leukocyte antigen (HLA) to peptides that are formed by the virus; the higher the binding affinity, the greater is the chance of tissue damage. HLA is the human version of major histocompatibility complex (MHC) which is an extremely polymorphic antibody. There are two classes of HLA molecules, class-I (HLA-I) and class-II (HLA-II), that are recognized by CD8 and CD4 T cells, respectively. HLA-I consists of the molecules HLA-A and HLA-B, that are ubiquitously expressed (Duquesnoy, 2014; Ali *et. al*., 2008; Apps *et. al*., 2009). Thus these are the two that are mostly used for analysis of the binding affinity of HLA-I to peptides.

1.5 Rationale of the Study

In Bangladesh, studies have been undertaken on HEV infection from socio-medical or public health points of view. But with further advancement in scientific methods at hand it is becoming increasingly necessary to delve into the molecular level of HEV to understand the genetic factors that prompt unexpected behavior and response to medication. As such, this study seeks to give a glimpse into the genetic buildup or status of currently circulating hepatitis E virus in Bangladesh, specially in Dhaka.

1.6 Objectives of the Study

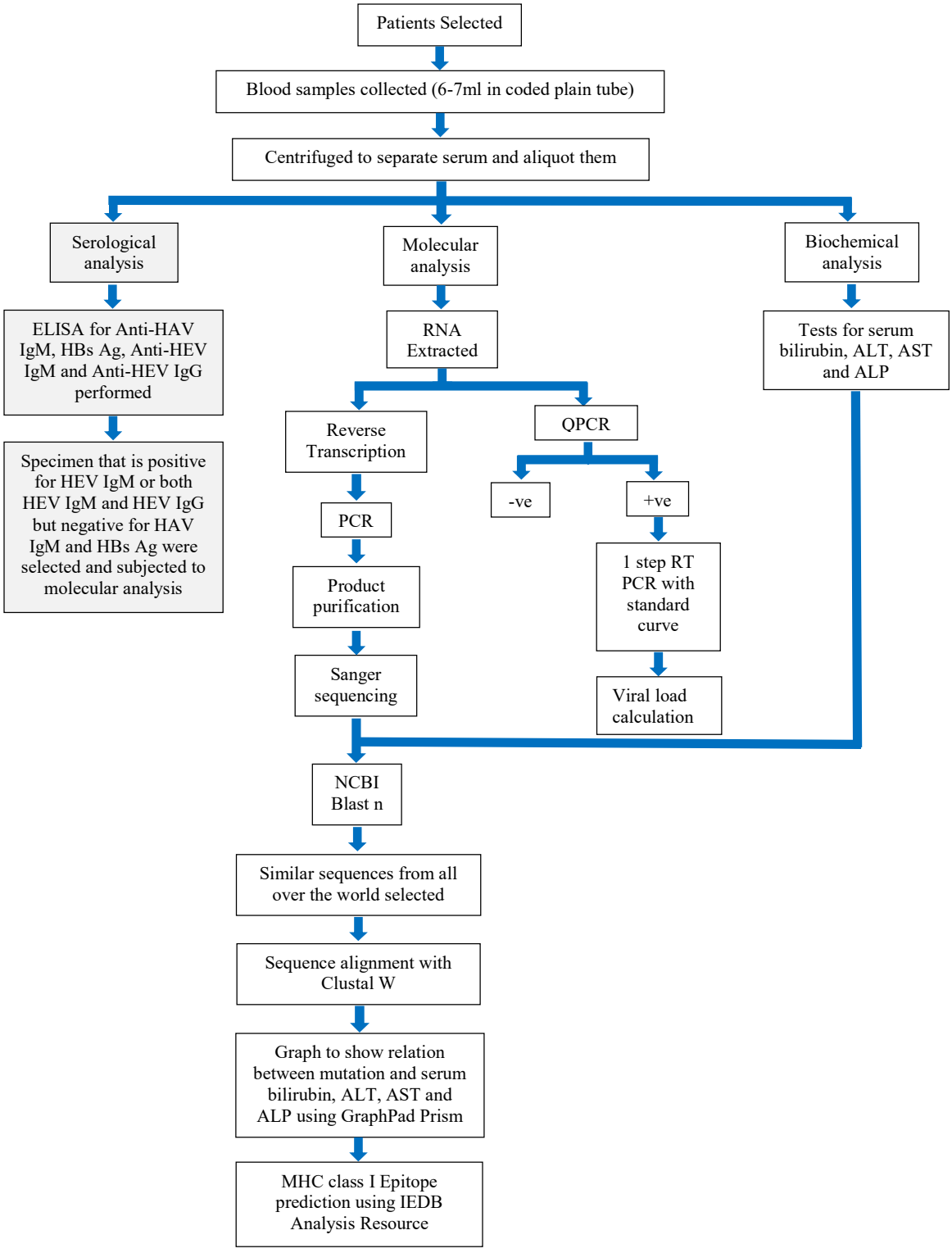
Hepatitis E viral infection is a rising public health concern in developing countries like Bangladesh. The aim of the present study was to establish a method to calculate viral load in infected patients and compare viral load between pregnant and non-pregnant women. In addition to viral load measurement, the study also focused to investigate the mutations in the nucleotide and protein sequences of the ORF 2 of hepatitis E viral genome and their effects on the patients and disease manifestation. It is expected that the genetic analysis of the virus may stimulate researchers to search

further in-depth and/or design assays to understand the different mutations and their effects on the disease prognosis.

Therefore, the specific objectives considered for this study were to:

- Determine the viral load in HEV positive patients
- Compare viral load between pregnant and non-pregnant women to understand the effect of viral load on disease manifestation
- Compare the nucleotide sequence of HEV circulating in Bangladesh with available data from other countries to identify the points of variation in protein sequence
- Measure level of liver damage biomarkers of patients
- Relate liver damage biomarkers with protein sequence variations
- Predict the effect and functional change in epitope-MHC class I interaction due to amino acid sequence variation and correlate changes in epitope-MHC class I interaction and liver damage markers in infected patients.

1.7 Conceptual Framework of the Study



Note: Shaded flow was not a part of this study.

CHAPTER TWO

MATERIALS AND METHODS

Materials and Methods

Keeping the research questions and objectives in mind, the details of the methods employed are penned below.

- **Research type:** Cross-sectional study
- **Study place:** The samples were collected from Dhaka Medical College Hospital (DMCH), Bangabandhu Sheikh Mujib Medical University (BSMMU) hospital and different urban healthcare centers of Dhaka city, and the laboratory work was done at the Institute for Developing Science and Health Initiative (ideSHi) Laboratory, Institute of Public Health Building, Mohakhali, Dhaka.
- **Time reference of data:** July 2016 to June 2017
- **Duration of study:** One year
- **Study population:** Selected hepatitis E virus infected pregnant and non-pregnant women who presented at DMCH and BSMMU
- **Data source:** Selected study population. Data collected from participants who have been originaly registered as respondents for a PhD project conducted by Dr Rozy Sulana titled "Influence of Hepatitis E viral load and genotypes on pregnant urban dwellers of Bangladesh". Only the first 42 participants have been included in this current study for analyzing genotype and determining viral load of hepatitis E virus among HEV infected pregnant and non-pregnant women in Bangladesh.
- **Site preparation:** All academic and administrative formalities were taken care of as part of the site preparation
- **Data collection technique and research instrument:**

Data collection technique	Research instrument	Data generated
Questioning	Researcher administered face-to-face open ended questionnaire	- Background history - Medical history - Socio-economic status
Laboratory based	Report sheets	- RNA profiling of HEV positive samples - Selected biochemical indicators

- **Sampling:** Purposive sampling technique was used to pick-up 42 blood samples from patients suffering from acute hepatitis. This was done as part of an ongoing

study at ideSHi. The samples were taken from pregnant,post-natal and non-pregnant women.

- **Sample size:** This academic study was a part of an ongoing extensive research. During this study, 42 blood samples were collected.
- **Data management:** The results from RNA profiling and biochemical analysis were collected and stored in the computer.
- **Ethical consideration:** After explaining the study objectives, potential comfort and discomfort, informed verbal consent was taken from human sample sources. They were free to retract their consent at any stage of their participation. Anonymity and confidentiality of the participants and records were maintained. No coercion or deception was done in any way. No physical, chemical, psychosocial or biological intervention was given to the study participants.

  Precaution was taken to meet the requirements to ensure safety aspects in handling blood samples. Adhering to the standard operating procedures (SOP) required for genetic analysis, laboratory analyses were carried out using BSL-1 and BSL-2 guidelines. Biochemical analysis was also done following the recommended SOP. Laboratory waste was treated and disposed as per standard guidelines. Plagiarism in any form was avoided by duly acknowledging, citing and referencing, and other issues related to source documentation.

2.1 Patient Selection

The samples were collected from DMCH, BSMMU hospital and different urban healthcare centers of Dhaka city. Pregnant, post-natal and non-pregnant female patients with acute hepatitis (history of jaundice for less than six weeks), who had no other co-morbid conditions such as respiratory and genito-urinary infection, autoimmune diseases… were enrolled in the study.

2.2 Sample Collection, Processing and Storage

With all aseptic precautions, the sample was collected from each participating patient through veni-puncture. Informed written consent was taken from each study subject before collection of blood sample. Six to eight ml of blood is collected in a coded sterile plain tube. This was kept in a cold box and quickly transported to the clinical laboratory for processing of the sample. The samples were centrifuged using

refrigerated centrifuge machine at 3000 rpm for 10 minutes at +4°C. The plasma layer was transferred to 1.5 mL micro-centrifuge tubes. These 1.5 mL micro-centrifuge tubes were further centrifuged at 3000 rpm for 5 minutes at +4°C. The plasma was then transferred in 200 µL aliquots in five 1.5 mL micro-centrifuge tubes for storage at -70°C. One of these was used for the biochemical test, one for serological analysis, and a third one was used for molecular analysis. The rest of the two aliquots are stored at -70°C for future analysis.

2.3 Biochemical Analysis

Biochemical analysis was done for the detection of liver damage to relate it with the affinity of peptides formed by the hepatitis E virus to the subclasses of HLA class 1 haplotype. For this, the serum bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkanine phosphatase (ALP) levels were detected. High levels of these in the blood serum are an indicator of damaged liver.

2.3.1 Serum bilirubin

The TBI method using the Dimension® Flex® reagent cartridge was used for quantitative estimation of total bilirubin in plasma samples. The TBI method is a modification of Doumas reference method. This *in vitro* method is used to detect the four distinct fractions of bilirubin in two distinct groups that make up the total bilirubin, viz-

- Conjugated: This is again composed of
  - mono and diconjugated bilirubin (β and γ-bilirubin)
  - delta fraction (δ-bilirubin) which is tightly bound to albumin
- Unconjugated: Unconjugated bilirubin (α-bilirubin) which is water insoluble

Measurement of total bilirubin is essential in the diagnosis, treatment and prognosis of liver, gallbladder, hemolytic, hematological, and metabolic disorders.

The unconjugated bilirubin is solubilized by dilution in a mixture of caffeine/benzoate/acetate/EDTA. Upon addition of the diazotized sulfanilic acid (formed by combining sodium nitrite and sulfanilic acid at low pH), the solubilized bilirubin, including conjugated bilirubin, is converted to diazo-bilirubin, a red

chromophore representing the total bilirubin which absorbs at 540 nm and is measured by bichromatic (540, 700 nm) endpoint technique.

Solubilized bilirubin + Diazotized sulfanilic acid $\longrightarrow$ Red chromophore

Strictly following the instructions accompanying TBI Flex® reagent cartridge (Cat. no. DF167), and using the suggested TBI Calibrator CAL (Cat. No. DC167) and purified water diluent (Cat no. 710615901), the steps quantitative determination of total bilirubin were done. For quality control, standard operating procedures of the laboratory were consistently followed.

2.3.2 Alanine aminotransferase (ALT)

The quantitative determination of alanine aminotransferase (ALT) activity in the serum from patients or plasma was performed. The process requires ALT reagents that are used to measure analyte activity by a kinetic rate method. In the reaction, alanine aminotransferase catalyzes the reversible transamination of L-alanine and alpha-ketoglutarate to pyruvate and L-glutamate. The pyruvate is then reduced to lactate in the presence of lactate dehydrogenase (LDH) with the concurrent oxidation of reduced β-nicotinamide adenine dinucleotide (NADH) to β-nicotinamide adenine dinucleotide (NAD).

$$\text{L-Alanine} + \alpha\text{-Ketoglutarate} \xleftrightarrow{\text{ALT}} \text{L-Glutamate} + \text{Pyruvate}$$

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xleftrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}^+$$

A proportion of one part sample to 11 parts reagent was put into the cuvette. The system monitors the change in absorbance at 340 nanometers. This change in absorbance is directly proportional to the activity of ALT in the sample and is used to calculate and express the activity of ALT.

2.3.3 Aspartate aminotransferase (AST)

The AST method using the Dimension® reagent cartridge for quantitative determination of aspartate aminotransferase (AST) in plasma samples was done. This method is an adaptation of the International Federation of Clinical Chemistry

recommendations. Here, the coenzyme pyridoxal-5-phosphate (PSP) activates apoenzyme and lactic acid dehydrogenase (LDH) to eliminate pyruvate interference. This was done to determine aspartate aminotransferase, an early indicator of liver function, which is elevated in conditions like hepatitis, hepatic necrosis, and cirrhosis. The principle of the method takes into consideration that AST catalyzes the transamination from L-aspartate to α-ketoglutarate, forming L-glutamate and oxaloacetate. The oxaloacetate is reduced to malate by malate dehydrogenase (MDH). The change in absorbance with time due to conversion of NADH to NAD is directly proportional to AST activity and is measured by using bichromatic (340, 700 nm) technique.

$$\text{L-aspartate} + \alpha\text{-ketoglutarate} \xrightarrow[\text{pH7.8}]{\text{AST}} \text{L-glutamate} + \text{Oxalacetate}$$

$$\text{Oxalacetate} + \text{NADH} \xrightarrow{\text{MDH}} \text{Malate} + \text{NAD}$$

Following strictly the instructions accompanying AST Flex® reagent cartridge, Cat. no. DF41A; and using the suggested Enzyme verifier, Cat. No.DC19, the steps of AST quantitative determination were done. For quality control, standard operating procedure of the laboratory, and comparison with known aspartate aminotransferase were diligently adhered to.

2.3.4 Alkaline phosphatase

The ALP method using the Dimension® reagent cartridge for quantitative determination of alkaline phosphatase (ALP) in plasma samples was done. This method is based on primary reference procedure for the measurement of catalytic activity of alkaline phosphatase at 37°C as recommended by the International Federation of Clinical Chemistry. This method responds to all alkaline phosphatase isoenzymes in human system, and is used in the diagnosis and treatment of diseases of liver, bone, parathyroid and intestines.

Alkaline phosphatase catalyzes the transphosphorylation of p-nitrophenylphosphate (p-NPP) to p-nitrophenol (p-NP) in the presence of transphosphorylating buffer 2-

amino-2-methyl-1-propanol (AMP). The reaction is enhanced by using magnesium and zinc ions. The change in absorbance due to formation of p-NP is directly proportional to ALP activity. Bichromatic (405, 510 nm) rate technique is used to measure the ALP content.

$$\text{p-NPP} + \text{AMP} \xrightarrow[\substack{\text{pH 10.25}\\ \text{Mg/Zn}}]{\text{ALP}} \text{p-NP} + \text{AMP} + \text{PO}_4$$

Following strictly the instructions accompanying ALP Flex® reagent cartridge (Cat. no. DF150); using required personal protection equipment (PPE), and using the suggested ALPI CAL (Cat. No. DC150) and enzyme diluent (Cat no. 790035901), the steps of ALP quantitative determination were done. For quality control, standard operating procedure of the laboratory was consistently adhered to.

2.4 Molecular Analysis

For the molecular analysis of Hepatitis E virus, blood samples that were collected were used to extract the ribonucleic acid (RNA) of the virus. This RNA was later used to detect the HEV RNA positive samples.

2.4.1 Isolation of hepatitis E virus (HEV) RNA from serum: Before the isolation process, a mixture of AVL Buffer and carrier RNA-buffer AVE was prepared. This was done by thoroughly dissolving equal volumes of Buffer AVE and lyophilized carrier RNA in a tube. This carrier RNA-buffer AVE mixture was aliquoted and stored at -30°C to -15°C. The buffer AVL and carrier RNA-buffer AVE was mixed as mentioned in the instruction manual accompanying the viral RNA extraction kit (QIAamp Viral RNA Mini Kit 52904), depending on the number of samples.

Precisely 560 µL of the prepared AVL Buffer containing carrier RNA was pipetted into a 1.5 mL micro-centrifuge tube. Then the 140 µL sample plasma was added to the Buffer AVL-carrier RNA into the micro-centrifuge tube and mixed thoroughly by pulse-vortexing for 15 seconds. After incubating for 10 minutes at room temperature (15-25°C), the tubes were spun to settle the contents and remove any droplets on the inner wall. Then 560 µL of 96-100% ethanol was added to the sample and again mixed by pulse-vortexing for 15 seconds. This was again spun briefly to remove any droplets on the inner wall. From this solution, 630 µL was carefully applied to the

QIAamp Mini column without wetting the rim. The column was then centrifuged at 6000×g for 1 minute. The QIAamp Mini column was placed in a clean 2 mL collection tube, while the previous collection tube containing the flow-through was discarded. These steps were repeated until all the sample mixture was passed through the QIAamp Mini column.

