SEROPREVALENCE OF HEPATITIS-E VIRUS INFECTION AMONG PATIENTS ATTENDING DIFFERENT HOSPITALS AT CHATTOGRAM, 2018

Name: Joyantee Sarkar

Roll no.: 0118/03

Registration no.: 600

Session: 2018-19

A thesis submitted in the partial fulfillment of the requirements for the degree of MPH (One Health)

One Health Institute

Chattogram Veterinary and Animal Sciences University

Chattogram-4225, Bangladesh

December 2019

Authorization

I hereby declare that I am the sole author of this thesis. I also authorize the Chattogram Veterinary and Animal Sciences University (CVASU) to lend this thesis to other institutions or individuals for the purpose of scholarly research. I further authorize the CVASU to reproduce the thesis by photocopying or by other means, in total or in part, at the request of other institutions or individuals for the purpose of scholarly research. I, the undersigned, and author of this work, declare that the **electronic copy** of this thesis has been provided to the CVASU Library, is an accurate copy of the print thesis submitted, within the limits of the technology available.

Joyantee Sarkar
December 2019

SEROPREVALENCE OF HEPATITIS-E VIRUS INFECTION AMONG PATIENTS ATTENDING DIFFERENT HOSPITALS AT CHATTOGRAM, 2018

Name: Joyantee Sarkar

Roll no: 0118/03

Registration no.: 600

Session: 2018-2019

This is to certify that we have examined the above MPH (One Health) thesis and have found that it is complete and satisfactory in all respects, and all revisions required by the thesis examination committee have been made.

----------	**----------**
Prof. Mohammad Mahmudul Hassan, PhD	**A/P Dr. Shahneaz Ali Khan, PhD**
Supervisor	**Co-supervisor**

Prof. Sharmin Chowdhury, PhD

Chairman of the Examination Committee

One Health Institute

Chattogram Veterinary and Animal Sciences University

Chattogram-4225, Bangladesh

December 2019

Acknowledgements

All praises to almighty Allah who gave me the opportunity to be enrolled in the **One Health Institute** for achieving Masters in Public Health. I would like to express my veneration to honorable supervisor Professor **Dr. Mohammad Mahmudul Hassan,** Head, Department of Physiology, Pharmacology and Biochemistry, Faculty of Veterinary Medicine, Chattogram Veterinary and Animal Sciences University (CVASU) for his coherent and articulated instructions. It would not be possible to complete such a laborious task without his scholastic guidelines. It was an exquisite experience for me to work under his supervision. I feel much pleasure to convey my gratitude to honorable co-supervisor Dr. Shahneaz Ali Khan for his valuable suggestions and inspiration. I am grateful to Dr. Md. Tarek-Ul Quader, Medical Officer, Department of Anesthesiology and Intensive Care Medicine, Chattogram Medical College Hospital for his supports in relation to data collection during the fieldwork.

I am grateful to my supervisor for providing all the laboratory facilities and other technical staffs for assistance. Specially thanks to **Dr. Ariful Islam and Dr Md. Kaisar Rahman** for their guidance and cooperation.

I would like to acknowledge the support and encouragement received during MPH program me from other teachers, technical and non-technical staffs of the One Health Institute, CVASU.

I am also grateful to my parents and family members for their support.

Table of Contents

List of Tables and Figures

List of Abbreviations

Abbreviation	Elaboration
ACLF	Acute chronic liver failure
CI	Confidence interval
COV	Cut-off value
CWASA	Chattogram Water Supply and Sewerage Authority
EDTA	Ethylene Diamine Tetra Acetic acid
ELISA	Enzyme-linked immunosorbent assay
FHF	Fulminant hepatic failure
HEV	Hepatitis E virus
HSPGs	Heparan sulfate proteoglycans
icddr,b	International Centre for Diarrhoeal Disease Research, Bangladesh
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
INSDC	International Nucleotide Sequence Database Collaboration
OR	Odds ratio
PCR	Polymerase chain reaction
ROC	Receiver operating characteristic curve
UK	United Kingdom

Abstract

Hepatitis E virus (HEV) infection is a growing cause of acute viral hepatitis and gastroenteritis around the world. Bangladesh is an endemic area of HEV associated with epidemic and sporadic infection and seasonal outbreak. The aim of the study is to better describe the sero-prevalence of hepatitis E virus in selected hospitals of Chattogram district among the admitted patients. A total of 505 patient data were collected from 10 different hospitals during the period of 12 months from January to December 2018. All the Blood samples were tested for HEV in Anti-HEV IgG and IgM through ELISA Procedure. The sero-prevalence of hepatitis E virus in Chattogram was recorded as 35.05% (n=177; 95% CI: 30.9-39.4). Prevalence in outbreak area was found higher 73.4% (n=80, 95% CI: 64.1-81.4) than non-outbreak area 24.5% (n=97; 95%CI: 20.3-29). The odds of infecting HEV in rainy season was significantly (2.2 times) higher (OR=2.2; Cl: 1.1-4.1, p=0.02) than the winter season. Male patients represented consistently higher infection over the year and they were 2.5 times more (OR=2.5; CI: 1.5-4.1, p=0.001) affected than the female patients. In case of age, HEV was significantly higher in 41-60 years category (OR=3.3; CI: 1.5-7.4; p=0.003).
The odds of HEV among the patients consuming municipal supply water (OR=13.5; CI: 6.8-26.7, p=0.000) and not treating with boiling (OR=40.9; CI: 18.5-161.6, p=0.000) were significantly higher. The result indicates the importance of modern molecular techniques such as polymerase chain reaction (PCR) for reliable diagnosis of hepatitis. Moreover, identification of further risk factors associated with the occurrence of hepatitis E virus would help to take proper strategies for the prevention and control in this area.

Chapter 1: Introduction

Hepatitis E virus (HEV) causes infections in both humans and animals round the world. It is the most obvious reason for acute viral hepatitis especially in developing countries such as Bangladesh, India, Pakistan and Nepal. The morbidity and mortality rate are higher compared to other infectious diseases. Hepatitis E, the endemic agent transmitted through the fecal-oral route via contaminated water. However, Poor sanitation and unhygienic conditions are responsible for the waterborne outbreak and multiple sporadic cases in the developing countries. Hepatitis E has one serotype and four genotypes namely genotype 1, 2, 3 and 4. Genotype 1 and 2 are responsible for acute viral hepatitis in human and endemic to Asia, Africa, and Central America, usually transmitted through contaminated water (Aggarwal, 2010; Teshale et al., 2010b). In contrast, genotype 3 and 4 are responsible for zoonotic diseases which are transmitted to humans by different reservoirs including rodents, pig, deer, dog (Schlauder et al., 1998; Meng et al., 1999; Okamoto, 2007). Not only humans are infected, but several animal species are at risk with this virus (Meng, 2010). The presence of HEV was suspected for the first time in 1978 in India during a large waterborne outbreak of hepatitis. The infection was spreading person to person and morbidity rate was found higher in adults. Interestingly, mortality rate was higher in pregnant women among infected adults (Khuroo et al., 1983).