Once all the mixture passed through the column, 500 µL of Buffer AW1 was added to the QIAamp Mini column. This was subjected to centrifugation at 6000×g for 1 minute and the collection tube containing the discarded flow-through was replaced. Then, 500 µL of Buffer AW2 was added to the column which was centrifuged at 20000×g for 3 minutes and the collection tube was replaced with a new one. This was centrifuged at the same speed for a minute and the collection tube was again replaced. Finally, 60 µL of Buffer AVE, equilibrated to room temperature, was added and left to incubate at room temperature. This was then centrifuged at 6000×g for 1 minute and the flow through stored at -70°C. The extracted viral RNA is stable for up to a year at this temperature.

Once the RNA was extracted, real time PCR was used to detect the presence of HEV RNA.

2.4.2 Real time PCR detection of HEV: The one step reverse transcription real time polymerase chain reaction (PCR) involves reverse transcription followed by real time PCR. This requires a forward and a reverse primer along with a probe that is tagged at the 5′ end with the fluorescent dye cy5, and at 3′ end with the quencher BHQ_2. TaqMan probe, a Taq polymerase enzyme that uses 5′→3′ exonuclease activity in the process, is also required. Initially the reverse primer produces cDNA from HEV RNA in the reverse transcription step. If the cDNA is produced, it is subjected to real time PCR which detects the presence of HEV in the serum sample. During the elongation process, the 5′→3′ exonuclease activity of Taq polymerase cleaves, and as a result frees the cy5 in the 5′ end, repeated cycles of which crosses the threshold value which results in the observance of a fluorescence.

For detection of the HEV RNA, the RT-PCR was carried out in the Bio-Rad's CFX96 Touch™ Real-Time PCR Detection System. The real time PCR process involves the preparation of reaction volume of 12.5 µL of master mix for each sample. The

reagents and enzymes in the master mix act for amplification of HEV, and direct detection of the specific amplicons in the fluorescence channels. The primers were designed to anneal to a nonvariable region of the ORF2 of all HEV genotypes. The master mix includes 6.25 µL of 2X reaction mix, 0.25 µL of the forward primer (nucleotide 5237-5255) HERTF (5' GGTGGTTTCTGGGGTGAC 3'), an equal volume of the reverse primer (nucleotide 5289- 5307) HERTR (5'AGGGGTTGGTTGGATGAA 3'), 0.125 µL of the probe (nucleotide 5260- 5278) HERTPR (5' Cy5/TGATTCTCAGCCCTTCGC/BHQ_2 3'), 0.25 µL of RNase OUT, 0.25 µL of SuperScript® III/Platinum Taq mix (cat no.: 11732-020), 150 ng of RNA sample, and water upto 12.5 µL. Internal controls provided by the manufacturer, and the positive and negative control samples were included in the amplification step as per SOP. The reaction tubes were put in a thermal cycler and incubated in the following thermal cycling profile:

Steps	Temperature	Time	Cycles
Reverse transcription	50°C	15 mins	1
Initial denaturation	95°C	2 mins	1
Denaturation	95°C	15 seconds	45
Annealing	55°C	30 seconds	

After the real time PCR, cDNA from the isolated HEV RNA virus was prepared.

2.4.3 Preparation of cDNA from isolated hepatitis E virus RNA: The preparation of cDNA involves reverse transcription which is a process of producing cDNA from mRNA using random hexamer or oligo-dT primer. After the production of cDNA, mRNA from cDNA-mRNA duplex is degraded by RNase H. Then using gene specific primer, PCR is accomplished to amplify the cDNA.

This process requires the use of the SuperScript® III First-Strand Synthesis System kit (Invitrogen: cat no. 18080051). As per the instruction manual, the RNA mixture was prepared by mixing 1.0 µL of random hexamers, 1.0 µL of 10 mM dNTPs (with SuperScript® III), 200 ng of the isolated viral RNA and nuclease free water upto10 µL. This mixture was heated at 65°C for 5 minutes in a PCR machine or heat block, and after incubation kept in ice for 2-3 minutes. Next the master mix was prepared, whose components were 2.0 µL of 10X RT buffer, 4.0 µL of 25mM $MgCl_2$, 2.0 µL of 0.1 mM DTT, 1.0 µL of SuperScript® III enzyme and 1.0 µL of RNase OUT. This

was added to the RNA mixture to prepare reverse transcription mixture which was incubated using a thermal profile in thermal cycler (Bio-Rad) that included 10 minutes at 25°C, 50 minutes at 50°C and 5 minutes at 85°C, and then cooled on ice followed by the addition of 1 µL of RNaseH. After an incubation at 37°C for 20 minutes, some of it was stored at -20°C while some were used immediately to perform PCR using the HEV cDNA as a template.

2.4.4 HEV cDNA amplification using PCR: Conventional PCR is a technique that is used to polymerize a nucleic acid sequence and to amplify single copy or few copies of target DNA. The process begins with the master mix. The preparation of the master mix involved a reaction volume of 10 µL for each sample. It was made by mixing 1.0 µL of 10X PCR buffer (Qiagen; cat no. 203203), 0.4 µL of 25 mM $MgCl_2$ (Qiagen; cat no. 203203), 1.6 µL of 2.5 mM dNTPs (Takara; cat no. 4030), 2.0 µL of 5X Q-solution (Qiagen; cat no. 203203), 0.5 µL of the forward primer (nucleotide 6554-6573) RfF_1 (5'-GCCGAGTATGACCAGTCCA-3'), 0.5 µL of the reverse primer (nucleotide 7084- 7104) RfR_1 (5'-ATAACTCCCGAGTTTTACCC-3'), 0.05 µL of HotStartTaq DNA polymerase (Qiagen: cat no. 203203), 1.0 µL of cDNA from RNA sample and finally water upto 10 µL. Such tubes were then put in a thermal cycler and incubated in the following thermal cycling profile:

Steps	Temperature	Time	Cycles
Initial denaturation	95°C	15 mins	1
Denaturation	94°C	30 seconds	45
Annealing	50 – 60°C	50 seconds	
Extension	72 °C	40 seconds	
Final extension	72 °C	10 minutes	

After the completion of each of the thermal cycles, the products from the three processes mentioned in sub-sections 2.4.2, 2.4.3 and 2.4.4 (real time PCR for HEV detection, preparation of cDNA from HEV RNA, and conventional PCR using HEV cDNA) were analysed using 1% agarose gel.

2.4.5 Preparation of 1% agarose gel and the loading of PCR products: Agarose gel was prepared by adding 1 gm of agarose powder in 100 ml of Tris/Borate/EDTA buffer (TBE buffer). Keeping this proportion of agarose powder and TBE buffer constant, agarose gel was prepared when required. The required amount of agar was prepared in a autoclaved conical flask, and put into an oven to boil till the agarose

powder was fully dissolved in the TBE buffer and no particle was observed. The flask was then removed from the oven and let to cool down for some time. The intercalating nucleic acid stain – gel red – was then added to this. Next the agarose solution was poured into previously ethanol washed gel casts with combs, and let to solidify. Once this solidified, the comb was removed and the gel was completely submerged into TBE buffer in the electrophoresis machine. Then the PCR products were loaded onto the gel wells.

To load the PCR products, 2 µL of the loading dye was put on a parafilm paper. On this, 3 µL of the PCR product was added. This was then mixed and added into the wells in the agarose gel alongside which the DNA ladder, consisting of reference bands, was also added. Next, the lid of the electrophoresis machine was closed and the machine turned on and a current with 150 V was passed through the solution. Once the gel run was complete, the gel was viewed in the Gel-Doc machine and straight bands were observed. Using the ladder as a reference, the length of the DNA was calculated.

Once the PCR products underwent gel electrophoresis, they were subjected to PCR product purification steps to obtain the products.

2.4.6 PCR product purification

The PCR products were purified on spin columns using the MinElute PCR Purification Kit (Qiagen, Cat.No. 28006). The PCR tubes containing the PCR products were spun and contents transferred to 1.5 mL Eppendorf tubes. Then, 5 volumes of Buffer PB was added to 1 volume of the PCR product and mixed thoroughly. The colour of the solution was checked. If orange/violet, then 10 µL 3 M sodium acetate (pH 5.0) was added and mixed by inverting. If yellow (similar to Buffer PB without the PCR sample), then sodium acetate was not added. The samples were then transferred to a 2 ml collection tube provided with MinElute spin column and incubated for 5 minutes at room temperature. The column was then subjected to centrifugation at 17900×g for 30-60 seconds. The flow-through was discarded and the MinElute column was placed back into the same collection tube. Then, 750 µL of Buffer PE was added to the MinElute column. The column was incubated at room temperature for 5 minutes and again centrifuged at 17900×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 17900×g for 1 minute. Each of the MinElute columns were 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 17900×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 freezer.

2.4.7 Sequencing of viral genome: The viral genome was sequenced using Sangers sequencing methods.

2.5 Determination of the Viral Load of the HEV RNA Positive Samples

To detect the HEV RNA content, the efficiency of the primers used and viral load in the samples for this study, it was necessary to develop a standard curve to determine the copy number. This standard curve was developed using synthetic viral RNA that was procured from Integrated DNA Technology (IDT), a supplier of custom nucleic acids, serving the areas of academic research, biotechnology, clinical diagnostics, and pharmaceutical development. The procured synthetic viral RNA contained 0.13 mg of 81 base pairs (bp) of the genotype 1 of hepatitis E virus ORF 2.

The calculation to determine the copy number of the standard was:

$$Number\ of\ copies = \frac{Amount\ of\ amplicon\ (ng) \times 6.0221 \times 10^{23} molecules/mole}{Length\ of\ dsDNA\ amplicon \times 660\ g/mole\ \times 10^{9}\ ng/g}$$

$$= \frac{13000\ ng \times 6.0221 \times 10^{23} molecules/mole}{81\ bp \times 660 \times 10^{9}\ ng/mole\ per\ bp}$$

$$= 1.46 \times 10^{15}\ copies\ of\ dsDNA$$

$$\cong 2.929 \times 10^{15}\ copies\ of\ ssDNA$$

Where the amount of amplicon = 0.13 mg = 130000 ng
Length of amplicon = 81 bp
Avogadro number = 6.0221×10^{23} molecules/mole
Average mass of 1 bp dsDNA = 660 g/mole

The synthetic viral RNA was diluted to form standards for certain copy numbers, followed by real time PCR to determine the Ct (threshold cycle) value. The 0.13 mg of the amplicon was dissolved in 540 µL of water. Therefore, in 540 µL, there was 2.929×10^{15} copies of ssDNA, making a copy number of [$(2.929 \times 10^{15}) / 540$] or 5.4×10^{12} copies per µL. For accuracy in measurement, this was further diluted to 5.4×10^{9} copies per µL by adding 1000 µL of water. From this, 1.85 µL was dissolved in water till the volume reached 1000 µL to get 1000 µL of 10^{7} copies/µL of ssDNA, which was stored as stock amplicon. The stock amplicon was serially diluted to obtain different copy numbers of ssDNA, as shown below:

Number of template copies and the amount of stock used to make it

Template copies	**Amount (stock=10^7 copies / 1 µL)**
1,000,000	1 µL stock + 9 µL nuclease free water
100,000	1 µL previous dilution + 9 µL nuclease free water
10,000	Serial dilution from formerly diluted sample
1,000	Serial dilution from formerly diluted sample
100	Serial dilution from formerly diluted sample
10	Serial dilution from formerly diluted sample
1	Serial dilution from formerly diluted sample

Once these dilutions were obtained, the amplicons were put through real time PCR to determine their C_t values. The values were used to draw a standard graph for the determination of the copy numbers of HEV RNA positive samples.

2.6 Databases, Tools, and Software Used in the Study

In this study, different databases and online tools were used to attain and analyse hepatitis E viral genome and protein sequences found in different parts of the world. Several online and offline software were also used for this purpose in line with the conceptual framework of this study.

2.6.1 National Center for Biotechnology Information (NCBI): This was established in 1988 as a modern molecular biology attempt to understand the molecular data that is encoded within the genome. It is a national resource for all molecular biology information that may affect the human health, where every new

discovery is recorded and stored. This computerized database contains software tools for analyzing genome data analysis tool; it also develops, and disseminates biomedical information.

Since NCBI is a database that contains all genome data that have been discovered, it was considered to be a good source of genome sequences of hepatitis E virus ORF 2 from across the world. Around 100 different nucleotide sequences from India, Nepal, Pakistan, Myanmar, Singapore, Thailand, Japan, Mongolia, China, Egypt, Germany, United Kingdom, Italy, France, Spain, United States, Canada, Morocco, Mexico and Chad were downloaded.

2.6.2 BLAST: The Basic Local Alignment Search Tool (BLAST) finds regions of local similarity between sequences. It takes an input of nucleotide and protein sequences and searches and compares it to nucleotide or protein sequences from sequence databases and calculates the statistical significance of the matches (Boratyn *et al.*, 2013). In this study blast N-suite (BLASTn) was used to retrieve the sequences of hepatitis E virus ORF 2.

2.6.3 Expert Protein Analysis System (ExPASy) Translate: ExPASy is a well known bioinformatics resource for proteomics. ExPASy translate is a tool from ExPASy that translates the nucleotide sequences to protein that were obtained from performing BLAST search (Artimo *et al.*, 2012). This is used in this study to translate the nucleotide sequences that were retrieved through NCBI BLAST.

2.6.4 MEGA 6: Molecular Evolutionary Genetics Analysis version six (MEGA6) software contains facilities for building sequence alignments, inferring phylogenetic histories, and conducting molecular evolutionary analysis (Tamura *et al*., 2013; Larkin *et al.*, 2007). This was used to align the nucleotide sequences obtained from BLAST and the protein sequences obtained from the ExPASy Translate.

2.6.5 T Cell Epitope Prediction Tools: The T Cell Epitope Prediction Tools are used for prediction of binding affinity of peptides to different subclasses of HLA class 1 haplotype. It uses the Arificial Neutral Network (ANN) and Stabilized Matrix Method (SMM) methods for prediction, which is given by IC_{50} values. Peptides with IC_{50} values less than 50 nM are considered high affinity, those between 50 nM and

500 nM are considered intermediate affinity and those between 500 nM and 5000 nM are considered low affinity. No known T-cell epitope has an IC_{50} value greater than 5000 nM.

2.6.6 GraphPad Prism 5: GraphPad Prism 5 is a software used for commercial scientific two dimensional graphing and statistics. It combines scientific graphing, comprehensive curve fitting (nonlinear regression), understandable statistics, and data organization. In this study, it is used to develop different graphs to find the relation between different variables and to measure the statistical significance.

These methods were followed to analyse the hepatitis E viral genome. The results are discussed in the following chapter.

CHAPTER THREE

RESULTS

Results

3.1 Selection Criteria of Study Participants

Blood samples were collected from hepatitis E patients selected from those attending Dhaka Medical College Hospital (DMCH) and Bangabandhu Sheikh Mujib Medical University (BSMMU) hospital or those from few local hospitals. The study participants were selected based on the level of serum bilirubin, alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP). Patients with a serum bilirubin level above 1.2 mg/dL, ALT above 40 U/L, AST above 37 U/L and ALP level above 120 U/L were selected. A total of 42 study participants were selected based on these criteria.

3.2 Age and Pregnancy Status of Study Participants

The study participants included antenatal, post-natal women, and non-pregnant women. The age of the ten antenatal women were between 18 – 38 years, with a mean of 25.6 years and a standard deviation of 6.53. The age range of 13 post-natal and 19 non-pregnant women were between 18 – 40 and 18 – 70 years, respectively; whereas their mean ages were 26.31 and 36.32 years respectively; while the standard deviation were 6.56 and 15.84 respectively (Table 3.1).

Table 3.1: Pregnancy status and average age of the study participants (N=42)

Pregnancy status	Number of participants	Age range (years)	Mean age (years)	Standard deviation of age
Pregnant	10	18-38	25.6	± 6.53
Post-natal	13	18-40	26.31	± 6.56
Non-pregnant	19	18-70	36.32	± 15.84

3.3 Determination of Standard Curve for Viral Load

To check the efficiency of primers used for detection of HEV RNA and to calculate the viral load, synthetic viral nucleic acid was used which contained the conserved region of HEV genome and the primers were designed form conserved region so that any of the well-known four genotypes could be detected using those primers. The synthetic viral nucleic acid 10^6, 10^5, 10^4, 10^3, 10^2, 10 and 2 copies/µL were amplified using the same reaction condition in one step reverse transcription real time PCR. Then, a graph was prepared plotting the cycle of threshold (C_t) in X-axis and the log of viral copy number in Y-axis (Figure 3.1). The correlation coefficient for C_t vs. log of viral copy number was r = -0.9997. Thus, using the obtained equation y = -3.8695x + 37.63 from the prepared standard curve, the primer efficiency was determined to be good and the standard curve could estimate the log of copy number in sample specimens with great efficiency.