The virus was first identified by Balayan and colleagues using immune electron microscopy in 1983. The full genome sequence of HEV was done in 1991 and identified it as a non-enveloped, single-stranded RNA virus with a positive sense genome, a sole member of the genus Hepevirus (Tam et al., 1991). The virus is relatively stable into the environment and is sensitive to heat, chlorination and ultraviolet light (Albinana-Gimenez et al., 2006; Girones et al., 2014). The existence of HEV in animals was first discovered in pig in 1995 and isolated in 1997 (Meng et al., 1999). Among 4 genotype of hepatitis E, HEV-3 has been isolated from various mammals' species such as wild boar (Sonoda et al., 2004), deer (Reuter et al., 2009; Forgách et al., 2010) rabbits (Zhao et al., 2009) and rats (Johne et al., 2010). HEV-4 has been isolated from different animal species in China (Zhang et al., 2008b). In addition new HEV and HEV like viruses with significant zoonosis have been also identified from different animal species such as ferret, moose, camel, fish and human as well (Batts et al., 2011; Raj et al., 2012; Lin et al., 2014; Woo et al., 2014).

Other modes of transmission of HEV infection include transmission from pregnant mothers to their fetuses, and rarely through blood transfusion (Boxall et al., 2006; Mansuy et al., 2009; Hewitt et al., 2014)

Clinical features of hepatitis E are indistinguishable from acute hepatitis caused by other hepato-tropic viruses with 15-60 days of incubation period and the mean value is 40 days. Infection is mainly subclinical and only 20-30% of patient shows signs and symptoms (Labrique et al., 2009). The ratio of symptomatic and asymptomatic infection may vary with different viral genotypes, environment, ecology and epidemiological setting. Patient of acute hepatitis E virus infection usually manifests with icterus, malaise, anorexia, fever, hepatomegaly, and occasionally pruritus. Immunosuppressed patients are unsuccessful to clear the virus which leads to chronic infection mostly with HEV-3 genotype (Dalton et al., 2009; Geng et al., 2014). Laboratory diagnosis of hepatitis E virus usually conducted based on ELISA-detection of HEV specific IgM antibodies or detection of RNA in clinical samples. HEV infection is also characterized by specific IgG antibodies against ORF2 (Khudyakov and Kamili, 2011).

The highest incidence of HEV infection was identified in Southeast Asia. Some seasonal outbreak has been recorded in India and Nepal and few studies have confirmed the HEV infection in Bangladesh. In spite of having seasonal outbreak in Bangladesh, the region is imagined as endemic for HEV with the range of sero-prevalence 27% to 60% (Labrique et al., 2009). The prevalence of HEV have found age specific which is different from the report for antibody to hepatitis A virus even though the infection route of both two viruses are similar in endemic countries. The sero-prevalence rate of anti-hepatitis A virus was found as about more than 95% in young age but anti-hepatitis E was less in children (Izopet et al., 2015). The most sporadic infection rate of HEV in endemic region usually occurs in 15-35 years of age group. In case of acute hepatitis, different age group showed somewhat similar prevalence in Nepal (Shrestha et al., 2003) but more than 30 years old women showed higher prevalence in Egypt (Stoszek et al., 2006). HEV infections are dominant in male than the female reported with a male-to-female ratio ranging from 1/1 to 3/1. The reason behind the more infection rate in male is not understandable. The mortality rate of HEV is very less but it depends on the situation, rates getting higher during the pregnancy period when disease becomes more severe. Report from develop countries present that, the HEV transmitted to the

travelers who travel the endemic areas and develop hepatitis and liver failure (Dalton et al., 2008).

Urban areas of developing country are in the big challenge of hepatitis E virus infection. Densely population and standard of living is the main reason for this. Previously reported outbreak suggests that the contaminated drinking water in municipal is the source and leads to the outbreak of HEV (Gurley et al., 2014). In addition, poor sanitation and personal unhygienic aggravate the condition. Study reveals that drinking water contaminated with fecal matter and HEV spread enterically through the fecal-oral route and 16.8% consumed boiled or filtered water while the rest consumed supplied water of city corporation or tube well and getting infection with hepatitis E virus (Gurley et al., 2014) in urban of Bangladesh. To control the vulnerability of this severe disease, proper strategies of vaccination should be used by taking into account the varying epidemiologic patterns across different parts of the world and populations.

This study represents the sero-prevalence of Hepatitis E virus infection among patients attending different hospital at Chattogram in 2018. The availability of the resulting comprehensive serological and patient data can be useful in formulating disease prevention policy and in developing novel antiviral compounds.

Aim of the Study

Liver diseases in Bangladesh mostly occur due to the hepatitis virus and poses enormous health burden to the countryman as well as the economy of the country. Out of all hepatitis viruses, hepatitis E is most dangerous and causes acute hepatitis, acute chronic liver failure (ACLF) and fatal condition in pregnancy period with maternal mortality 20 to 45 percent (Khuroo et al., 2009; Mamun-Al-Mahtab et al., 2009; Borkakoti et al., 2013). There are very few studies about the prevalence of hepatitis E virus and its distribution in Bangladesh. Therefore, this study was undertaken to know about the sero-prevalence of Hepatitis E virus, to gain information about the distribution of HEV and risk factors in selected hospitals of Chattogram District in Bangladesh.

The aim of this study was as below:

- To evaluate the sero-prevalence of HEV in Chattogram District in Bangladesh
- To evaluate the outbreak of HEV and to know the distribution of the virus
- To know the risk factors associated with HEV infection in Chattogram.

Chapter 2: Review of the literature

2.1 History of Hepatitis E Virus

Hepatitis E virus (HEV) is the most recently invented of the 5 well-recognized hepatotropic viruses, named A to E. The earliest well-documented report of this infection was a deadly disease of acute viral hepatitis, that had occurred in New Delhi, India in 1955–1956 that was at the start thought to be associated with viral hepatitis (Khuroo et al., 2009). It was first recognized throughout an epidemic of liver disease, that occurred in Kashmir valley in 1978 when the stored sera from the Kashmir outbreak and the epidemic in Delhi were tested (Aggarwal and Naik, 2009). Serum not expressed specific markers for any Hepatitis whether it's A or B, and therefore was provisionally named as enterically transmitted non-A, non-B Hepatitis virus. These viruses were identified from a water-borne epidemic of non-A hepatitis volunteer after inspection in the fecal sample using immune electron microscopy after self-ingestion of acute phase stool suspensions in Central Asia.