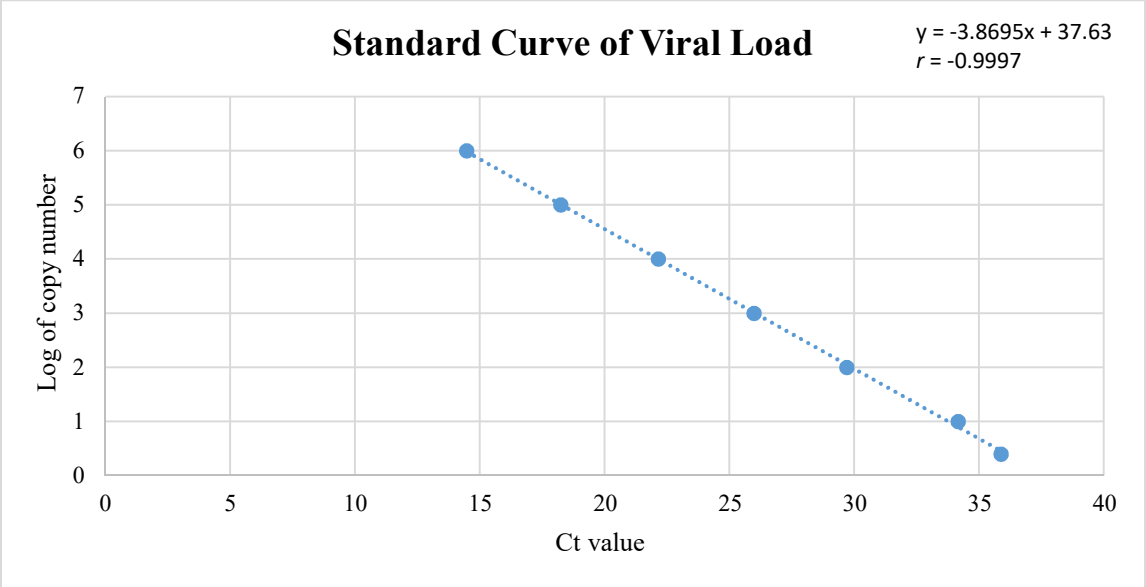


Figure 3.1: Graph showing the standard curve for the detection of viral load

3.4 Detection of HEV Positive Samples and Viral Load Using Real Time Polymerase Chain Reaction (RT-PCR)

After confirming that the primer could efficiency amplify HEV viral nucleic acid as low as two copies in reaction mixture, we then used them to detect the presence of HEV in viral hepatitis patients. One-step reverse transcription real time polymerase chain reaction (PCR) was performed for the detection of HEV RNA positive cases among study participants. As the detection method was probe based, any evidence of amplification was considered as a positive result. Three replicates were used for each sample. The readings obtained from the real time PCR is given in Table 3.2. Out of the 42 samples, 15 (S1 through S15) were positive for HEV. Two of these samples (S1 and S10) were collected from BSMMU; two (S12 and S15) from the local hospitals; and the rest of the positive samples (11 specimens) were from DMCH (S2 to S9, S11, S13 and S14).

The equation y = -3.8695x + 37.63 was used to determine the log of copy number (x) from the Ct value (y). Initially, the RNA isolation was started from 140 µL of serum and finally the RNA was eluted using 60 µL of nuclease free water. As 2.5 µL of sample elution was used for reverse transcription real time polymerase chain reaction, the log copy number got from the calculation was then converted to total HEV genome in 60 µL extraction or 140 µL of serum specimen. Finally, the amount of viral genome in copies/1mL of serum was estimated (Table 3.2).

Table 3.2: The HEV copy number in 1000 µL of serum from each patient along with their pregnancy status

Sample number	C_t value (Mean ± SD)	Log of copy number	Copy number in 2.5 µL of dissolved RNA	Copy number in 140 µL of serum	Copy number in 1000 µL of serum	Pregnancy status
S1	36.05 ± 0	0.41	2.55	61.31	437.91	Non-Pregnant
S2	36.65 ± 1.02	0.43	2.69	64.62	461.55	Non-Pregnant
S3	35.12 ± 0.27	0.65	4.46	107.04	764.54	Non-Pregnant
S4	26.99 ± 0.10	2.75	560.90	13461.53	96153.81	Pregnant
S5	37.84 ± 0	-0.06	0.88	21.13	150.90	Non-Pregnant
S6	29.54 ± 0.09	2.09	123.49	2963.85	21170.33	Non-Pregnant
S7	34.57 ± 0.59	0.79	6.16	147.95	1056.77	Pregnant
S8	37.19 ± 0.27	0.11	1.30	31.13	222.36	Non-Pregnant
S9	35.71 ± 0.52	0.50	3.14	75.41	538.63	Post-Natal
S10	32.54 ± 0.30	1.31	20.63	495.18	3537.02	Post-Natal
S11	33.92 ± 0.24	0.96	9.11	218.70	1562.16	Post-Natal
S12	34.27 ± 0.29	0.87	7.38	177.23	1265.94	Pregnant
S13	28.95 ± 0.12	2.24	175.06	4201.41	30010.07	Post-Natal
S14	37.2 ± 0	0.11	1.29	31.00	221.42	Pregnant
S15	36.59 ± 1.45	0.27	1.86	44.56	318.31	Non-Pregnant

After initially calculating the copy numbers in HEV infected women, who was either in pregnant, post-natal or non-pregnant state, comparisons were made among the groups to find out whether there were differences in viral copy numbers during or post-pregnancy period compared to non-pregnant women. Among the fifteen HEV RNA positive cases, four were pregnant, four were post-natal, and seven were non-pregnant women. The four pregnant women had viral load 96153.81 copies/mL, 1056.77 copies/mL, 1265.94 copies/mL, and 221.42 copies/mL, respectively with an average viral load of 24674.49 ± 41270.46 copies/mL (mean ± SD). In the four post-natal women, viral copy numbers were 538.63 copies/mL, 3537.02 copies/mL, 1562.16 copies/mL and 30010.07 copies/mL of serum. The mean of viral load was 8911.97 ± 12228.58 copies/mL (mean ± SD). On the other hand, the seven non-pregnant women had a viral count of 437.91 copies/mL, 461.55 copies/mL, 764.54 copies/mL, 150.90 copies/mL, 21170.33 copies/mL, 222.36 copies/mL and 318.31 copies/mL with an average of 3360.84 ± 7273.03 copies/mL.

The viral copy numbers did not show statistically significantly difference (p = 0.41) between pregnant and non-pregnant women (Figure 3.2) as were the cases for post-natal versus non-pregnant (p = 0.17) and pregnant versus post-natal (p = 0.86). Although no significant difference were observed in the copy number in different groups of women infected with HEV virus, the average copy numbers for the pregnant (24674.49 ± 41270.46 copies/mL) and post-natal (8911.97 ± 12228.58 copies/mL) women were higher than that of the non-pregnant women (3360.84 ± 7273.03 copies/mL).

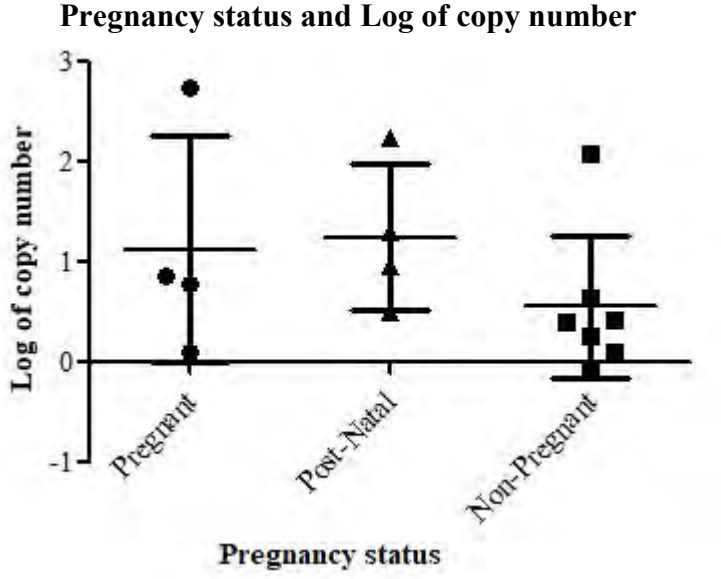


Figure 3.2: Vertical scatter diagram showing log of copy number with pregnancy status

3.5 Demography of HEV RNA Positive Cases

Among the fifteen HEV RNA positive cases, four were pregnant, four were post-natal, and seven were non-pregnant women. The pregnant and post-natal women were within the age range of 18-25 and 18-28 years, respectively. Their mean and standard deviation of age were 21 ± 2.74 years and 23.5 ± 3.57 years respectively. On the other hand, age of non-pregnant women ranged between 19-70 years with a mean and SD of 34 ± 16.35 years. The household income of these women was used as a proxy indicator of their socio-economic status. This statistic helped in identifying any correlation between the

socio-economic status and the onset of hepatitis E infections. The means and SDs of the annual income of pregnant, post-natal, and non-pregnant women's households were BDT 675000 ± 525000, 192000 ± 108000, and 320,800 ± 343445 correspondingly.

The durations of jaundice of these three groups of women were 16.25 ± 7.26, 16.75 ± 3.83, and 26.71 ± 11.77 days, respectively and their pulses per minute were 79 ± 1.73, 84 ± 6.93, and 70.29 ± 5.5, respectively. In that order, the respirations per minute of these women were 17.5 ± 0.87, 25 ± 13.30, and 15.71 ± 2.25.

Table 3.3: Pregnancy status, and mean and standard deviation of some selected participants (N=15)

Pregnancy status	Number of participants	Age range (years)	Mean ± SD of age (years)	Mean ± SD income/ annum (BDT)	Mean ± SD of duration of jaundice (days)	Mean ± SD of pulse (per minute)	Mean ± SD of respiration (per minute)
Pregnant	4	18-25	21 ± 2.74	675,000 ± 525000	16.25± 7.26	79± 1.73	17.5± 0.87
Post-natal	4	18-28	23.5 ± 3.57	192,000 ± 108000	16.75 ± 3.83	84± 6.93	25 ± 13.30
Non-Pregnant	7	19-70	34 ± 16.35	320,800 ± 343445.1	26.71 ± 11.77	70.29 ± 5.5	15.71 ± 2.25

Of these fifteen women, six lived in tin shed houses, whilst nine lived in buildings. With regard to their source of drinking water, seven of the women took water supplied from municipality, two drank tube-well water, five drank boiled water, and one woman drank filtered water. In relation to the consumption of street food, three women were found to consume street foods frequently, while only two women had never eaten street food.

Table 3.4: Distribution of selected socio-economic features of the women (N=15)

Pregnancy status	Housing type		Drinking water source				Street food habit		
	Tin shed	Building	Filter	Municipal supply	Boiled	Tube-well	Yes, frequent	Yes, infrequent	No
Pregnant	1	3	0	2	2	0	1	3	0
Post-Natal	1	3	0	3	0	1	1	3	0
Non-Pregnant	4	3	1	2	3	1	1	4	2

3.6 Biochemical Findings

The findings of selected biochemical tests – serum bilirubin, alanine transaminase, aspartate transaminase and alkaline phosphatase, performed on serum samples from the fifteen HEV positive patients are shown in the Table 3.5. The mean ± SD of serum bilirubin in HEV positive samples was 8.45 ± 4.24 (mg/dL) and that of ALT was 529.93 ± 529.78 (U/L). The average AST level of the samples was 324.13 ± 323.74 (U/L) (mean ± SD) and that of ALP was 186.07 ± 76.56 (U/L) (mean ± SD).

The data presented in Table 3.5, had elaborated the fact that the liver damage markers were higher in some patients (S3, S4, S6, and S15), whereas they were comparatively lower in other patients. These differences could be due to variations in host immune responses to the HEV infections and may involve inter-individual difference in MHC class-1 haplotype or difference in the natural killer (NK) cell responses. Involvement of these probable reasons would be further elaborated in epitopes, haplotype and immune response section of results.

Table 3.5: Distribution of biochemical findings of HEV positive samples (N=15)

Sample number	**Serum bilirubin** (mg/dL)	**ALT** (U/L)	**AST** (U/L)	**ALP** (U/L)
S1	6.1	321	250	217
S2	8.9	205	109	117
S3	11	1011	980	137
S4	6.8	1286	936	293
S5	3.6	84	71	234
S6	15.6	1607	709	152
S7	10.3	253	149	129
S8	6.4	82	53	88
S9	5.4	256	103	133
S10	13.9	514	333	218
S11	15.1	513	321	344
S12	2.4	29	33	109
S13	7.6	223	100	295
S14	2	50	31	209
S15	11.6	1515	684	116
Mean ± SD	8.45±4.24	529.93±529.78	324.13±323.74	186.07±76.56

Note: The HEV positive samples have been labeled as S1–S15

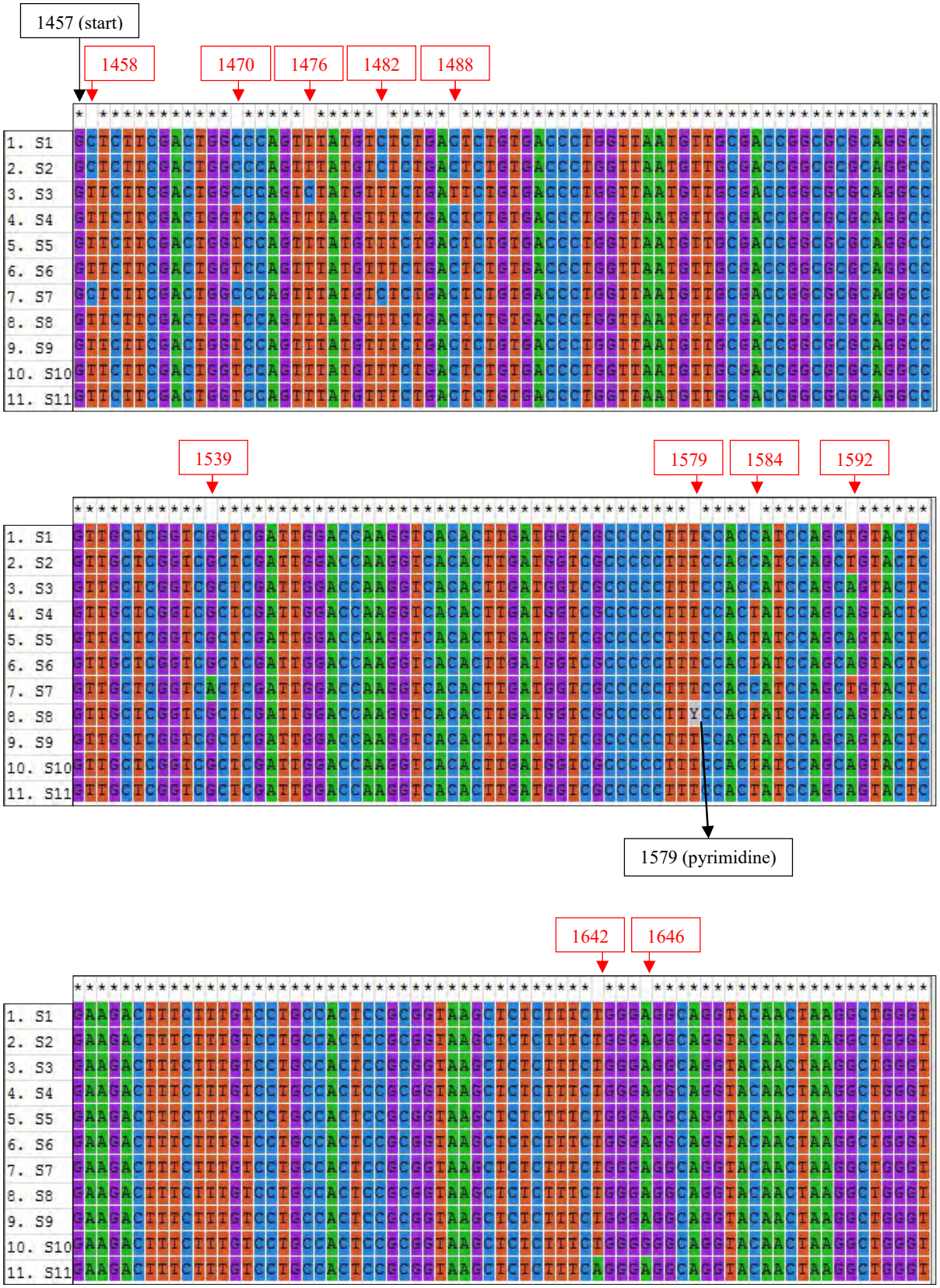
3.7 Sequence Variation in Study Samples

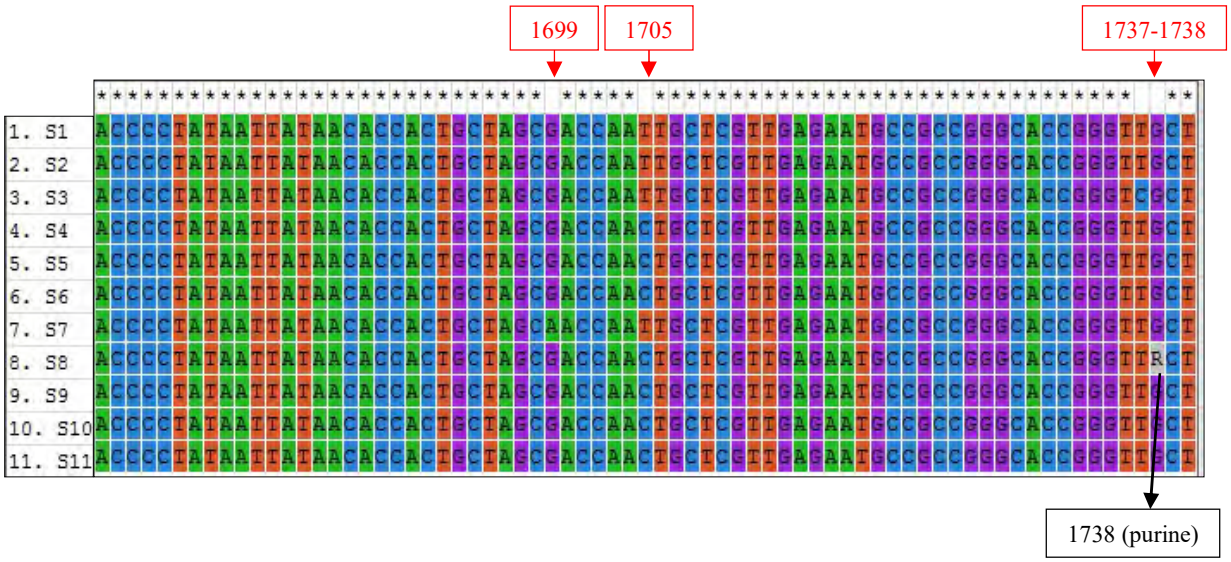
Of the fifteen HEV positive study samples, the first eleven were sequenced using Sanger DNA sequencing approach. The following figure (Figure 3.3) represents the dissimilarities found after the alignment of the nucleotide sequence of these samples.