Based on the enteric route of transmission and capacity of causing epidemic infection, the viruses were named Hepatitis E virus. After that, in 1990 first the hepatitis E virus was cloned and sequenced (Khuroo et al., 2009). Hepatitis E infection has been seen most prevalently in the unhygienic and poor sanitation areas of the world such as biggest part of Asia, Africa, the Mediterranean region, Mexico and South America. Acute sporadic viral hepatitis and fulminant hepatic failure (FHF) are the main outcome of medium to large-sized waterborne epidemics and which ultimately happened by HEV in the endemic regions. In the developed countries HEV infection occurred different ways; it may have zoonotic origin of infection and carried virus by the travelers and tourists from the disease-endemic countries. In the 19^{th} century and early time of 20^{th} century sanitary conditions of Europe was not good so there numerous hepatitis outbreak happened with similar features which currently occurring in the developing countries and now those Europe countries are non-endemic.

2.2 Molecular virology of Hepatitis E virus

Molecular virology of HEV consists of morphological and genomic organization. In addition to life cycle make it better insight.

2.2.1Morphology and genomic organization of Hepatitis E virus

HEV is the only representative of the genus Hepevirus in the family of Hepeviridae (Tam et al., 1991). HEV is small, size of 27-34 nm round non-enveloped with a genome of nearly 7.2kb in length viral particle which is polydenylated. It is consisting of single strand RNA molecules, which have 3 partially overlapping and discontinuous open reading frames (ORFs). It has important roles to play in replication and transcription due to its 5′ and 3′ cis-acting elements (Fig. 1). The nonstructural proteins helpful for replication and protein processing, including an RNA helicase, an RNA-dependent RNA polymerase, a methyltransferase, and a cysteine protea. The nonstructural proteins consist ORF1 extends 5 kb from the 5′ end, consists of 5,079 nt and encodes (Mushahwar, 2008; Wedemeyer et al., 2012). Other part ORF2, which is of 1,980 nt in length contain helpful epitope at its 3′ end with a signal sequence and viral capsid protein. The smallest part are ORF and ORF3 which contain 372 nt. Its overlaps ORF1 by one nucleotide at the 5′ end and extensively overlaps ORF2 by 328 nt at the 3′ end. The very current study found that the ORF3 contain encoded a protein which function is as ion channel and viroporin activity (Ding et al., 2017). Now, there are nearly 1600 hepatitis E virus sequence. At present, at the International Nucleotide Sequence Database Collaboration (INSDC, previously known as DDBJ/EMBL/GenBank) there are approximately 1,600 HEV sequences are availableand the number of sequences is expanding at a great rate(Mushahwar, 2008).

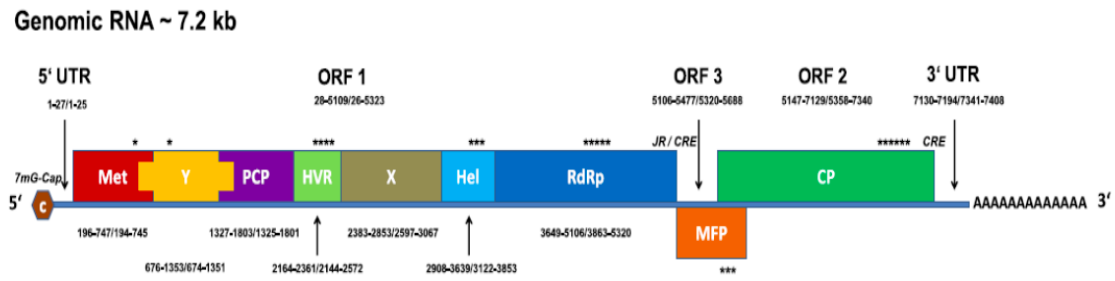

Figure 1: Schematic description of the HEV genome and viral proteins.

The Figure shows linear, ssRNA (+) genome of ~7.2 kb HEV genome and corresponding viral proteins. The 5′-end is capped and the 3′-terminus is polyadenylated. ORF1 encodes the nonstructural polyprotein, including methyltransferase (Met), Y-domain (Y), papain-like cysteine protease (PCP), hypervariable region (HVR), macro-domain (X), RNA helicase (Hel) and RNA-dependent RNA polymerase (RdRp). The ORF2 encodes the capsid protein

(CP). The ORF3 encodes a small multifunctional protein (MFP). CRE is cis-reactive element; Nucleotide positions are relative to the HEV-1 Burmese strain (Acc. No. M73218)/HEV-3 47832 strain (Acc. No. KC618402). Asterisk (*) indicates the hot spot region for clinical mutations.

2.2.2 Life Cycle of Hepatitis E Virus

The life cycle of Hepatitis E Virus (HEV) is related to other single stranded RNA (Fig. 2). Hepatitis E virus attaches to the cell by interaction of ORF2 as for example the capsid protein with attached receptors eg. heparan sulfate proteoglycans (HSPGs) and heat shock cognate protein 70 (HSC70) and enters the cells by dynamin-2, clathrin,membrane cholesterol and actin dependent endocytosis (Cao and Meng, 2012). After entrance, in the cytoplasm the virion uncoated and release the viral RNA where the 7-methylguanosine cap structure in the 5' non-coding region (NCR) of the HEV genome recruits the 40S ribosomal subunit to initiate codependent translation of ORF1 polyproteins which include viral enzymes. The viral RNA helicase and RdRp replicated the viral genome. The viral sub genomic RNA also translated the ORF2 and ORF3 proteins (Debing et al., 2016).

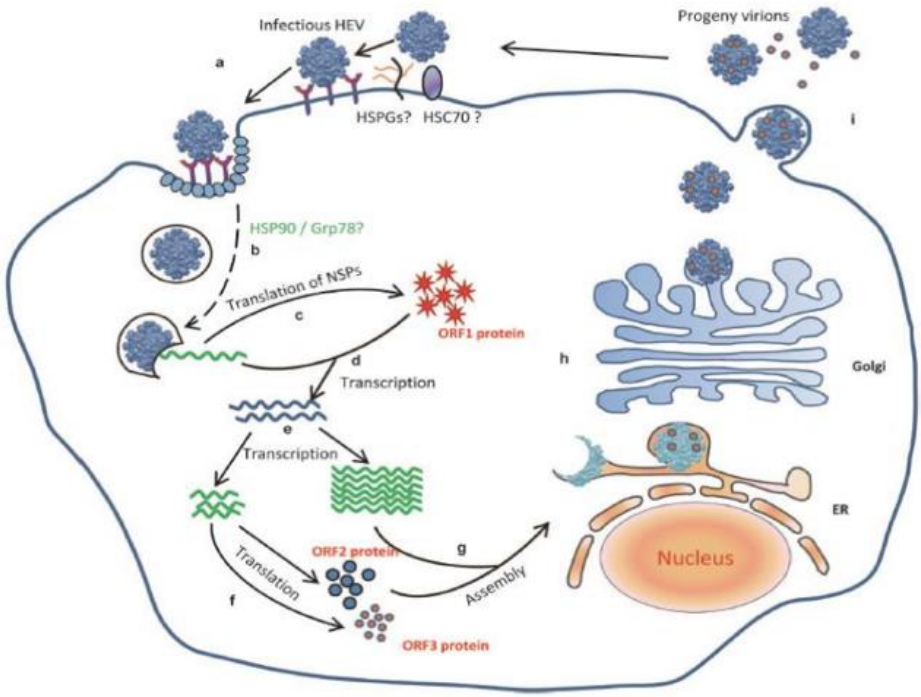

Figure 2: Life cycle of Hepatitis E virus

The viral proteins and positive single stranded RNA could be localized at the ER Golgi intermediate compartment but there replication complex of HEV is located (Perttilä et al., 2013). The association of RNA and ORF2 protein arrangement the breed of viral particles, which are then released from the host cells via the ORF3 protein which has viroporin activity that is critical for viral egress (Ding et al., 2017)

Step a: HSPGs, HSC70 or other putative connection receptor(s) attaches to the cell external of HEV and then enters the cell through an unknown definite cellular receptor.
Step b: The cell invaded by the HEV virion and go through the cells. HSP90 and Grp78 may be associated in this transport. Into the cytoplasm, the virions then open and discharge the positive-sense genomic RNA.
Step c: The positive-sense genomic viral RNA serves as the pattern to translate the ORF1 nonstructural polyprotein in the cytoplasm.
Step d: From the positive-sense genomic RNA the viral RdRpincorporates an intermediate, replicative negative-sense RNA that (**Step e**) acts as the template to produce of positive-sense, progeny viral genomes.
Step f: The sub genomic helps to translate the ORF2 and ORF3 proteins, positive stranded RNA, and (**Step g** the genomic viral RNA packaged the ORF2 capsid protein and bring together new virions.
Step h: The cell membrane transport nascent virions; the virion than trafficked the ORF3 protein facilitates.
Step i: From the infected cells the nascent virions are released;

2.3 Transmission of HEV:

2.3.1 Waterborne Transmission

In the developing countries, the most important route of transmission of virus is waterborne transmission (Boccia et al., 2006; Hazam et al., 2010; Howard et al., 2010). The fecal contamination in water supply chain is the main reason for the spill over the epidemics. Other environmental source of water also responsible such as river has proven earlier as a contaminated source for HEV. In the study of Mutha river, a representative number of positive samples were found positive compared to earlier years (Verma and Arankalle, 2010). In the monsoon season, contamination became increase due to the high flow of water and

poses an elevated threat. The G1 and G2 genotypes are associated with waterborne epidemics where G1 is important for Asia and G2 for Africa and Mexico (Teshale et al., 2010a).

2.3.2 Direct transmission

The direct transmission of hepatitis E from human to human or even animal to human is not clear. Previous report provided evidence of direct transmission of HEV between the farmers and swine also the veterinarians (Christensen et al., 2008; Lee et al., 2013). Another report provide result of direct transmission within a butcher in England and a slaughterhouse worker in Spain who have been diagnosed with acute HEV infection (Pérez-Gracia et al., 2007).Intra familial transmission was negligible even during epidemics (Aggarwal and Naik, 1994). The human also acts as a poor vector for spread hepatitis E virus. However, a prolong epidemic was happen in 2008 due to the result of another infection previously. The origin of infection was removed by the installation program of proper sanitation. Therefore, Person to person infections is the key factors for long time duration of the epidemic (Teshale et al., 2010a). Previous epidemiological studies showed a relationship between seroconversion of owner with the rearing of pets (Widdowson et al., 2003; Kuniholm et al., 2009). A Japanese patient found positive with anit-HEV antibodies proof of HEV infections (Kuno et al., 2003). Another report of pets shows that about 33% of cats was positive for anti-HEV Ab's (Okamoto et al., 2004). However, there is no specific study that show the link behind the transmission of HEV into pets. Rats might be a source of infection as they can carry the G3 genotype of HEV. The level of infection of anti-HEVAb's in rats have been found in different geographic locations of the world between 4.5% to 90% (Favorov et al., 2000).

2.3.3 Vertical transmission (Parenteral Transmission)

The risks of HEV infection in pregnant women are more than the normal peoples due to the different complications. The HEV infection transmitted vertically to the offspring from the mother and there was strong evidence. If the baby survive than it develop hepatitis after birth (Khuroo et al., 2009). Nourishing the child with breastfeeding was not detected as a risk factor for the transmission of HEV (Chibber et al., 2004).

2.3.4 Blood transfusion and Organ transplants

There are several cases documented as transmission of HEV through blood transfusion where genomic sequences were matched between donor and recipient (Boxall et al., 2006; Colson et al., 2007; Matsubayashi et al., 2008). Earlier a study from United Kingdom, the presence of HEV RNA was found 880 minipools associated to the 42000 individual donors were tested and 6 were found positive. All the samples were positive for IgG testing. About 75% immunosuppressed patients in the UK have taken donated blood and who showed chronic infection previously and developed risk (Bihl and Negro, 2009). A study from USA in 2013 revealed a low prevalence of HEV were a total 1939 individual samples were tested but no one was positive for HEV RNA but 16% sample was tested positive for anti-HEV IgG (Xu et al., 2013).

2.3.5 Foodborne zoonotic transmission

There are lots of evidences of animal to human transmission of Hepatitis E virus in many countries in the world. In japan, HEV infection was found due to consumption of uncooked or half cooked wild boar and deer meat. For the confirmation genetic sequence was done from both meat and the patient and found the similar viruses (Inagaki et al., 2015). In USA, Japan and Netherlands, contamination of HEV was detected from retail pig liver sample but same time virus was absent in the sample of pigs from UK. HEV found even after heating in USA; virus detected after heating to 56ºC for 1 hour and virus became inactivated at the temperature of 71ºC for 5 min which indicate half cooking might not eliminate the virus from contaminated food (Cossaboom et al., 2016). In Canada and French, a strong association of HEV and pork consumption has been found in chronic liver diseases. Another research from 18 developed countries showed that the hepatitis E infection is related with the pig farming and meat consumption and development of chronic liver disease (Berto et al., 2012). The food supply chain has been infected with the HEV in some articles (Leblanc et al., 2010; Di Bartolo et al., 2012). Due to long incubation periods after taking the infected food, it became more difficult to detect the source of infection of HEV. As it takes long time to expose the clinical signs, the food left over disposed within the time limit. But there are very rare case of frozen foods (Tei et al., 2003). A few cases have been found with low incubation period and severe infection. A well-established example of foodborne zoonotic transmission of HEV is Figatelli liver sausage from south French, peoples traditionally consumed raw. Which led to develop hepatitis who consumed that sausage (Colson et al., 2010). Figatelli liver sausage

from pig in French later isolated virus presented as 3D cell culture systems and proof the infectivity (Berto et al., 2013). Two man from Japan were admitted in the hospital with severe hepatitis and after collecting history it was revealed that infection came from uncooked liver of a boar (Matsuda et al., 2003).