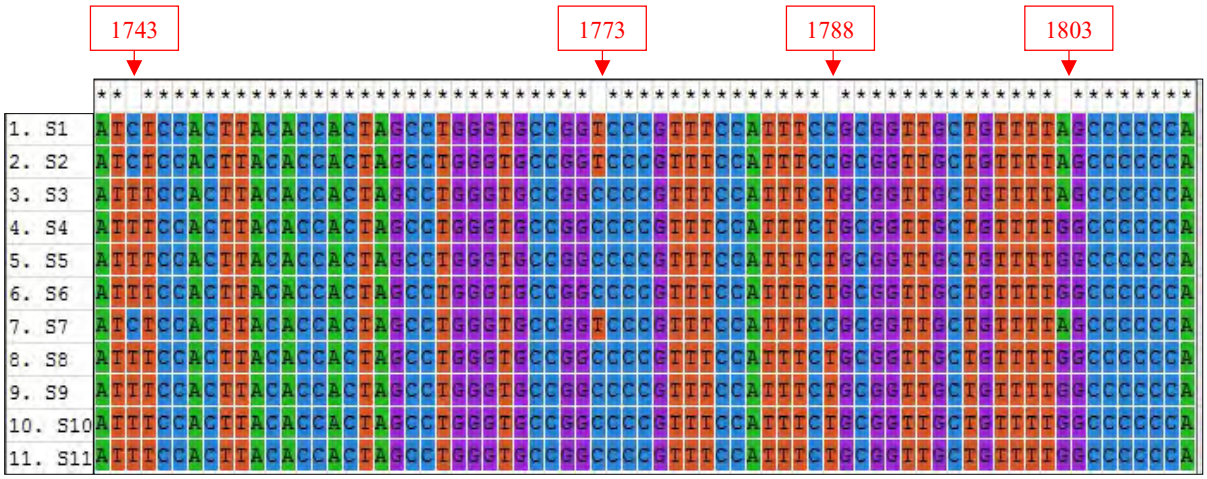
Within the segment of the ORF 2 region of the viral genome that was sequenced, there were altogether thirty two nucleotides that were not similar. Of these, two were from sample S8 in which the nucleotide base at the 1579th position had both cytosine (C) and thymine (T) – pyrimidine bases, and at the 1738th position had both adenine (A) and guanine (G) – purine bases. This maybe because the patients were simultaneously infected by two different hepatitis E viruses, or the patients may have already been chronically infected with one virus and the onset of the second infection with different genome of the virus might have been responsible for the chronic infection.

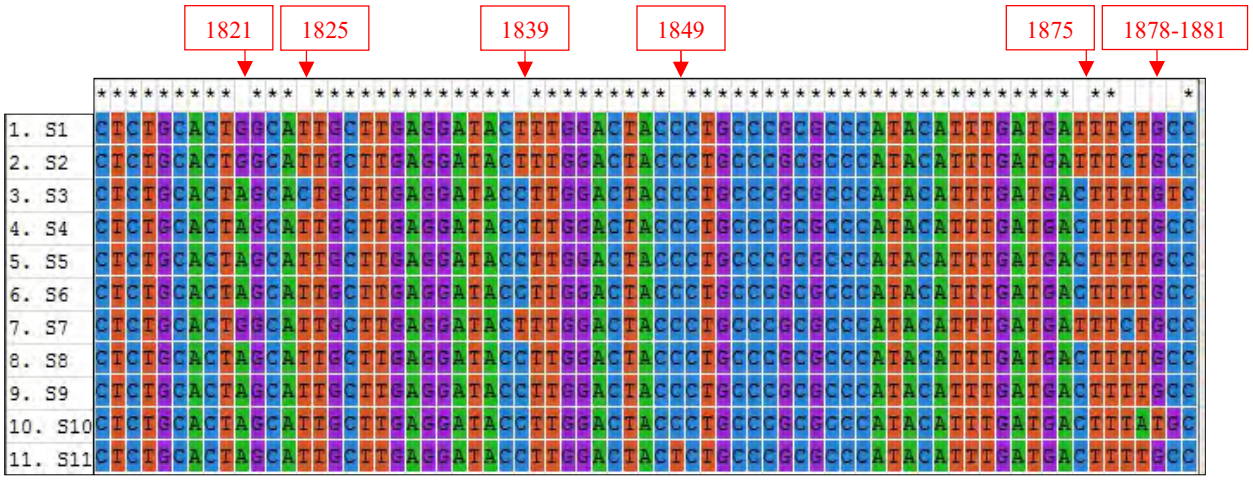
There was T ↔ C substitution at positions 1458, 1470, 1476, 1482, 1584, 1737, 1743, 1788, 1825, 1878, 1926, 1488, 1705, 1773, 1839, 1849, 1875 and 1899 of the ORF 2 in Hepatitis E viral genome, while a C ↔ G substitution was observed at 1897th position. It was noted that at 1881st nucleotide position, there was both T ↔ C and C ↔ G substitutions. Similarly, it was observed that at the positions 1539, 1699, 1803, 1910, 1646 and 1821, there was a replacement of the nucleotide bases G and A (G ↔ A). The nucleotides A and T substituted each other at 1592, 1642 and 1879 positions (A ↔ T). Finally, at 1880, there was a G ↔ T replacement.

Multiple Sequence Alignment (MSA) of nucleotide sequence of first eleven samples




1699
1705
1737-1738
1. S1
2. S2
3. S3
4. S4
5. S5
6. S6
7. S7
8. S8
9. S9
10. S10
11. S11
1738 (purine)


1743
1773
1788
1803
1. S1
2. S2
3. S3
4. S4
5. S5
6. S6
7. S7
8. S8
9. S9
10. S10
11. S11


1821
1825
1839
1849
1875
1878-1881
1. S1
2. S2
3. S3
4. S4
5. S5
6. S6
7. S7
8. S8
9. S9
10. S10
11. S11

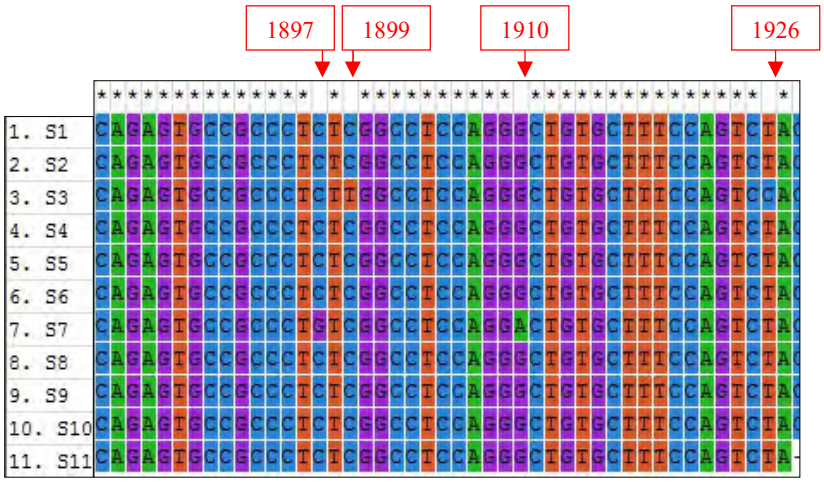


Figure 3.3: Dissimilarities found after the alignment of the nucleotide sequence of HEV positive (N=11)

The nucleotide sequences of Hepatitis E virus found in HEV positive samples were translated to amino acid sequences with the aid of the web-based tool ExPASy translate. Figure 3.4 represents the dissimilarities found after the alignment of the amino acid sequences. The amino acids in the 527th and 580th positions were the ones corresponding to the nucleotides at the 1579th and 1738th positions which had two bases at the same position, as mentioned in Fig 3.2. At the 527th position of amino acid, it was either serine (when thymine is present at the 1579th position in nucleotide sequence) or proline (when cytosine is present at the 1579th nucleotide position). At the 580th position, it was either alanine (when guanine is present at the 1738th position in nucleotide sequence) or threonine (when alanine is present at the 1738th nucleotide position).

From the Figure 3.4, it is noted that some variations are present in different amino acid positions in any one sample. The 548th position of the amino acid sequence, the sample S11 had arginine (R) while the rest of the samples had tryptophan (W). This was due to the A ↔ T substitution at the 1642 nucleotide position. At the 617th position, S11 had serine (S), while the other samples had proline (P) due to the C ↔ T nucleotide substitution at the 1849th position.

Similarly, S10 had glycine (G) at the 549th amino acid position, whereas the rest of the samples had glutamic acid (E) at the same position. This was because of the 1646th nucleotide substitution of A ↔ G.

The amino acids at the 567th, 633th and 637th positions in the protein sequence of S7 were asparagine (N), valine (V) and aspartic acid (D) respectively, while the other samples had aspartic acid (D), leucine (L) and glycine (G) at the corresponding positions. These were due to the A ↔ G, C ↔ T and A ↔ G substitutions at the 1699, 1899 and 1910 nucleotide positions, respectively.

In case of the amino acid at the 531st position, three samples – S1, S2 and S7 – were different from the rest of the samples. These three samples had leucine (L) at the 531st position, while the rest of the samples had glutamine (Q) at this position.

Multiple Sequence Alignment (MSA) of amino acid sequence of first eleven samples

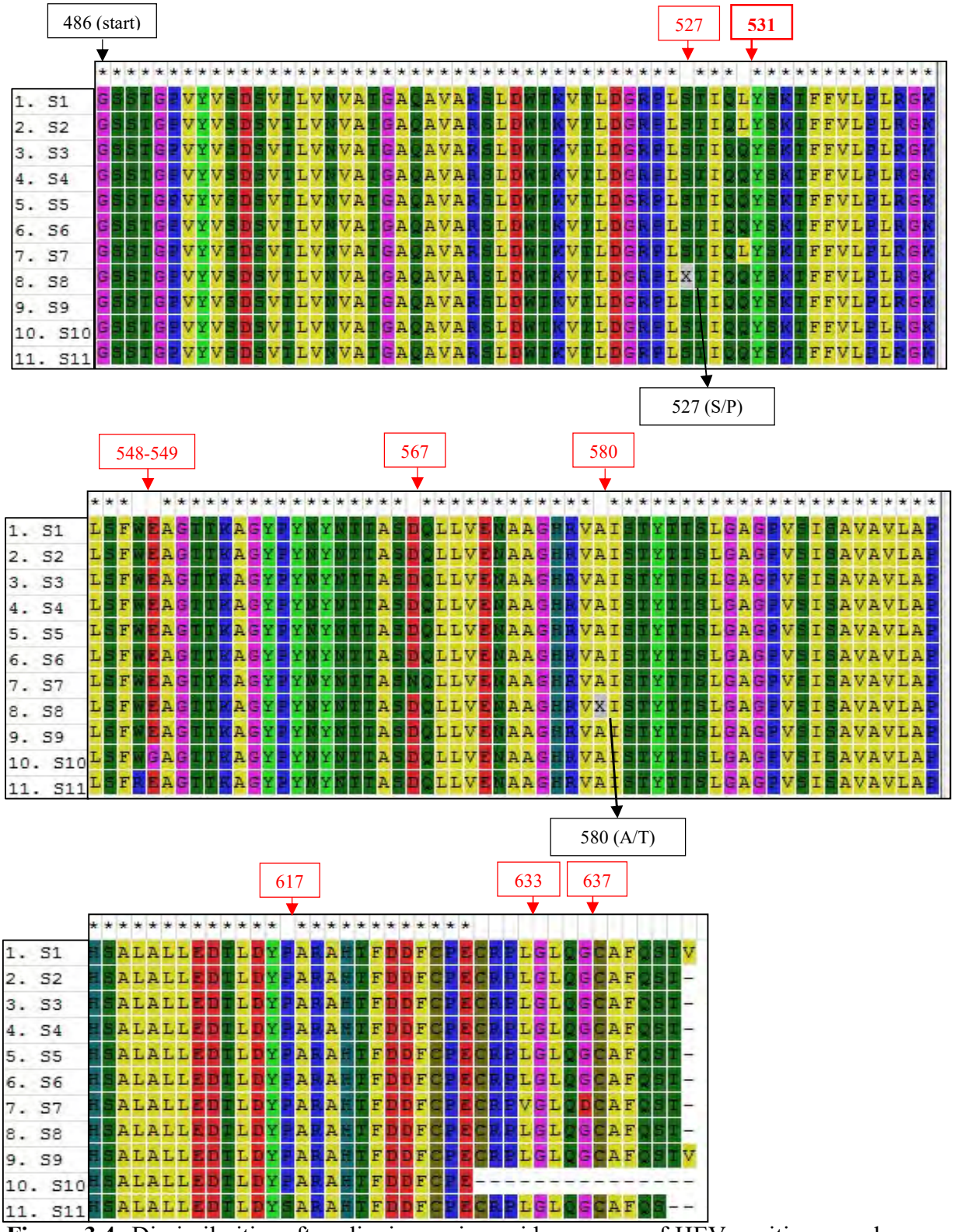


Figure 3.4: Dissimilarities after aligning amino acid sequence of HEV positive samples (N=11)

3.8 Analysis of Biochemical Results:

The level of certain biochemical components, namely serum bilirubin, alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) were analyzed. The level of these parameters in the blood directly correlates with the extent of liver damage. The damaged liver cannot form and/or eliminate bilirubin, and thus serum bilirubin acts as an indicator of extent and/or severity of hepatic damage. When the hepatic cells are damaged, ALT, AST and ALP are released into the blood. Thus these are also an indicator of the severity of hepatic damage.

The leucine (L) residue at 531^{st} position in ORF2 might indicate onset of a new mutation which had not been found in any other HEV. Glutamine (Q) is the residue found at the 531^{th} position of ORF2 in all HEV sequenced till date (according to NCBI database). The change in amino acid might play role in attenuation or enhancement of viral infectivity or host liver cell damage. To learn the effect of 531^{th} amino acid change in HEV genome to patients' outcomes after infections, a comparison of liver damage markers (serum bilirubin, AST, ALT and ALP) was made among HEV infected participants based on difference in amino acids at 531^{th} position of ORF2.

The average bilirubin for three HEV^{531Leu} positive specimens was 8.43 ± 1.75 (mg/dl). On the other hand, HEV^{531Gln} positive participants had mean serum bilirubin level 9.73 ± 4.45 (mg/dl) (Figure 3.5 (a)). Statistical analysis revealed no significant ($p = 0.9862$) difference in serum bilirubin level between these two groups of HEV infected patients.

As presented in Figure 3.5 (b), the average ALT level for the HEV^{531Leu} positive participants was 259.67 ± 47.59 (U/L), and that for HEV^{531Gln} positive participants was 669.13 ± 534.23 (U/L). The p value was found to be 0.0856, which means that the difference between these groups of patients was not statistically significant. But seemingly HEV^{531Gln} infection had higher chance of liver damage compared to HEV^{531Leu} infection. The HEV^{531Gln} infection related immune response-mediated damage could be due to higher prevalence of a MHC class 1 haplotype(s) in Bangladeshi population that may exert hyper-immunity in response to 531^{Gln} containing epitope(s) compared to epitope(s) containing 531^{Gln}.

The average AST level for the participants who were HEV^{531Gln} positive was found to be 438.25 ± 359.70 (U/L), while the average AST level for the samples that were HEV^{531Leu} positive was found to be 169.33 ± 59.33 (U/L), as illustrated in Figure 3.5 (c). The difference in AST level between the two groups was not statistically significant since the p-value was 0.0954. Similar to ALT level, AST-related information supports that HEV^{531Gln} positive participants had higher chance of liver damage.

Finally, the mean ALP level for the three HEV^{531Leu} positive samples was 154.33 ± 44.58 (IU/L). Conversely, HEV^{531Gln} positive participants had a mean ALP level of 199.88 ± 82.29 (IU/L). Statistical analysis did not show any significant difference ($p = 0.3434$) between HEV^{531Gln} and HEV^{531Leu} positive groups (Figure 3.5 (d)).

Distribution of different biochemical factors in patients with leucine or glutamine in 531 position of amino acid

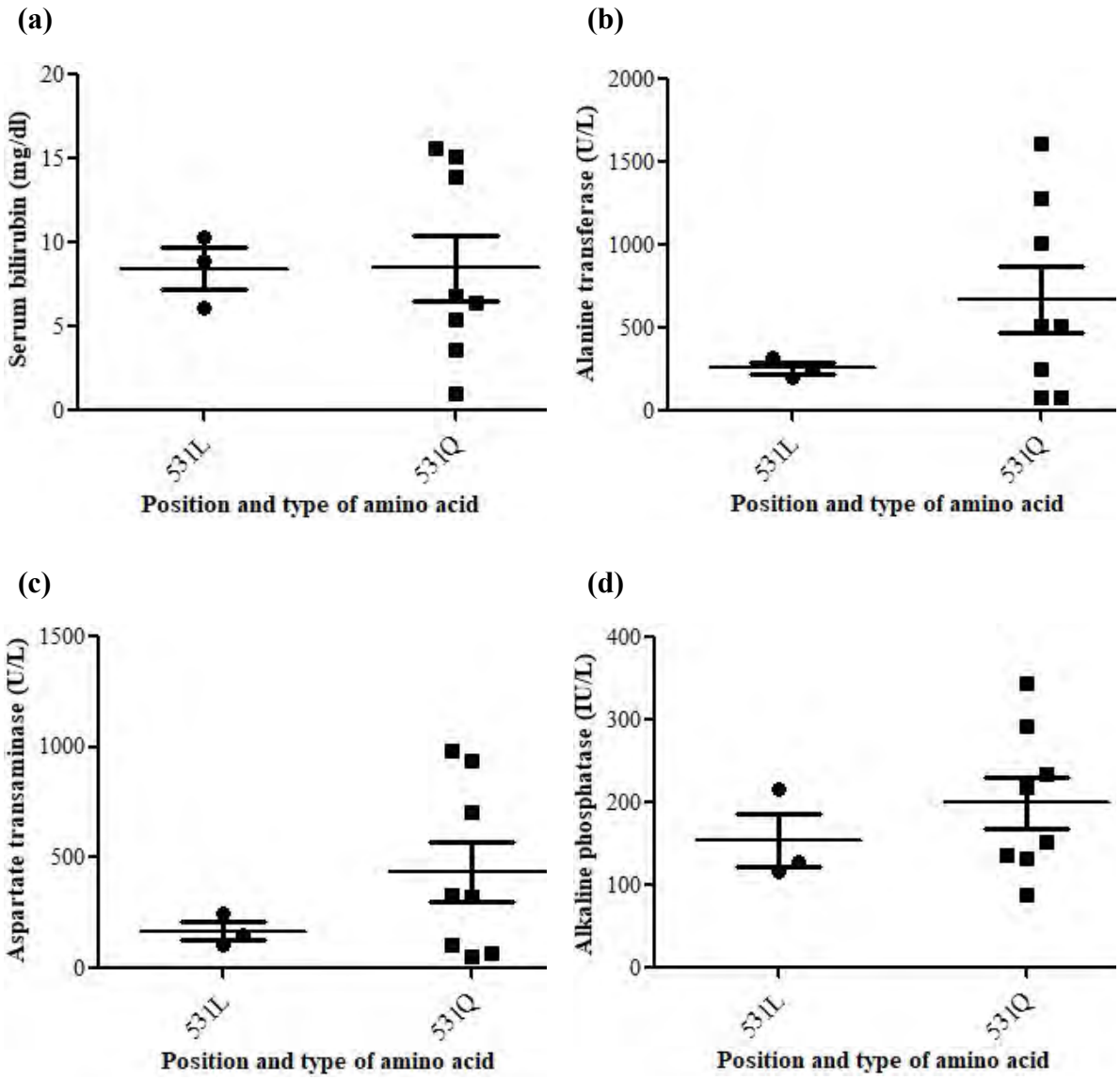


Figure 3.5: The distribution of selected biochemical indicators in patients with Leucine or Glutamine at the 531st position of ORF 2. **(a)** Serum bilirubin, **(b)** alanine transaminase (ALT), **(c)** aspartate transaminase (AST) and **(d)** alkaline phosphatase (ALP)

Figure 3.6 is a graph that shows the log of copy number for patients with glutamine or leucine at the 531st position in the amino acid sequence. From the graph, it was observed that the log of copy number for patients with glutamine varied more than that with leucine. The average for Q is much higher than that for L. The p value was 0.2122.

Distribution of log of viral copy number in patients with leucine or glutamine in 531 position of amino acid

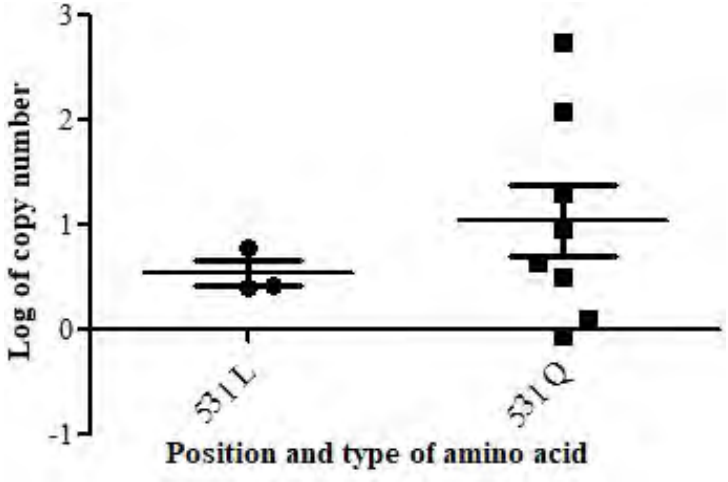


Figure 3.6: The vertical scatter diagram showing the log of viral copy number in patients with 531 Land 531 Q

3.9 Peptide Epitopes, HLA Haplotype (MHC class 1) and Immune Responses

We have already seen in biochemical findings in section 3.6 that the liver damage markers vary from one infected individual to another. Also in previous section 3.8 (Analysis of biochemical results) it was evident that amino acid change in 531st position does not increase liver damage markers in all patients infected with HEV^{531Gln} virus. Some of the individuals infected with HEV^{531Gln} had higher liver damage markers; others had similar level of liver damage markers to that of HEV^{531Leu} infected participants. Based on these observations, we wanted to perform bioinformatics-based study to see whether 531^{Gln} and 531^{Leu} containing HEV peptides could show various levels of affinity to different subclasses of HLA class 1 haplotype, which could help to explain differences in immune response-related outcomes in HEV infected participants.

As it was found that HEV^{531Gln} infections had shown involvement with much more higher levels liver damage markers in some cases but none of the HEV^{531Leu} infected participants showed such results, we started to look for epitopes and corresponding MHC class 1 haplotypes involving the corresponding 531th residue in ORF2 that differ in binding affinity and promote liver damage when Gln is the 531th residue. Epitopes QL^{531}YSKTFFV, IQL^{531}YSKTFF, IQL^{531}YSKTF and QL^{531}YSKTFF has lower affinity for MHC class 1 haplotype HLA-A*02:06, HLA-B*15:01, HLA-B*15:01, and HLA-B*15:01 respectively, than QQ^{531}YSKTFFV, IQQ^{531}YSKTFF, IQQ^{531}YSKTF and QQ^{531}YSKTFF (Table 3.6). Even though the affinity values were not significantly different, but it still could cause different levels of liver damage in *in vivo* system in response to single epitope-MHC class 1 haplotype or multiple epitopes-MHC class 1 haplotypes interaction(s).

Table 3.6: Affinity of different allele to predicted peptides including 531th residue of ORF2

Allele	Peptide	Arificial Neutral Network (ANN) IC50 (nM)	Stabilized Matrix Method (SMM) IC50 (nM)	Serum bilirubin (Mean ± SD)	ALT(Mean ± SD)	AST(Mean ± SD)	ALP(Mean ± SD)
HLA-A*02:06	QL^{531}YSKTFFV	High (8.34)	High (18.82)	8.43± 1.75	259.67 ± 47.59	169.33 ± 59.33	154.33 ± 44.58
	QQ^{531}YSKTFFV	High (4.37)	High (4.45)	9.73 ± 4.45	669.13 ± 534.23	438.25 ± 359.70	199.88 ± 82.29
HLA-B*15:01	IQL^{531}YSKTFF	Moderate (93.17)	Moderate (172.98)	8.43± 1.75	259.67 ± 47.59	169.33 ± 59.33	154.33 ± 44.58
	IQQ^{531}YSKTFF	Moderate (50.08)	Moderate (76.38)	9.73 ± 4.45	669.13 ± 534.23	438.25 ± 359.70	199.88 ± 82.29
HLA-B*15:01	IQL^{531}YSKTF	Low (1455.69)	-	8.43± 1.75	259.67 ± 47.59	169.33 ± 59.33	154.33 ± 44.58
	IQQ^{531}YSKTF	Low (780.21)	-	9.73 ± 4.45	669.13 ± 534.23	438.25 ± 359.70	199.88 ± 82.29
HLA-B*15:01	QL^{531}YSKTFF	Low (5627.30)	-	8.43± 1.75	259.67 ± 47.59	169.33 ± 59.33	154.33 ± 44.58
	QQ^{531}YSKTFF	Low (1043.40)	-	9.73 ± 4.45	669.13 ± 534.23	438.25 ± 359.70	199.88 ± 82.29

The following Table 3.7 shows the p-values of the epitopes with 531^{Leu} and 531^{Gln} for the serum bilirubin, ALT, AST and ALP levels. The values show that there was no significant differences between the liver damage markers for the two groups. The

findings could demonstrate the fact that Q^{531} containing peptides could be damaging to liver (Table 3.7).

Table 3.7: Statistical analysis of the biochemical results

Biochemicals	Peptides with		p-values
	L^{531}	Q^{531}	
Serum bilirubin (Mean ± SD)	8.43 ± 1.75	9.73 ± 4.45	0.5530
ALT (Mean ± SD)	259.67 ± 47.59	669.13 ± 534.23	0.0856
AST (Mean ± SD)	169.33 ± 59.33	438.25 ± 359.70	0.0954
ALP (Mean ± SD)	154.33 ± 44.58	199.88 ± 82.29	0.3434

Most of the study participants were infected with HEV^{527Ser} and HEV^{531Gln} virus. However, three patients were infected with HEV^{527Ser} and HEV^{531Leu} virus. These samples were found to have relatively less liver damage biochemical markers than the ones that were infected with HEV^{527Ser} and HEV^{531Gln} virus (Table 3.6). The mean serum bilirubin, ALT, AST and ALP levels of patients with leucine at 531^{st} position in amino acid sequence was found to be 8.43 ± 1.75, 259.67 ± 47.59, 169.33 ± 59.33 and 154.33 ± 44.58, respectively, while that of patients with glycine at the same position was found to be 9.73 ± 4.45, 669.13 ± 534.23, 438.25 ± 359.70 and 199.88 ± 82.29, respectively. Furthermore, one sample was found to be infected with a hepatitis E virus that contained 527^{Pro} and HEV^{531Gln}. The level of serum bilirubin, ALT, AST and ALP was found to be the lowest in this patient (6.4 ± 0, 82 ± 0, 53 ± 0 and 88 ± 0 respectively). Even though HEV^{531Gln} containing patients had higher bilirubin level than HEV^{531Leu}, the presence of 527^{Pro} with 531^{Gln} resulted in reduction in liver damage marker. So, we looked for epitopes that involves both of those residues to interact with MHC class 1 haplotypes. Our investigation for MHC class 1 haplotypes and epitopes with 527^{Pro} and 531^{Gln} could reveal that epitopes $RPLS^{527}TIQL^{531}Y$, $RPLS^{527}TIQQ^{531}Y$ and $RPLP^{527}TIQQ^{531}Y$ to interact differently with MHC class 1 haplotypes, namely HLA-B*53:01, HLA-A*11:01, and HLA-A*68:01. Even though $RPLS^{527}TIQL^{531}Y$, $RPLS^{527}TIQQ^{531}Y$ epitopes could interact with moderate affinity (IC50 < 500) with HLA-B*53:01, HLA-A*11:01, and HLA-A*68:01, however, for same haplotypes the affinity was low (IC50 > 500) for $RPLP^{527}TIQQ^{531}Y$ epitope. This phenomenon indicates the fact that a single amino acid change can cause significant change in affinity of epitope-MHC class 1 haplotype

interaction and outcome of viral infection. However, to confirm that the effect of 527^{Pro} in ORF2, further study is needed as only one sample was found with such genotype. If the finding turns out to be true it can be a significant breakthrough for development of HEV vaccination strategy.

Table 3.8: Affinity of different alleles to predicted peptides

Allele	Peptide	Arificial Neutral Network (ANN) IC50 (nM)	Stabilized Matrix Method (SMM) IC50 (nM)	Serum bilirubin (Mean ± SD)	ALT (Mean ± SD)	AST (Mean ± SD)	ALP (Mean ± SD)
HLA-B*53:01	RPLS^{527}TIQL^{531}Y	Moderate (257.01)	Moderate (273.45)	8.43 ± 1.75	259.67 ± 47.59	169.33 ± 59.33	154.33 ± 44.58
	RPLS^{527}TIQQ^{531}Y	Low (758.03)	Moderate (351.46)	10.2 ± 4.56	753 ± 519.51	493.29 ± 351.62	215.86 ± 75.47
	RPLP^{527}TIQQ^{531}Y	Low (1697.04)	Low (1179.99)	6.4 ± 0	82 ± 0	53 ± 0	88 ± 0
HLA-A*11:01	LS^{527}TIQL^{531}YSK	Moderate (94.39)	Moderate (161.18)	8.43 ± 1.75	259.67 ± 47.59	169.33 ± 59.33	154.33 ± 44.58
	LS^{527}TIQQ^{531}YSK	Moderate (197.08)	Moderate (188.06)	10.2 ± 4.56	753 ± 519.51	493.29 ± 351.62	215.86 ± 75.47
	LP^{527}TIQQ^{531}YSK	Low (12058.03)	Low (871.57)	6.4 ± 0	82 ± 0	53 ± 0	88 ± 0
HLA-A*30:01	LS^{527}TIQL^{531}YSK	Low (1116.43)	Moderate (225.93)	8.43 ± 1.75	259.67 ± 47.59	169.33 ± 59.33	154.33 ± 44.58
	LS^{527}TIQQ^{531}YSK	Low (1185.97)	Moderate (200.9)	10.2 ± 4.56	753 ± 519.51	493.29 ± 351.62	215.86 ± 75.47
	LP^{527}TIQQ^{531}YSK	Low (14827.85)	Low (4073.52)	6.4 ± 0	82 ± 0	53 ± 0	88 ± 0
HLA-A*68:01	LS^{527}TIQL^{531}YSK	Moderate (364.05)	Moderate (411.61)	8.43 ± 1.75	259.67 ± 47.59	169.33 ± 59.33	154.33 ± 44.58
	LS^{527}TIQQ^{531}YSK	Moderate (298.02)	Moderate (377.13)	10.2 ± 4.56	753 ± 519.51	493.29 ± 351.62	215.86 ± 75.47
	LP^{527}TIQQ^{531}YSK	Low (7246.04)	Low (1141.54)	6.4 ± 0	82 ± 0	53 ± 0	88 ± 0
HLA-B*53:01	LS^{527}TIQL^{531}YSK	Low (31085.87)	Low (18402.63)	8.43 ± 1.75	259.67 ± 47.59	169.33 ± 59.33	154.33 ± 44.58
	LS^{527}TIQQ^{531}YSK	Low (30497.86)	Low (14450.4)	10.2 ± 4.56	753 ± 519.51	493.29 ± 351.62	215.86 ± 75.47
	LP^{527}TIQQ^{531}YSK	Low (3712.46)	Moderate (438.41)	6.4 ± 0	82 ± 0	53 ± 0	88 ± 0

The following Table 3.9 shows the p-values of the epitopes RPLSTIQL^{531}Y and RPLSTIQQ^{531}Y for the serum levels of bilirubin, ALT, AST and ALP. The values show that there was statistically no significant difference between the liver damage markers, but the findings could demonstrate the fact that RPLSTIQL^{531}Y epitopes could be more damaging to liver compared to RPLSTIQL^{531}Y if HLA-B*53:01, HLA-A*11:01, and

HLA-A*68:01 haplotypes were involved in immune responses against HEV infections (Table 3.9).