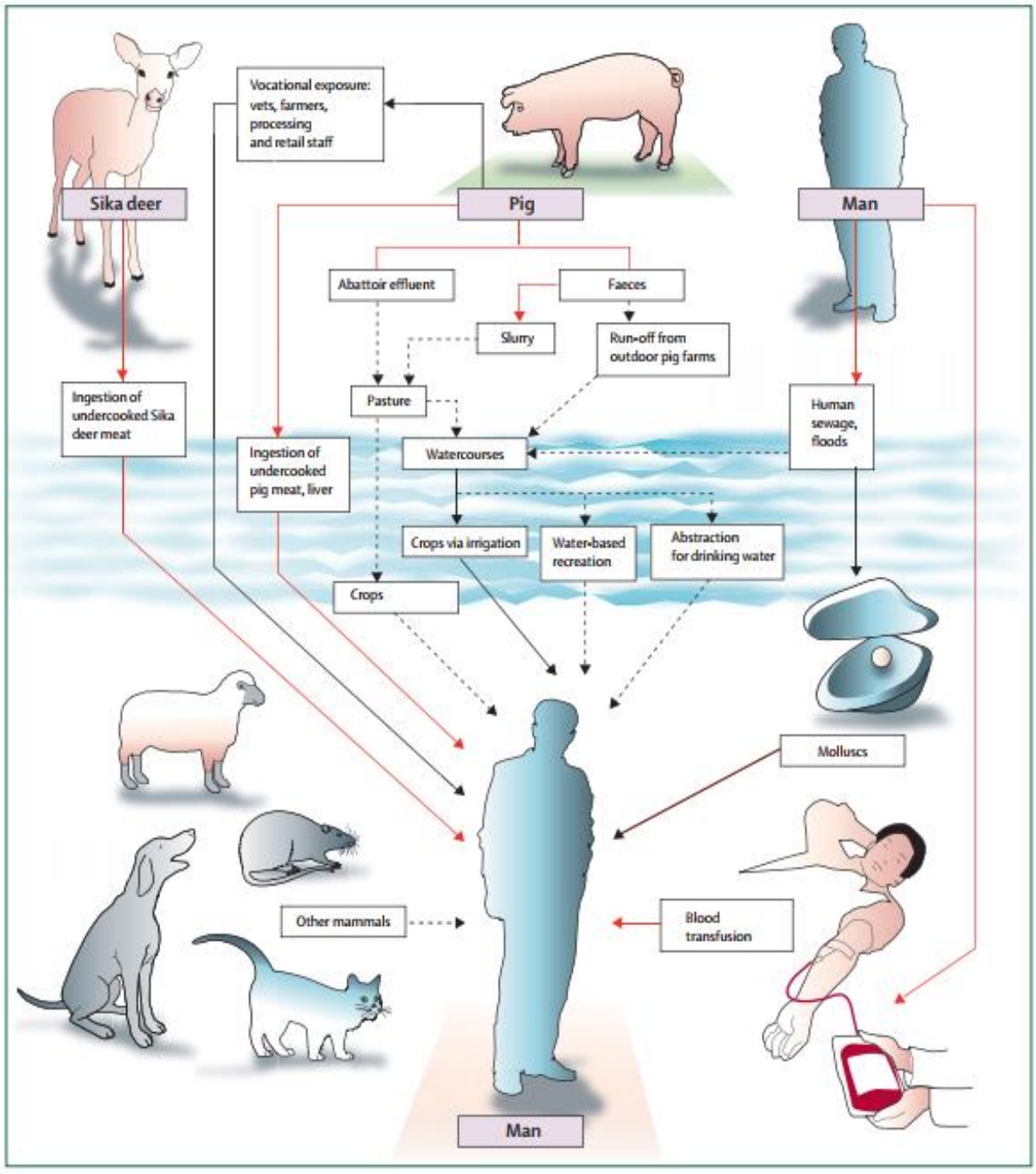

Figure 3: Transmission cycle of Hepatitis Virus

Several studies provide evidence of presence of HEV in grocery meat store in many countries such as Germany (4%), India (0.8%), Netherlands (4%), France (4%), and Japan (2%) (Bouwknegt et al., 2007; Kulkarni and Arankalle, 2008; Bouquet et al., 2011; Wenzel et al., 2011). In USA, much higher percentage of HEV was found positive (11%) in the meat and liver (Feagins et al., 2008). Multiple studies provide example of exposed to HEV with consumption of swine liver, meat and other types of organs (Kuniholm et al., 2009). Previous studies showed that the outbreak of HAV and NoV were correlated with the consumption of frozen berries. The contamination in berries might come to the production areas through poor sanitation systems so the quality of water supply and sanitation has been questioned later. The investigating study revealed that, there was no infection of HEV in the irrigated water samples but strawberries were found positive for HEV which proved the foodborne zoonotic transmission (Brassard et al., 2012).

2.4 Genotypes and Subtypes of Hepatitis E virus

To date, Hepatitis E virus have identified one serotype with four genotypes (1, 2, 3 and 4). it was demonstrated unequivocally that the use of this region in phylogenetic analysis generates a tree consistent with full length sequences. According to analysis of either the complete genome sequence and/or the 371 base region, the largest base region from ORF1, spanning nucleotide 80–450 (Mushahwar, 2008; Rein et al., 2012). The distribution of HEV of different genotypes spread throughout the world. In Asia, genotype 1 is more prevalent in developing countries. On the other hand, in Mexico and central African countries the genotypes 2 were more found. Genotype 1 and 2 both are infectious exclusively in human populations (Lam et al., 2009). The mostly wide distributed genotype is 3 which has been isolated different places such as Europe, North and south America, Japan and pacific region. Specially the domestic pig was mostly infected except in Africa. On the other hand, genotype 4 has been identified from different countries such as Japan, China, Vietnam and Taiwan. Genotype 3 and 4 have been isolated from several animals eg. domestic pigs, boar, and deer so, this two-genotype known as zoonotic. Recently a new genotype of HEV was detected with splenomegaly syndrome in chicken and suggested as a new genotype 5. Genotype 1 have 5 subtypes1a, 1b, 1c, 1d, and 1e; Genotype 2 has two subtypes: 2a and 2b; on the other hand 3 and 4 genotype are diverse with respectively ten and seven subtypes: 3a, 3b, 3c, 3d, 3e, 3f, 3g, 3h, 3i, and 3j and 4a, 4b, 4c, 4d, 4e, 4f, and 4g (Mushahwar, 2008). The genotypes

and subtypes are very essential to understand the disease distribution, ecology and epidemiology.