Table 3.9: Statistical analysis of the biochemical results

Biochemicals	Peptides		p-values
	$S^{527}$and L^{531}	$S^{527}$and Q^{531}	
Serum bilirubin (Mean ± SD)	8.43 ± 1.75	10.2 ± 4.56	0.4551
ALT (Mean ± SD)	259.67 ± 47.59	753 ± 519.51	0.0613
AST (Mean ± SD)	169.33 ± 59.33	493.29 ± 351.62	0.0735
ALP (Mean ± SD)	154.33 ± 44.58	215.86 ± 75.47	0.2216

CHAPTER FOUR

DISCUSSION

Discussion

At present, one of the most rising concerns in public health for developing countries is the occurrence of hepatitis E infection. The 7.2 kb long hepatitis E viral genome contains three open reading frames (ORF1, ORF2, and ORF3), and 5′- and 3′-untranslated regions (UTRs), with a polyA-tract at the 3′-end. The virus is a positive-sense single-stranded RNA virus and causes sporadic and water-borne infections. It is related to poor hygiene and sanitation of the people from low socio-economic class and is the cause for this particular infection. The infection is a chronic, self-limiting disease and is usually enterically transmitted. Suspected cases of hepatitis E have been found in Asia, Africa, the Middle East, Mexico, and Central America (Kar *et al.*, 2008; Borkakoti *et al*., 2013; Huang *et al.*, 1992; Tong *et al*., 2016). The virus can cause some serious infections during pregnancy with a high maternal mortality rate and can also be vertically transmitted to cause intrauterine fetal infections and fetal deaths (Khuroo and Khuroo, 2008). Recent studies have shown the presence of antibodies against the genotypes 1 and 2 of hepatitis E virus in human and genotypes 3 and 4 in animals such as pigs and rats. These are known to be transmitted to humans through inter-species transmission (Vasickova *et al.*, 2007; Abravanel *et al.*, 2011).

There were a total of 42 study participants, in the present study from different socio-economic backgrounds, of whom 15 were HEV RNA positive. Among these 15 HEV-RNA positive women, four were antenatal, four were post-natal and seven were non-pregnant. The household income of these women was used as a proxy indicator to identify any existing correlation between the socio-economic status and the onset of hepatitis E infections. According to data from The World Bank, people with an income of Taka 82589.38 per annum or less is called low class, those with Taka 82589.38 to 325120.13 per annum is called low-middle class, upper middle class are those with gross national income (GNI) per capita between BDT 325120.13 and 1005173.13 per annum. Finally the high-income people are those with a GNI per capita of Taka 1005173.13 or more per annum (WDI 2017 Maps). As per this classification, the study participants were in the low-middle income class.

It was seen that the women who drank water supplied by municipality were more likely to be affected by hepatitis E virus, while filtered water resulted in less chance of being infected. This implies that the immunocompromised patients who drink contaminated water are at higher risk of developing hepatitis E infection than the alternative as stated in researches conducted by Legrand-Abravanel and her team (Legrand-Abravanel *et al.*, 2010). Also, a study in France showed that chronic HEV infection is mostly reported in immunocompromised individuals alongside recipients of organ transplantation and chemotherapy, and patients infected with HIV (Kamar *et al*., 2008).

A standard graph for the detection of the viral copy number was developed using synthetic viral RNA. Through calculations and serial dilutions, synthetic viral genome of 10^6, 10^5, 10^4, 10^3, 10^2, 10 and 2 copies/µL were developed and later amplified using reaction condition for one step reverse transcription real time PCR. The HEV-RNA content in the samples was detected using TaqMan® RT-PCR assay which is a one step process that can be used for the detection of HEV in environmental and clinical samples. This assay was validated using environmental and clinical specimens in researches conducted by Jothikumar *et al*., 2005. They showed that the method is sensitive and specific for the detection of HEV genotypes 1–4. The standard curve was used to determine the copy number of the viral genome in the HEV RNA positive samples.

In the present study, the viral copy number for the HEV RNA positive women were linked with their pregnancy status. It was observed that the non-pregnant women had a relatively lower copy number than the pregnant or post-natal women. This may be because pregnant women visit the hospital much earlier than non-pregnant women do. Therefore, the samples collected from the pregnant women were usually at the time when the viral loads were at their peaks but it was not the case for non-pregnant women (Kar *et al.*, 2008). The study by Borkakoti and his team of researchers showed similar results with statistically significant difference of the viral copy number between pregnant and non-pregnant patients.

Biochemical tests performed on the samples from the HEV-RNA positive patients showed that the levels of serum bilirubin, ALT, AST and ALP for each of the 15 HEV-

RNA positive patients were more than the reference ranges (serum bilirubin – 0.2 mg/dl to 1.2 mg/dl; ALT – upto 40 U/L; AST – upto 37 U/L; and finally ALP – upto 120 IU/L). Studies by other researchers show that there is a strong correlation between high ALT, AST and serum bilirubin levels and HEV infections (Legrand-Abravanel *et al*., 2010; Panda, Thakral and Rehman, 2006; Dalton *et al*., 2009; Kamar *et al*., 2008). An increase in serum levels of these enzymes is associated with increased liver damage in infected animals. The ALT level usually starts to increase by the second week of the onset of hepatitis and reaches the peak 100 days after the onset of hepatitis (Panda and Verma, 2013; Takahashi *et al*., 2007).

The HEV-RNA from the first eleven out of the fifteen samples were sequenced, and they were translated using ExPASy translate to obtain the corresponding amino acid sequence. The nucleotide and amino acid sequences were aligned using the program Clustal W which calculates the best match and shows the identities, similarities and differences of the selected sequences. Through the alignment, it was observed that there were some mutations in the nucleotide that resulted in changes in the amino acid sequences that coded for the capsid protein of the ORF 2 in hepatitis E genotype 1. It has been shown that selective host immune pressure may give rise to such naturally occurring mutations that may influence the function of some viral proteins which results in decreased replication and infectivity of the hepatitis E virus (Huang *et al*., 2005). Mutations in the ORF 2 of hepatitis E virus may be related to the progression of liver diseases (Suneetha *et al*., 2012).

T580A is a substitution that have occurred in one of the samples. Studies have shown that such mutations may have resulted in a drastic attenuation in hepatitis E virus and may affect the packaging of the viral genomic RNA (Cordoba *et al*., 2011). Another substitution was N567D. Such substitutions have been shown to glycosylate, dimerize and neutralize to alter the structure of epitopes of the capsid protein encoded by ORF 2. This may enable the hepatitis E virus to successfully adapt and/or escape the host immune system, resulting in chronic infections (Xu *et al*., 2016; Todt *et al*., 2016). This assumption that may be accepted for this study too.

One of the most prominent substitutions found was Q531L as a result of the nucleotide mutation of A1592T in three out of the eleven samples. Similar mutations – F51L, T59A, and S390L – were observed in the study conducted by Cordoba and his team of researchers. The study showed that the mutations F51L and S390L might contribute to the attenuation in the production of HEV. S390L mutation may change the structure of antigenic epitopes to prevent interactions between virus and host cell receptor (Cordoba *et al*., 2011). It can be assumed that Q531L mutation may result in similar changes since glutamine (Q) and serine (S) have similar properties, in regards that both are polar, uncharged and hydrophilic in nature. This can imply that the effect of such mutations may be close to each other. In fact, results in this study do suggest that there are some, although not significant, changes in the binding properties of antigenic epitopes to the host cell receptor. Generally, the presence of HEV^{531Leu} correlates to a higher binding affinity than the presence of HEV^{531Gln}.

Analysis of the biochemical components in patients with 531^{Leu} or 531^{Gln} show that although there is no significant difference in these liver damage markers amongst the patients, there are some observable differences nonetheless. The viral load between the two groups of patients was not significantly different either, but the viral load in patients with 531^{Leu} is lower than that in patients with 531^{Gln}. The log of copy number was also relatively lower in patients with 531^{Leu} than in those with 531^{Gln}.

Polymorphic genes are vital for studies on genetic analysis. Human leukocyte antigen (HLA) is the human version of major histocompatibility complex (MHC) which is extremely polymorphic in nature. It determines immune function and susceptibility to complex diseases. HLA polymorphisms can be detected using serological and DNA typing methods. These methods were used in a study to determine the HLA-A and HLA-B polymorphisms among the people of Bangladesh. Out of the 16 different HLA-A alleles that were detected in the study, A*33 was the most frequent one (17.02%), followed by A*24 (16.31%), A*11(15.60%) and A*02 (14.54%) (Ali *et al*., 2008). A*33 was also found to be one of the most frequent allele amongst Pakistani people. On the other hand, HLA-A*24 was the most common allele in Indian communities (Thomas and Banerjee, 2005; Mohyuddin *et al*., 2002).

Bioinformatics-based study was performed to see if 531^{Gln} and 531^{Leu} containing HEV peptides showed various levels of affinity to different subclasses of HLA class 1 haplotype. The results from this analysis and the information mentioned above could help to explain differences in immune responses-related outcomes in HEV infected participants. It was observed that the patients with 531^{Leu} had a lower affinity for MHC class 1 haplotype than the patients with 531^{Gln}. The former also had a lower level of liver damage biomarkers than the latter. This implies that there is a lower chance of liver damage in patients infected with HEV^{531Leu} than those infected with HEV^{531Gln} virus. However, the difference in the biomarkers between patients infected with HEV^{531Leu} and HEV^{531Gln} viruses was not statistically significant, as suggested by the respective p-values. These results may indicate that a mutation which causes replacement of glutamine by leucine at the 531^{st} position, the level of liver damage biomarkers and consequently the chance of liver damage decreases.

The findings of this study are may not be generalizable because of the small sample size with limited diversity, and only few bioinformatics tools were used. Data from large, diverse sample analyzed with varieties of bioinformatics tools and further in-depth intricate assays may help to expand the knowledge regarding different mutations and their effects on the prognosis of HEV infection in Bangladesh.

References

Abravanel, F., Sandres-Saune, K., Lhomme, S., Dubois, M., Mansuy, J. M. and Izopet, J. (2011). Genotype 3 Diversity and Quantification of Hepatitis E Virus RNA. *Journal of Clinical Microbiology*, *50*(3), 897–902. doi:10.1128/JCM.05942-11

Ahmad, I., Holla, R. P. and Jameel, S. (2011). Molecular virology of hepatitis E virus. *Virus Research*, *161*(2011), 47– 58. doi:10.1016/j.virusres.2011.02.011

Ali, M. E., Ahmed, M. U., Alam, S. and Rahman, M. H. (2008). HLA-A, -B and -DRB1 allele frequencies in the Bangladeshi population. *Tissue Antigens*, *72*, 115–119. doi: 10.1111/j.1399-0039.2008.01079.x

Apps, R., Murphy, S. P., Fernando, R., Gardner, L., Ahad, T. and Moffett, A. (2009). Human leucocyte antigen (HLA) expression of primary trophoblast cells and placental cell lines, determined using single antigen beads to characterize allotype specificities of anti-HLA antibodies. *Immunology*, *127*(1), 26 – 39. doi: 10.1111/j.1365-2567.2008.03019.x

Artimo, P., Jonnalagedda, M., Arnold, K., Baratin, D., Csardi, G., de Castro, E., Duvaud, S., Flegel, V., Fortier, A., Gasteiger, E., Grosdidier, A., Hernandez, C., Ioannidis, V., Kuznetsov, D., Liechti, R., Moretti, S., Mostaguir, K., Redaschi, N., Rossier, G., Xenarios, I. and Stockinger, H. (2012). ExPASy: SIB bioinformatics resource portal. *Nucleic Acids Research*, *40*(W1), W597-W603. doi: https://doi.org/10.1093/nar/gks400

Balayan, M. S. (1997). Epidemiology of hepatitis E virus infection. *Journal of Viral Hepatitis*, *4*(3), 155–166. doi: 10.1046/j.1365-2893.1997.00145.x

Baylis, S. A., Mizusawa, S., Okada,Y., Hanschmann, K. M. O. (2011). Collaborative study to establish a World Health Organization international standard for hepatitis E virus RNA for nucleic acid amplification technology (NAT)-based assays. *Expert Committee on Biological Standardization*,

Behrendt, P., Bremer, B., Todt, D., Brown, R. J. P., Heim, A., Manns, M. P., Steinmann, E. and Wedemeyer, H. (2016). Hepatitis E Virus (HEV) ORF2 Antigen Levels Differentiate Between Acute and Chronic HEV Infection. *The Journal of Infectious Diseases*, *214* (3), 361-368. doi: 10.1093/infdis/jiw161

Boratyn, G. M., Camacho, C., Cooper, P. S., Coulouris, G., Fong, A., Ma, N., Madden, T. L., Matten, W. T., McGinnis, S. D., Merezhuk, Y., Raytselis, Y., Sayers, E. W., Tao, T., Ye, J. and Zaretskaya, I. (2013). BLAST: a more efficient report with usability improvements. *Nucleic Acids Research*, *41*(Web Server issue), W29-W33. doi: 10.1093/nar/gkt282

Borkakoti, J., Hazam, R. K., Mohammad, A., Kumar A. and Kar, P. (2013). Does High Viral Load of Hepatitis E Virus Influence the Severity and Prognosis of Acute Liver Failure During Pregnancy? *Journal of Medical Virology, 85*, 620–626

Bouquet, J., Cheval, J., Rogée, S., Pavio, N. and Eloit, M. (2012). Identical consensus sequence and conserved genomic polymorphism of hepatitis E virus during controlled interspecies transmission. *Journal of Virology*, *86*(11), 6238 – 6245. doi:10.1128/JVI.06843-11.

Rosa, G. L., Fratini, M., Muscillo, M., Iaconelli, M., Taffon, S., Equestre, M., Chionne, P., Madonna, E., Pisani, G., Bruni, R. and Ciccaglione, A. R. (2014). Molecular characterisation of human hepatitis E virus from Italy: comparative analysis of five reverse transcription-PCR assays. *Virology Journal*, *11*(72). doi:10.1186/1743-422X-11-72

Co´rdoba, L., Huang, Y.W., Opriessnig, T., Harral, K. K., Beach, N. M., Finkielstein, C. V., Emerson, S. U. and Meng, X. J. (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), 5338–5349. doi: 10.1128/JVI.02278-10

Dalton, H.R., Bendall, R.P., Keane, F.E., Tedder, R.S., Ijaz, S. (2009). Persistent carriage of hepatitis E virus in patients with HIV infection. *The New England Journal of Medicine*, *361*(10), 1025 – 1027

Desmet, V. J., Gerber, M., Hoofnagle, J. H., Mans, M. and Scheuer, P. J. (1994). Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatolo1ogy*, 19, 1513 – 1520

Ding, Q., Heller, B., Capuccino, J. M. V., Song, B., Nimgaonkar, I., Hrebikova, G., Contreras, J. E. 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), 1147–1152.

Doceul, V., Bagdassarian, E., Demange, A. and Pavio, N. (2016). Zoonotic hepatitis E virus: classification, animal reservoirs and transmission routes. *Viruses*, *8*(270), 1 – 24. doi:10.3390/v8100270

Dufour, D. R., Lott, J. A., Nolte, F. S., Gretch, D. R., Koff, R. S. and Seeff, L. B. (2000). Diagnosis and monitoring of hepatic injury. i. performance characteristics of laboratory tests. *Clinical Chemistry*, *46*(12), 2027 – 2049.

Duquesnoy, R. J. (2014). Human leukocyte antigen epitope antigenicity and Immunogenicity. *Current Opinion*,*19*(00), 1 – 8. doi: 10.1097/MOT.0000000000000100

Emerson, S. U. and Purcell, R. H. (2003). Hepatitis E virus. *Reviews in Medical Virology¸ 13*, 145–154. doi: 10.1002/rmv.384

Guu, T. S. Y., Liu, Z., Ye, Q., Mata, D. A., Li, K., Yin, C., Zhang, J. and Tao, Y. J. (2009). Structure of the hepatitis E virus-like particle suggests mechanisms for virus assembly and receptor binding. *Proceedings of the National Academy of Sciences*, *106*(31), 12992 – 12997. doi:10.1073/pnas.0904848106.

Huang, C. C., Nguyen, D., Fernandez, J., Yun, K. Y., Fry, K. E., Bradley, D. W., Tam, A. W. and Reyes, G. R. (1992). Molecular cloning and sequencing of the Mexico isolate of hepatitis E virus (HEV). *Virology*, *191*, 550 – 558.

Huang, Y. W., Haqshenas, G., Kasorndorkbua, C., Halbur, P. G., Emerson, S. U. and Meng, X. J. (2005). Capped RNA transcripts of full-length cDNA clones of swine hepatitis E virus are replication competent when transfected into huh7 cells and infectious when intrahepatically inoculated into pigs. *Journal of Virology*, *79*(3), 1552 – 1558. doi:10.1128/JVI.79.3.1552–1558.2005

Iloeje, U. H., Yang, H., Su, J., Jen, C. L., You, S. L., Chen, C. J. and The Risk Evaluation of Viral Load Elevation and Associated Liver Disease/Cancer-In HBV (the REVEAL-HBV) Study Group. (2006). Predicting Cirrhosis Risk Based on the Level of Circulating Hepatitis B Viral Load. *Gastroenterology*, *130*(3), 678–686. doi:10.1053/j.gastro.2005.11.016

Jothikumar, N., Cromeans, T. L., Robertson, B. H., Meng, X. J. and Hill, V. R. (2005). A broadly reactive one-step real-time RT-PCR assay for rapid and sensitive detection of hepatitis E virus. *Journal of Virological Methods, 31*(2006), 65 – 71. doi:10.1016/j.jviromet.2005.07.004

Kamar, N., Dalton, H. R., Abravanel, F. and Izopet, J. (2014). Hepatitis E virus infection. *Clinical Microbiology Reviews*, *27*(1), 116 – 138. doi:10.1128/CMR.00057-13.

Kamar, N., Selves, J., Mansuy, J. M., Ouezzani, L., Peron, J.M., Guitard, J., Cointault, O., Esposito, L., Abravanel, F., Danjoux, M., Durand, D., Vinel, J.P., Izopet, J. and Rostaing, L. (2008). Hepatitis E virus and chronic hepatitis in organ-transplant recipients. *The New England Journal of Medicine*, *358*(8), 811 – 7

Kar, P., Jilani, N., Husain, S. A., Pasha, S. T., Anand, R., Rai, A., and Das, B. C. (2008). Does hepatitis E viral load and genotypes influence the final outcome of acute liver failure during pregnancy? *American Journal of Gastroenterology*, 103, 2495 – 2501. doi: 10.1111/j.1572-0241.2008.02032.x.

Khuroo, M. S. and Khuroo, M. S. (2008). Hepatitis E virus. *Current Opinion in Infectious Diseases*, *21*, 539–543. doi:10.1097/QCO.0b013e32830ee08a

Khuroo, M. S., Kamili, S. and Jameel, S. (1995). Vertical transmission of hepatitis E virus. *Lancet*, *345*(8956), 1025-6.

Kim, W. R., Flamm, S. L., Di Bisceglie, A. M. and Bodenheimer, H. C. (2008). Serum activity of alanine aminotransferase (ALT) as an indicator of health and disease. *Hepatology*, *47*(4), 1363 – 1370. doi: 10.1002/hep.22109.

Komurian-Pradel, F., Paranhos-Baccala, G., Sodoyer, M., Chevallier, P., Mandrand, B., Lotteau, V. and Andre, P. Quantitation of HCV RNA using real-time PCR and fluorimetry. *Journal of Virological Methods*, *95*(2001), 111–119

Koonin, E. V., Gorbalenya, A. E., Purdy, M. A., Rozanov M. N., REYES, G. R., and Bradley, D. W. (1992). Computer-assisted assignment of functional domains in the nonstructural polyprotein of hepatitis E virus: Delineation of an additional group of positive-strand RNA plant and animal viruses. *Procedings of National Academy of Sciences of the United States of America, 89*, 8259 – 8263.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G.. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics*, *23*(21), 2947-2948. doi: https://doi.org/10.1093/bioinformatics/btm404

Legrand-Abravanel, F., Kamar, N., Sandres-Saune, K., Garrouste, C., Dubois, M., Mansuy, J. M., Muscari, F., Sallusto, F., Rostaing L. and Izopet,J. (2010). Characteristics of autochthonous hepatitis E virus infection in solid-organ transplant recipients in france. *The Journal of Infectious Diseases*, *202*(6), 835 – 844. doi: 10.1086/655899.

Meng, X. J., Wiseman, B., Elvinger, F., Guenette, D. K., Toth, T. E., Engle, R. E., Emerson, S. U. and Purcell, R. H. (2002). Prevalence of antibodies to hepatitis E virus in veterinarians working with swine and in normal blood donors in the united states and other countries. *Journal of Clinical Microbiology*, *40*(1), 117–122. doi: 10.1128/JCM.40.1.117–122.2002

Mohyuddin, A., Ayub, Q., Khaliq, S., Mansoor, A., Mazhar, K., Rehman, S. and Mehdi, S. Q. (2002). HLA polymorphism in six ethnic groups from Pakistan. *Tissue Antigens*, *59*, 492 – 501

National Center for Biotechnology Information. Programs & Activities. https://www.ncbi.nlm.nih.gov/home/about/programs/

Panda, S. K., Thakral, D. and Rehman, S. (2006). Hepatitis E virus. *Reviews in Medical Virology*, *17*, 151–180. doi: 10.1002/rmv

Panda, S. K., Varma, S. P. K. (2013). Hepatitis E: molecular virology and pathogenesis. *Journal of Clinical and Experimental Hepatology*, *3*(2), 114–124. doi: 10.1016/j.jceh.2013.05.001

Piiparinen, H., Hockerstedt, K., Lappalainen, M., Suni, J. and Lautenschlager, I. (2002). Monitoring of Viral Load by Quantitative Plasma PCR during Active Cytomegalovirus Infection of Individual Liver Transplant Patients. *Journal of Clinical Microbiology*, *40*(8), 2945–2952. doi: 10.1128/JCM.40.8.2945–2952.2002

Rein, D. B., Stevens, G. A., Theaker, J., Wittenborn, J. S. and Wiersma, S. T. (2012). The global burden of hepatitis E virus genotypes 1 and 2 in 2005. *Hepatology*, *55*(4), 988 – 997. doi: 10.1002/hep.25505

Schlecht, N. F., Trevisan, A., Duarte-Franco, E., Rohan, T. E., Ferenczy, A., Villa, L. L. and Franco, E. L. (2003). Viral load as a predictor of the risk of cervical intraepithelial neoplasia. *International Journal of Cancer,* 103, 519–524

Smith, D. B., Purdy, M. A., Simmonds, P. (2013). Genetic Variability and the Classification of Hepatitis E Virus. *Journal of Virology*, *87*(8), 4161–4169. doi:10.1128/JVI.01092-13

Suneetha, P.V., Pischke, S., Schlaphoff, V., Grabowski, J., Fytili, P., Gronert, A., Bremer, B., Markova, A., Jaroszewicz, J., Bara, C., Manns, M. P., Cornberg, M. and Wedemeyer, H. (2012). Hepatitis E virus (HEV)-specific T-cell responses are associated with control of HEV infection. *Hepatology*, *55*(3), 695–708.

Surjit, M., Jameel, S. and Lal, S. K. (2004). The ORF2 protein of hepatitis E virus binds the 5' region of viral RNA. *Journal of Virology*, *78*(1), 320–328. doi: 10.1128/JVI.78.1.320–328.2004

Swift, M. L. (1997). GraphPad Prism, data analysis, and scientific graphing. *Journal of Chemical Information and Computer Sciences*, *37*(2), 411-412.

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), 3671–3679. doi:10.1128/JCM.01086-07

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution*, *30*(12), 2725-9. doi: 10.1093/molbev/mst197

Teshale, E. H., Grytdal, S. P., Howard, C., Barry, V., Kamili, S., Drobeniuc, J., Hill, V. R., Okware, S., Hu, D. J. and Holmberg, S. D. (2010). Evidence of Person-to-Person Transmission of Hepatitis E Virus during a Large Outbreak in Northern Uganda. *Clinical Infectious Diseases*, *2010*(50), 1006–1010.

Teshale, E. H., Hu, D. J. and Holmberg, S. D. (2010). The two faces of hepatitis E virus. *Clinical Infectious Diseases*, *51*(3), 328–334. doi: 10.1086/653943

Thomas, R. and Banerjee, M. (2005). HLA-A allele frequency and haplotype distribution in the Dravidian tribal communities of south India. *Indian Journal of Human Genetics*, *11*, 140 – 4

Todt, D., Gisa, A., Radonic, A., Nitsche, A., Behrendt, P., Suneetha, P. V., Pischke, S., Bremer, B., Brown, R. J. P., Manns, M. P, Cornberg, M., Bock, C. T., Steinmann, E. and Wedemeyer, H. (2016). In vivo evidence for ribavirin-induced mutagenesis of the hepatitis E virus genome. *Gut*, *65*, 1733 – 1743. doi:10.1136/gutjnl-2015-311000

Tong, H. V., Hoan, N. X., Wang, B., Wedemeyer, H., Bock, C. T., Velavan, T. P. (2016). Hepatitis E virus mutations: functional and clinical relevance. *EBioMedicine*, (2016). doi: org/10.1016/j.ebiom.2016.07.039

Vasickova, P., Psikal, I., Kralik, P., Widen, F., Hubalek, Z. and Pavlik, I. (2007). Hepatitis E virus: a review. *Veterinarni Medicina*, *52*(9), 365–384.

Vollmer, T., Diekmann, J., Johne, R., Eberhardt, M., Knabbe, C. and Dreier, J. (2012). Novel approach for detection of hepatitis E virus infection in german blood donors. *Journal of Clinical Microbiology*, *50*(8), 2708–2713. doi:10.1128/JCM.01119-12

Watzinger, F., Suda, M., Preuner, S., Baumgartinger, R., Ebner, K., Baskova, L., Niesters, H. G. M., Lawitschka, A. and Lion, T. (2004). Real-time quantitative PCR assays for detection and monitoring of pathogenic human viruses in immunosuppressed pediatric patients. *Journal of Clinical Microbiology*, *42*(11), 5189–5198. doi: 10.1128/JCM.42.11.5189–5198.2004

WDI 2017 Maps | Data. 2017. WDI 2017 Maps | Data. [ONLINE] Available at http://data.worldbank.org/products/wdi-maps

Wolf, P. L. (1999). Biochemical diagnosis of liver disease. *Indian Journal of Clinical Biochemistry, 14*(1), 59 – 90.

Xu, M., Behloul, N., Wen, J., Zhang, J. and Meng, J. (2016). Role of asparagine at position 562 in dimerization and immunogenicity of the hepatitis E virus capsid protein. *Infection, Genetics and Evolution*, *37*, 99 – 107

Appendix – I A

Consent Form

(Adapted from the PhD project by Dr. Rosy Sultana)

গবেষণার বিষয়ে বিস্তারিত তথ্য ও সম্মতি পত্র

গবেষণার শিরোনামঃ

বাংলা - গর্ভাবস্থায় হেপাটাইটিস-ই ভাইরাস জনিত জন্ডিসে আক্রান্ত বাংলাদেশের শহরে বসবাসকারী মায়েদের রক্তে ভাইরাসের সংখ্যা এবং এর জেনোটাইপের প্রভাব অনুসন্ধানে গবেষণা।

English- Influence of Hepatitis E viral load and genotypes on pregnant urban dwellers of Bangladesh

গবেষকের নাম: ডাঃ রোজী সুলতানা, সহযোগী অধ্যাপক, ইমিউনোলজি বিভাগ, বাংলাদেশ ইউনিভার্সিটি অব হেল্‌থ সায়েন্সেস (বিইউএইচএস), ১২৫/১ দারুস সালাম রোড, মিরপুর-১, ঢাকা-১২১৬।

তত্ত্বাবধায়কবৃন্দঃ

(১) অধ্যাপক মুহাম্মদ মনজুরুল করিম, মাইক্রোবায়োলজি বিভাগ, ঢাকা বিশ্ববিদ্যালয়।

(২) ডঃ ফিরদৌসী কাদরী, এডজাঙ্কট্ ফ্যাকাল্টি, ইমিউনোলজি বিভাগ, বাংলাদেশ ইউনিভার্সিটি অব হেল্‌থ সায়েন্সেস (বিইউএইচএস), ১২৫/১ দারুস সালাম রোড, মিরপুর-১, ঢাকা-১২১৬; প্রধান, ইমিউনোলজি ইউনিট এবং পরিচালক, ভ্যাকসিন সায়েনন্স সেন্টার, আইসিডিডিআর,বি। **গবেষণা প্রতিষ্ঠানের নামঃ**

যে সকল ল্যাবরেটরীতে গবেষণার কাজ হবেঃ

(১) মাইক্রোবায়োলজি বিভাগ, ঢাকা বিশ্ববিদ্যালয়, (২) ইমিউনোলজি বিভাগ, বাংলাদেশ ইউনিভার্সিটি অব হেল্‌থ সায়েন্সেস (বিইউএইচএস), মিরপুর-১; (৩) ইনস্টিটিউট ফর ডেভেলপিং সায়েন্স এবং হেলথ ইনিশিয়েটিভস (আইদেশী) ল্যাবরেটরী, সেন্টার ফর মেডিকেল বায়োটেকনোলজি, জনস্বাস্থ্য প্রতিষ্ঠান (আইপিএইচ), ঢাকা; এবং (৪) ইমিউনোলজি ইউনিট এবং ভ্যাকসিন সায়েন্স সেন্টার, আইসিডিডিআর,বি।

গবেষণা প্রকল্পের বিবরণঃ

হেপাটাইটিস ভাইরাস (হেপাটাইটিস এ, বি, সি এবং ই) আমাদের দেশে যে কোন বয়সের মানুষের জন্ডিসের অন্যতম কারণ। হেপাটাইটিস-এ ও হেপাটাইটিস-ই ভাইরাস দুষিত খাবার এবং পানির মাধ্যমে ছড়ায়। হেপাটাইটিস-এ এর সংক্রমণ প্রধানতঃ শিশু ও বৃদ্ধদের মধ্যে প্রকট। হেপাটাইটিস-বি এবং হেপাটাইটিস-সি রক্তের মাধ্যমে ছড়ায়। হেপাটাইটিস-বি ভ্যাকসিন প্রয়োগ এবং মানুষের সচেতনতা বৃদ্ধির ফলে বি-ভাইরাসের সংক্রমণ অনেকটা নিয়ন্ত্রনে আছে। সঞ্চালনের পূর্বে বাধ্যতামূলক ভাবে রক্তের হেপাটাইটিস-সি ভাইরাসের পরীক্ষা করা হয় যার ফলে সি-ভাইরাসের সংক্রমণ আমাদের দেশে অনেক কম। হেপাটাইটিস-ই ভাইরাস জনিত জন্ডিসের লক্ষণ অনেক সময় অলখ্যেই থেকে যায় বা খুব মৃদু মাত্রার হয়ে থাকে এবং ফলত: সংক্রমিত ব্যক্তির স্থায়ী প্রতিরোধ ক্ষমতা তৈরী হয়। দেখা গেছে যে আমাদের দেশে ২৫% সাধারণ মানুষের হেপাটাইটিস-ই ভাইরাস প্রতিরোধ ক্ষমতা আছে।

গর্ভবতী নারীর রক্তে অনেক হরমোনের মাত্রা কম বেশী হয় ও রোগ প্রতিরোধ ক্ষমতা কমে যায়। গর্ভস্থ ভ্রুনের স্বাভাবিক বেড়ে ওঠার জন্য গর্ভবতী মায়ের নিজের সুস্বাস্থ্য অত্যন্ত গুরুত্বপূর্ন। যথেষ্ট সতর্ক থাকার পরেও গর্ভবতী মা বিভিন্ন সংক্রমনের শিকার হয়ে থাকে। যার মধ্যে বিভিন্ন ধরনের হেপাটাইটিস ভাইরাসের সংক্রমণ অন্যতম। এ সময়ে জন্ডিসে আক্রান্ত রোগীদের মধ্যে ৬০% ক্ষেত্রে সংক্রমনের কারণ হেপাটাইটিস-ই ভাইরাস। এই সব গর্ভবতী মায়েদের অসুস্থতার মাত্রা মৃদু থেকে মারাত্মক হয়ে থাকে এবং মায়ের জীবন সংকটাপন্ন হয়ে শতকরা ১৯-২৫ ভাগ মাতৃ মৃত্যু ও ৭-১৩ ভাগ নবজাতকের মৃত্যুর কারণ হয়ে দাড়ায়।