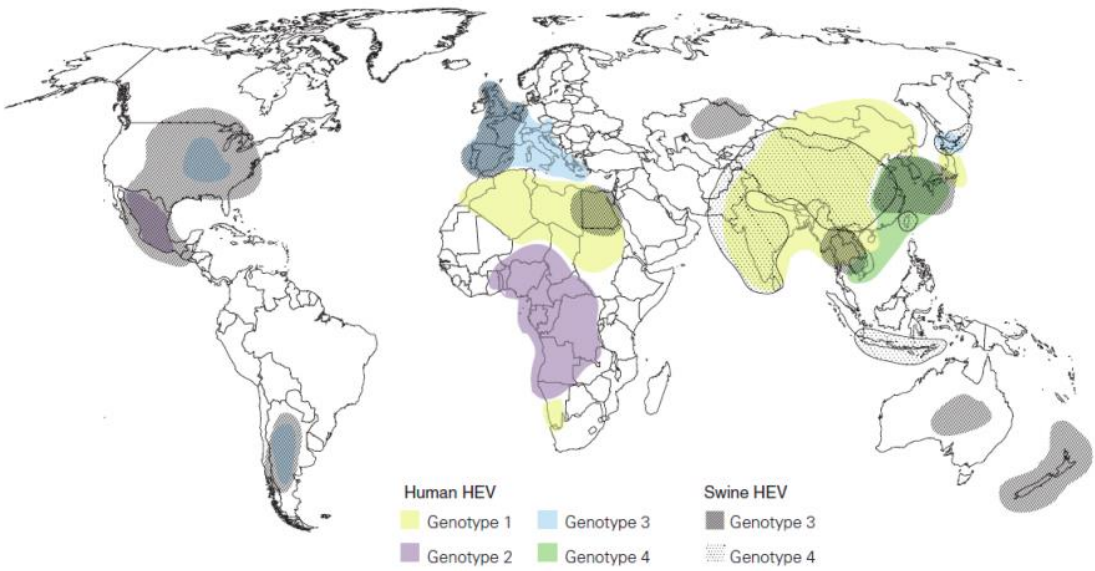

Figure 4: Map showing geographical distribution of hepatitis E virus genotypes

Among (a) human isolates, and (b) swine isolates

2.5 Epidemiology of Hepatitis E virus

The epidemiologies of hepatitis E virus are two different patterns based on geographic locations where prevalence is associated. Many outbreaks have been reported in the developing countries with thousands of infected people. Countries are mainly in south and central Asia, most part of middle east, Mexico and Africa (Aggarwal and Naik, 2009). The main transmission route of infection is fecal-oral transmission which means via contaminated water consumption. Other types of transmission are zoonotic transmission by food, blood transfusion, vertical transmission from mother to children (Navaneethan et al., 2008). The most infection rate was found in fifteen to forty years age group in case of sporadic infection in epidemic regions. In the beginning the manifestation of HEV was very low with self-limiting, icteric hepatitis and very low mortality rate. However, with the time, the attack rate of HEV increased in a level of 20% to 45% specially in third trimester of pregnant women (Khuroo et al., 2009). About 55% fulminant hepatic failure (FHF) were developed in HEV infected women than the non-infected. Maternal death also noticed in higher rate in higher

secondary to FHF (HEV infected) 41% on the other hand 7% in non-hepatitis E infected group (Navaneethan et al., 2008). In case of immune suppressed patients faced chronicity of the diseases specially organ transplanted patient and HIV co-infected persons (Ding et al., 2017). In developing countries high sero-prevalence of hepatitis E virus infection have been reported in the epidemiological studies suggesting poor sanitation and hygiene leads the infection much more frequently and urban areas are mostly vulnerable due to high population density.

2.6 Hepatitis E Virus Infection in Bangladesh

Seasonal factor is the well-known causes for the epidemic of hepatitis E infection in south and south east Asia, especially Bangladesh, India and Nepal. However, the epidemiological study of HEV in Bangladesh has not done broadly. In 2008, a study was conducted by International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) was reported jaundice outbreak in a large population more than 4000 cases in the urban community. It reported high risk of HEV in low income and densely populated areas where mostly mans are working outside and poses risk of illness. on the other hand, high fatality rate was observed in pregnant women with their neonates (Gurley et al., 2014). Some travelers of Bangladesh diagnosed with HEV infections followed by an outbreak of hepatitis E virus in Bangladeshi UN peacekeepers in 1990s (Labrique et al., 2009). Between 2003 and 2005, another study provide the baseline of sero-prevalence of hepatitis E virus in rural areas of Bangladesh where found 22.5% positive and result was similar to other south Asian countries (Labrique et al., 2010). Bangladesh is a land of river and mostly are populated near the urban side. Flood occurs annually and water become contaminated mainly by fecal. The municipal water supply quality is not well thus leading the outbreak of HEV very highly. The poor sanitation is the same risk factor was found in neighboring countries such as India and Nepal. HEV is the most important infection due to high outbreak in the urban areas, death of pregnant women and infant. Better study is needed to find out the burden and endemicity of HEV in Bangladesh.

2.7 Clinical Course and Pathogenesis of HEV Infection

Hepatitis E infection is well known to its clinically quiet course and hibernating nature. The clinical sign and symptoms of HEV are infrequent in case of children but anti-HEV antibodies were commonly detected among the adult person of endemic region though there was no sign of acute hepatitis which indicates the asymptomatic infection of HEV (Aggarwal and Naik, 2009; Wedemeyer et al., 2012). In case of asymptomatic cases, longer incubation period range of 2 to 8 weeks (mean of 40 days), although some person has continuous illness with outstanding cholestatic phenomenon. An experimental study was done with cynomolgus monkeys and infected with HEV, the virus was first isolated from the serum of monkey on the day 9 and after that on day 14 and 23 the titer of the virus was peak on PCR. On day 27 monkeys appeared with anti-HEV IgG serum at the height of ALT elevation and on the day 28 the virus departed (Mushahwar, 2008). The starting symptoms of acute hepatitis E are not specific, its include weakness, nausea, vomiting, jaundice, dark urine, anorexia, flulike myalgia, arthralgia, enlarged tender liver, abdominal pain, and tenderness in addition to increases of liver bilirubin, alkaline phosphatase, transaminases, and γ-glutamyltransferase (Wedemeyer et al., 2012). The infection may increase its severity from sub-clinical to fulminant in infected patients with predominant chronic liver diseases and death rate might be increased during pregnancy 20%-45% (Khuroo et al., 2009). The severity of infection in pregnant women may be due to imbalance of hormone and complexity in immunity in the pregnancy period. Progesterone receptor reduced in hepatitis E infection in pregnant women and T cell response got weaker for a huge viral load which aggravate the death during pregnancy period (Wedemeyer et al., 2012). The emergence of neurotropic HEV variants affected extrahepatic tissue and can causes damage and inflammation of local tissue. The main role of this thing happened by invention of HEVquasispecies in serum and cerebrospinal fluid. However, Better explanation is needed in the system of extrahepatic manifestations by HEV(Kamar et al., 2010).

2.8 Laboratory Diagnosis of HEV Infection

The main routine diagnosis in the laboratory of Hepatitis E is on nucleic acid amplification and serology procedures. The serological assays were most popular for diagnosis of serum antibodies to HEV (IgA, IgM, and IgG). Commercially available diagnosis assay for anti-

HEV antibodies are made of based on recombinant proteins or synthetic peptides which are derived from ORFs2 and ORFs3 (Dawson et al., 1992; Anderson et al., 1999). In case of identifying acute HEV infection or onset of sign and symptoms or degeneration of liver function usually HEV specific IgM serology is useful (Dalton et al., 2008). This technique is useful in case of immediate onset of infection, after 3 months, later detection of strong positive result is very rare sometime it is impossible. There is lot of chance of false positive result (Lin et al., 2000; Takahashi et al., 2005). HEV antigen-specific IgA is consider as a highly specific by many scientists but it is used sometimes as additional confirmatory antibody in the diagnosis purpose of acute hepatitis infection (Mushahwar, 2008). On the other hand, HEV antigen-specific IgG became most popular assay for the diagnosis after the onset of symptoms and continues for years (Dalton et al., 2008). To detect HEV a very useful and feasible ELISA has been established which have the capacity to neutralizing antibodies and particular power to quantify the hormonal immune response against HEV for future vaccine trails and sustainability of the immune response (Mushahwar, 2008). There are two hepatitis E antibody are commercially available: Genelabs IgG Assay and Abbott Immunoglobulin G Assay (Myint et al., 2006). More sensitive and effective assay of diagnosis of HEV RNA from clinical specimens is PCR than serology. Two PCR protocol are available: conventional and real-time (RT-PCR) assays (Dalton et al., 2008). If there is no contamination to confirm the sample as positive further analysis needed including sequencing and genotyping of the infected virus. The critical thing is that, the viral load of HEV in samples stay for a range of 17 to 48 days (mean 28 days). So, there is a big chance to get negative result from the infected patients for the presence of HEV due to narrow window after the onset of illness (Takahashi et al., 2007). Therefore, HEV specific IgM and IgA are the procedure of diagnosis of hepatitis E infection but not that level of IgG. In case of HEV specific IgG the serum transaminase concentrations rises a higher level for identifying HEV so it could be a useful option for diagnosis (Dalton et al., 2007).

2.9 Treatment and Prevention of Hepatitis E virus

Hepatitis E is one of the most considerable reasons for the high morbidity and mortality of peoples in the developing countries so effective treatment and prevention measures need to be taken. Body immune response act as an important factor for hepatitis E infection though it is a self-limiting disease. The immune-competent persons do not require any treatment for this because active immune system is able to low the virus load in circular system. Treatment

procedure for chronic patient include treatment of ribavirin or pegylated interferon alfa and reduction of immunosuppression (Wedemeyer et al., 2012). Some parents have pre-existing hepatitis and chronic liver disease, those patients should follow liver transplantation but outcome is grave (Dalton et al., 2008). Implementation of proper sanitary measures are necessary in the developing world where the infection is sporadic to avoid transmission of HEV virus among the peoples via fecal-oral route. Better treatment and proper disposal of waste and human excreta, supplementation of pure drinking water are some protective measures for the infection. To avoid foodborne zoonotic transmission, proper sanitation of food, avoid consumption of uncooked or undercooked food is necessary. HEV is heat labile virus so appropriate heating can destroy organisms from food (Aggarwal and Naik, 2009). Another effective preventive measure is passive immune prophylaxis which has been proved of neutralization of virus after inoculation of HEV into laboratory animals so, it is a protective measurement. However, in some cases of human studies this protective measure failed to prevent protectivity against the disease. Effective and main target of vaccine development in the world is ORF2 (Zhang et al., 2015). Consequence of HEV infection is chronic liver disease in patients and targeted prevention of those individuals is vaccination. However, some records provide evidence of failure of cellular immune response from vaccinated personals for the mutation of ORF2 (van Tong et al., 2016). Therefore, the main challenge of producing effective vaccines is the mutation of ORF2 protein structure. In addition to diverse number of mutations taking place in HEV attenuation (e.g., L477T, L613T, HVR, F51L, T59A, S390L and N562Q/D/P/Y). Live attenuated vaccines against hepatitis E virus developed based on mutation (Zhang et al., 2008; Pudupakam et al., 2009; Córdoba et al., 2011). To develop an effective vaccination program some other factors needs to be considered such as protection duration should be long term, cost effective, suitable for pregnant women and young children (Mushahwar, 2008). Therefore, the control and eventual elimination of this disease is very attainable if highly effective vaccine is developed.

Chapter 3: Materials and Methods

3.1 Description of the study areas

Chattogram is the commercial capital of Bangladesh and located southeastern part of Bangladesh. The district's area is 5282.98 sq km and lies between 21°54' and 22°59' north latitudes and 91°17' and 92°13' east longitudes. It is bounded by Khagrachhari and Rangamati districts and Tripura state of India on the north, Cox's Bazar district on the south, Bandarban, Rangamati and Khagrachari districts on the east and Noakhali district and the Bay of Bengal on the west.

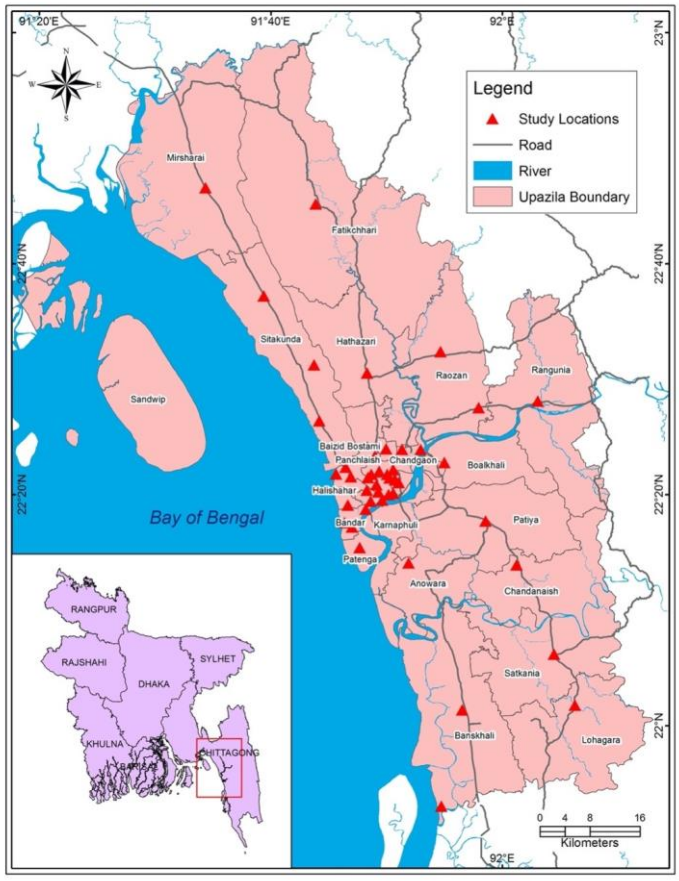

Figure 5: Map of the study site

Chattogram is subdivided into 18 Administrative locations or upazillas and Chattogram Metropolitan Area. The average temperature of this district ranges from 13º to 32ºC, humidity 70 to 85%. The average rainfall varies from 5.6 mm to 727.0 mm. The total human population of Chattogram district is around 76,16,352 and population density of Chattogram