বিশ্বে হেপাটাইটিস-ই ভাইরাস (ঐঊঠ)-এর চারটি ধরণ বা টাইপ (টাইপ -১, টাইপ -২, টাইপ -৩ এবং টাইপ -৪) 'এর উপস্থিতি বর্তমান। প্রখম তিনটি টাইপ মূলত এশিয়ার কয়েকটি দেশ সহ আফ্রিকা, ল্যাটিন আমেরিকার দেশ, মার্কিন যুক্তরাষ্ট্র, অষ্ট্রেলিয়া, নিউজিল্যান্ড, ইউরোপের অনেক দেশে দেখা যায়। আর চতুর্থ ধরণ চীন-এর কয়েকটি অঞ্চলে এবং এশিয়ার বেশীরভাগ দেশে দেখা যায়। ধারনা করা হয় যে সংক্রমণকারী হেপাটাইটিস-ই ভাইরাসের বিভিন্ন ধরনের কারনেই জন্ডিসের মাত্রা বিভিন্নতর হয়ে থাকে। কয়েকটি দেশে হেপাটাইটিস-ই ভাইরাস প্রতিরোধী টিকা আবিষ্কারের গবেষণা অনেকদূর এগিয়ে গেছে। বাংলাদেশে হেপাটাইটিস-ই ভাইরাস সংক্রান্ত গবেষণা অনেক পিছিয়ে আছে। **আর্থসামাজিক** অবস্থার কারণে আমাদের দেশে হেপাটাইটিস-ই ভাইরাস সংক্রমণের প্রাদূর্ভাব অত্যন্ত বেশী। কিন্তু এখন পর্যন্ত বাংলাদেশে রোগ সংক্রমনকারী হেপাটাইটিস-ই ভাইরাস এর ধরণ আবিস্কারের জন্য কোন গবেষণা করা হয় নাই। অধিকন্তু উলে-খ্য যে, আমাদের দেশে হেপাটাইটিস-ই সংক্রমিত রোগীদের রক্তে ই-ভাইরাসের সংখ্যা নিরূপন পরীক্ষাও করা হয় নাই। সুতরাং, দেশে গর্ভবতী নারীদের হেপাটাইটিস-ই ভাইরাস সংক্রমনের ভয়াবহতা বিবেচনায় নিয়ে আক্রান্ত রোগীর রক্তে ভাইরাসের সংখ্যা, এর জেনোটাইপ ও সাব-টাইপ নিরূপন করা একান্ত প্রয়োজন।

গবেষণার উদ্দেশ্যঃ

বাংলাদেশের গর্ভবতী এবং গর্ভবতী নয় উভয় ক্ষেত্রে নারীদের রক্তে সংক্রমকারী হেপাটাইটিস-ই ভাইরাস-এর সংখ্যা, এর জেনোটাইপ ও সাবটাইপ সনাক্ত করা। হেপাটাইটিস-ই ভাইরাস-এর সংখ্যা, এর জেনোটাইপ ও সাবটাইপের সাথে আক্রান্ত রোগীদের অসুস্থতার মাত্রা তুলনা করা। হেপাটাইটিস-ই ভাইরাস-এর সংখ্যা, এর জেনোটাইপ ও সাবটাইপের সাথে গর্ভাবস্থায় আক্রান্ত নারীদের বয়স, গর্ভ সংখ্যা, গর্ভের বয়স (জেষ্টেশানাল এইজ) ও **আর্থসামাজিক** অবস্থার সম্পর্ক খতিয়ে দেখা এবং হেপাটাইটিস-ই ভাইরাস প্রতিরোধী টিকা আবিষ্কারের পথ প্রশস্ত করা।

গবেষণায় অন্তর্ভুক্তির নৈতিক দিকঃ

জন্ডিসে আক্রান্ত গর্ভবতী ও গর্ভবতী নয় যাদের রক্তে উচ্চ মাত্রায় বিলির"বিন (>১.২ মিলিগ্রাম /১০০ মিলি: রক্ত) এবং এসজিপিটি (>৮০ আইউ/লিটার রক্ত) থাকবে এমন নারী-দের গবেষণার বিষযে বিস্তারিত জানানো হবে। গবেষণায় অন্তর্ভুক্তির জন্য প্রত্যক্ষ বা পরোক্ষ চাপ প্রয়োগ করা হবে না। গবেষণায় অংশগ্রহনে সম্মতি প্রদানকারী রোগীদের নিকট থেকে লিখিত অনুমতি নেয়া হবে। গুর"তর অসুস্থ / স্বেচ্ছাপ্রনোদিত সম্মতি জ্ঞাপনে অক্ষম রোগীর ক্ষেত্রে তার নিকট আত্মীয়কে বিস্তরিত জানানো হবে। তিনি অসম্মত হলে ঐ রোগীকে গবেষনায় অন্তর্ভুক্ত করা হবে না। সম্মতি প্রদানকারী রোগী/ রোগীর আত্মীয় পরবর্তীতে গবেষণা থেকে নাম প্রত্যাহার করে নিতে পারবেন। গবেষণা প্রকল্পে অংশ গ্রহণে সম্মতি দেয়ার বিষয়ে রোগী সম্পূর্ণ স্বাধীন এবং দেয় মতামত কোন আপত্তি ছাড়াই গ্রহণ করা হবে। এই গবেষণায় রোগী সংগ্রহের জন্য সংশি-ষ্ট হাসপাতাল প্রধানের নিকট থেকে অনুমতি নেয়া হবে।

রোগীর সংগৃহীত রক্তের সেরাম হেপাটাইটিস-ই ভাইরাসের আরএনএ প্রস্তুত করার কাজে ব্যবহার করা হবে। রোগীর রক্তের ডিএনএ প্রস্তুত করা হবে না। সংগৃহীত রক্তের সেরাম প্রস্তুতির পরে ঐ রক্ত যথাযথ ভাবে নষ্ট করা হবে ফলে অন্য কোন অননুমোদিত কাজে ব্যবহৃত হওয়ার আশংকা থাকবে না। যে সকল হাসপাতাল থেকে রোগী সংগ্রহ করা হবে ঐ হাসপাতালের বিভাগীয় প্রধান সব নিয়ম মেনে রোগীকে এই গবেষণায় অন্তর্ভুক্ত করা হচ্ছে কি না তার তদারকী করতে বা করাতে পারবেন।

এ বিষয়ে গবেষণা প্রকল্পের **তত্ত্বাবধায়কবৃন্দ** গবেষক এবং হাসপাতালের বিভাগীয় প্রধানের সাথে সার্বক্ষনিক যোগাযোগ বজায় রাখবেন।

গবেষণায় অর্ন্তভূক্ত রোগীর গোপনীয়তা রক্ষা প্রসংগঃ

গবেষণায় অংশগ্রহনকারী রোগীদের রোগ সংক্রান্ত তথ্য চিকিৎসক এবং গবেষণা সংশি- ষ্ট ব্যক্তিদের মধ্যে সীমাবদ্ধ থাকবে। কোন ভাবেই রোগ সংক্রান্ত কোন তথ্য তৃতীয় কোন ব্যক্তিকে জানানো হবে না। গবেষণায় প্রাপ্ত-উপাত্ত কেবল মাত্র বৈজ্ঞানিক প্রবন্ধ/ নিবন্ধ লেখার কাজে ব্যবহার হবে। এখানে উলে- খ্য যে প্রবন্ধে/নিবন্ধে রোগীর নাম ও পরিচয় প্রকাশ করা হবে না।

গবেষণায় অর্ন্তভূক্তির প্রক্রিয়াঃ

স্বেচ্ছাপ্রনোদিত হয়ে সম্মতি প্রদানকারী রোগীদের আর্থসামাজিক অবস্থা ও মাতৃত্বকালীন সময়ের বিষয়ে প্রয়োজনীয় (ডাটা কালেকশান ফরম সংযুক্ত) প্রশ্ন করা হবে। বিভিন্ন পরীক্ষা -নিরীক্ষার জন্য রোগীর ৬/৮ মি:লি রক্ত সংগ্রহ করা হবে। রক্তের নমুনা সংগ্রহ করার সময় প্রয়োজনীয় সর্তকতা অবলম্বন করা হবে।

স্বাস্থ্য ঝুঁকিঃ

জন্ডিসে আক্রান্ত গর্ভবতী রোগীদের গবেষণায় অংশগ্রহনে কোন স্বাস্থ্য ঝুকির আশংকা নাই। গবেষণার জন্য রোগীর শিরা থেকে ৬/৮ মি:লি: রক্ত সংগ্রহ করা হবে। সাধারনত: রোগ নির্ণয় এবং চিকিৎসার অগ্রগতি পর্যবেক্ষণের জন্য পরীক্ষা-নিরীক্ষার নিমিত্তে ৮-১০ মি:লি: রক্ত সংগ্রহ করা হয়। সাধারণ মানুষের মধ্যে এ বিষয়ে সম্যক ধারণা ও গ্রহন যোগ্যতা আছে। সুতরাং প্রস্তাবিত গবেষণায় একজন রোগীর থেকে ৬/৮ মি:লি: রক্ত সংগ্রহ করা হলে স্বাস্থ্য ঝুঁকির কোন আশংকা নাই।

উপকারিতাঃ

এই গবেষণায় রোগীর রক্তে হেপাটাইটিস-ই ভাইরাসের সংখ্যা (ভাইরাল লোড) পরিমাপ করা হবে এবং এই নিরীক্ষার ফল চিকিৎসককে জানানো হবে যা প্রয়োজনীয় ক্ষেত্রে রোগীর চিকিৎসা ব্যবস্থায় গুর"ত্বপূর্ন ভুমিকা পালন করতে পারে। এখানে উলে- খ্য যে,এই নিরীক্ষার ব্যয় গবেষক নিজেই বহন করবেন।

কোন বিষয়ে গবেষণা একটি চলমান প্রক্রিয়া। রোগ সম্পর্কে দীর্ঘ দিনের গবেষণা লব্ধ তথ্য-উপাত্ত সঠিক রোগ নির্নয় ও চিকিৎসা ব্যবস্থায় সম্যক অগ্রগতির প্রতিফলন ঘটায়। প্রস্তাবিত গবেষনা হেপাটাইটিস-ই ভাইরাস সংক্রমনের ধরণ ও রোগের মাত্রা বিষয়ে নূতন তথ্য-উপাত্তের আলোকপাত করবে। হেপাটাইটিস-ই ভাইরাসের প্রাপ্ত জেনোটাইপ ও সাবটাইপ জন্ডিসের বিভিন্ন মাত্রার যোগসূত্র বুঝতে সাহায্য করবে এবং লব্ধ জ্ঞান গবেষক ও চিকিৎসকদের ভবিষ্যতে রোগীদের চিকিৎসায় প্রয়োজনীয় উন্নতি বিধানে নিয়ামক হবে। অধিকন্তু ,বাংলাদেশে বিদ্যমান হেপাটাইটিস-ই ভাইরাসের টাইপ ও সাবটাইপ সনাক্ত করা সম্ভব হলে এর প্রতিরোধী টিকা আবিষ্কারের প্রচেষ্টা ত্বরান্বিত হবে। সফল টিকা আবিষ্কারে মধ্য দিয়ে হেপাটাইটিস-ই ভাইরাস সৃষ্ট জন্ডিস প্রতিরোধ করা সম্ভব হবে।

প্রয়োজনীয় ক্ষেত্রে যোগাযোগঃ

গবেষণার বিষয় ও উদ্দেশ্য সম্পর্কে যদি কোন প্রশ্ন থাকে তবে অনুগ্রহ করে যোগাযোগ করুনঃ

ডাঃ রোজী সুলতানা। ফোন- ০১৭১১০১৪৮০৬।

সম্মতি পত্র

(ডাঃ রোজী সুলতানা-র পি-এইচ.ডি প্রোজেক্ট-এর সম্মতিপত্র এই থেসিস-এর প্রয়োজনে ব্যবহার করা হয়েছিল)

গবেষণার শিরোনামঃ

বাংলা - গর্ভাবস্থায় হেপাটাইটিস-ই ভাইরাস জনিত জন্ডিসে আক্রান্ত বাংলাদেশের শহরে বসবাসকারী মায়েদের রক্তে ভাইরাসের সংখ্যা এবং এর জেনোটাইপের প্রভাব অনুসন্ধানে গবেষণা।

English- Influence of Hepatitis E viral load and genotypes on pregnant urban dwellers of Bangladesh

আমি নিশ্চিত করছি যে গবেষণা তথ্যপত্র আমি পড়েছি এবং গবেষণা বিষয়ে বিস্তারিত জেনেছি। গবেষণা বিষয়ে আমার প্রশ্নের উত্তর

পেয়েছি। আমি বুঝতে পেরেছি যে গবেষণায় অংশ নিলে আমার কোন স্বাস্থ্য ঝুঁকি নেই।

আমি স্বেচ্ছায় গবেষণা প্রকল্পে অংশ নিতে এবং আমার স্বাস্থ্য সংক্রান্ত প্রয়োজনীয় তথ্য গবেষক ও গবেষণা সহকারীকে জানাতে ইচ্ছুক বলে সম্মতি জ্ঞাপন করছি।

রোগীর নামঃ ----------; রোগীর স্বাক্ষরঃ ----------; তারিখঃ ----------

গবেষকের নামঃ ডাঃ রোজী সুলতানা; স্বাক্ষরঃ

গবেষণা সহকারীর নামঃ স্বাক্ষরঃ

Appendix – I B

Data Collection Form

(Adapted from the PhD project by Dr. Rosy Sultana)

গবেষণা প্রকল্পের শিরোনাম -	গর্ভাবস্থায় হেপাটাইটিস- ই ভাইরাস জনিত জন্ডিসে আক্রান্ত বাংলাদেশের শহরে বাসকারী মায়েদের রক্তে ভাইরাসের সংখ্যা এবং এর জেনোটাইপের প্রভাব অনুসন্ধানে গবেষণা।
Title of the proposal-	Influence of Hepatitis E viral load and genotypes on pregnant urban dwellers of Bangladesh

Principal Investigator: Dr Rosy Sultana, Associate Professor, Dept of Immunology, Bangladesh University of Health Sciences (BUHS), 125/1 Darus Salam, Mirpur-1, Dhaka-11216;
Cell Phone: 01711014806

Name of the Centers for collection of samples: BSMMU/DMC/ SSMC/ MCH Azimpur/ BIHSGH/ Other

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A. <u>Personal information</u>

1. **Name**: 2. **Age**: yrs

3. **Pregnancy**: Yes/ No

4. **Family member**:

5. **Education level**: PSC/ JSC/ SSC/ HSC/ Graduate/ Higher

6. **Husband's Name**:

7. **Education level**: PSC/ JSC/ SSC/ HSC/ Graduate/ Higher

8. **Present Address**:

9. **Duration of stay at the present address**:

10. **Permanent address**:

B. Socio-demographic status

11. Area of residence: Rural/ Semi urban/ Urban

12. Types of housing: Slum/ Tin shade shared facilities/ Tin shade individual facilities/ Building-shared facilities/ Building separate facilities

13. Annual family income (Approx): **Tk**

14. **Source of drinking water:** Tube well shallow/ Tube well deep/ Municipal supply/ Surface-pond/ Surface- canal/ Surface river/ Untreated/ Traditionally treated/ Boiled/ Filtered

15. **Habit of eating street food**: Yes / No; **If yes**: Frequent/ Infrequent

C. <u>Obstetric History:</u>

16. Para: 17: Gravida:

18. Duration of pregnancy: wks

19. Bad obstetric history: Abortion/ Miscarriage/ Still birth

D. <u>Clinical Data:</u>

20. **History of previous jaundice**: Yes/ No

21. **Pulse:** /min**;** 22. **BP:** mmHg**;** 23. **Respiration:** /min

24. **Serum bilirubin:** mg/dl**;** µmol/l; 25. **SGPT**: IU/l

26. **Liver enlargement:** Yes/ No; 27. **If Yes**: Size:

28. **Diagnosis:** Acute viral hepatitis (AVH)/ Fulminant hepatic failure (FHF)

29. **If FHF:** Stage of encephalopathy: Stage 1/ Stage 2/ Stage 3/ Stage 4

E. <u>Laboratory Investigation:</u>

30. **Serum Bilirubin:** mg/dl**;** 31. **SGPT:** IU/l

32. **SGOT:** IU/l**;** 33. **Alkaline phosphatase:** IU/l

34. **Anti-HAVIgM:** Yes/ No; 35. **HBsAg:** Yes/ No

36. **Anti-HCV:** Yes/ No 37. **Anti-HEVIgM:** Yes/ No

38. **Anti-HEVIgG:** Yes/ No

F. Molecular Analyses

39. **HEV-RNA, Viral count** (Real-Time PCR)**:** copy number/ ml

40. **HEV Genotyping:** Type 1/ Type 2/ Type 3/ Type 4

41. **HEV sub-types:**

Hepatic Encephalopathy and Cerebral Edema

	Stage I	**Stage II**	**Stage III**	**Stage IV**	**Brain Edema**
Symptoms	Insomnia Difficulty in concentration	Drowsiness Confusion	Somnolence Stupor	Coma	Coma ICP, HTN Convulsion
Signs	Sluggish speech	Flapping tremor	Flapping tremor		Herniation

Appendix – II

Instruments

The important equipment used through the study are listed below:

Instrument	Manufacturer
Autoclave	Daihan, Korea
Freeze (-20ºC)	EQUiTEC
Micropipette (10-100µl)	Gilson, France
Micropipette (20-200 µl)	Gilson, France
Micropipette (2-20 µl)	Gilson, France
Oven	Panasonic, Japan
Refrigerator (4ºC)	Walton
Class II Biological Safety Cabinet	BIOBASE, Germany
Vortex Mixture	Wisd laboratory instruments, Germany
Weighing Balance	Wisd laboratory instruments, Germany
PCR machine	Bio-Rad Laboratories, USA
Gel electrophoresis machine	Bio-Rad Laboratories, USA
Centrifuge machine	Eppendorf, Germany
Micro-centrifuge machine	Wisd laboratory instruments, Germany
Gel Doc	Bio-Rad Laboratories
Nano drop machine	Thermo Scientific