city corporation is 20,190 person per square kilometer. Population and housing census, national Vol. 3, urban area report, Bangladesh Bureau of Statistics. Ministry of Planning. Government of the People's Republic of Bangladesh, Dhaka, Bangladesh.) Overcrowded and densely populated city always face public health challenges and outbreak due to different viral infections. Due to geographical position, the water level in Chattogram district is very lower. To meet up the requirement of water Chattogram Water Supply and Sewerage Authority (CWASA) is supplying water through its distribution network after treatment of water from the Halda River and underground water sources (Zuthi et al., 2009).

The ArcGIS map software (Version 10.2; Environmental System Research Institute, Redlands, CA, USA) was used to display the study area from where the patients come to the hospital for their treatment.

3.2 Study design

A cross sectional study was done in 10 medical hospital of Chattogram to investigate the sero-prevalence of hepatitis E virus in Chattogram. Hospital record book was used to select the patients for this study. Sample was selected according to the case definition.

3.3 Case definition

Suspected patients were chosen by case definition of Hepatitis. Suspected patients were further referred for the serological test to the laboratory for the confirmation of Hepatitis E. Following signs were considered to suspect a patient as Hepatitis:

- Fever persist for 2 to 10 days
- Headache
- Retro orbital pain
- Myalgia
- Arthralgia/severe backache/ bone pains
- Rash
- Bleeding manifestations (Epistaxis, hematemesis, bloody stools, menorrhagia, hemoptysis)
- Abdominal pain
- Decreased urinary output despite adequate fluid intake

- Irritability in infants

3.4 Study period

All hospital data of the suspected patients and laboratory results were collected over the period of twelve months (January to December) in 2018.

3.5 Sample collection

A total of 505 patients were selected from 10 hospitals of Chattogram Districts for the period of 01 year January and December 2018. Blood samples were collected in EDTA vacutainer tubes during the period of one to two weeks of the onset of clinical symptoms. Blood samples were centrifuged at 3000 rpm for 10 minutes to separate the serum. Sterile 1.5 ml Eppendorf tubes were used to store the individual serum sample in 4˚C for further analysis.

3.6 Data Collection

A detailed data was collected in predetermined questionnaire from the patient at the time of registration and sample collection. The questions were aimed to see the association between the variable and hepatitis E virus in Chattogram. The variables were Season, Location, Outbreak area, Sex, Age, Source of water, Boiling of water. Season were divided into three types (Summer: March to June; Rainy: July to October and Winter: November to February) (Joshi et al., 2009). Statistical Pocket Book of Bangladesh, (Statistics Division, Government of the People's Republic of Bangladesh). Location is subdivided into 3 areas (Chattogram City corporation, Halishahar: Hepatitis E outbreak area and Outside Chattogram city corporation). There were two types of location: outbreak and non-outbreak area. There were four type of age categories patients (1 to 20 years, 21 to 40 years, 41 to 60 years and 61 to older). People of Chattogram drink water from three different water sources (Deep tubewell, Shallow tubewell and supplied water by WASA). Some people use to drink water by boiling and some are not.

3.7 Serological Analysis

Standard serological procedure used to detect hepatitis E virus using commercially available ELISA kits. Every ELISA kit has individual manufacturer’s manual. Different types of

serological tests were done in this study. Those were: detection of anti HAV IgM using WANTAI HAV-IgM ELISA diagnostic kit (Beijing Wantai Biological Pharmacy Enterprise Co., Beijing, CN), detection of HBsAg using bioelisa HBsAg 3.0 (BIOKIT, S.A., Barcelona, Spain), detection of anti-HCV antibodies using bioelisa HCV 4.0 (BIOKIT, S.A., Barcelona, Spain), detection of anti-HEV IgM using WANTAI HEVIgM ELISA diagnostic kit (Beijing Wantai Biological Pharmacy Enterprise Co., Beijing, CN) and detection of anti-HEV IgG using HEV-IgG ELISA diagnostic kit (Beijing Wantai Biological Pharmacy Enterprise Co., Beijing, CN). The sample were analyzed further very carefully if the result found positive for both hepatitis E IgM and IgG or HEV IgM or HEV IgG. Hepatitis E virus positive sample were evaluated and correlated clinically and categorized as acute viral hepatitis and acute liver failure patients. All positive samples were stored in -4°C for further analysis for the presence of HEV RNA.

3.7.1 Anti-HEV IgG and IgM ELISA Procedure

WANTAI HEV-IgG ELISA employed solid phase, indirect ELISA method for detection of IgG-class antibodies to HEV (anti-HEV) in two-step incubation procedure. The wells were first prepared by marking three wells for negative control, two wells for positive control and one well for blank. Then, 100µL of the specimen diluent was added into each well except the blank. 10 microliter of each positive control, negative control and specimen were added to their respective wells. The plate was then covered and incubated for 30 minutes at 37°C. At the end of the incubation, the plated cover was removed and the each of the wells was washed 5 times with Wash Buffer, allowing the microwells to soak for 30-60 seconds after every wash. After the final wash cycle the plate was turned down onto a paper towel and tapped to remove any remainders. Then, 100µL of HRP-Conjugate was added into each well except the blank. The plate was covered and incubated for 30 minutes at 37°C. At the end of the incubation, the wells were again washed 5 times with Wash Buffer, allowing the microwells to soak for 30-60 seconds after every wash. At the end of the wash cycle, the plate was turned down onto a paper towel and tapped to remove any remainders. For coloring, Chromagen A and Chromagen B solutions, 50µL each, was added into each well including the blank. The plate was incubated at 37°C for 15 minutes, making sure that light is avoided. The enzymatic reaction between the Chromagen solutions and the HRP- Conjugate produced blue color in positive control and in HEV IgG positive sample wells. The reaction was stopped by adding 50µL of Stop Solution into each well. The solution turned yellow in

Positive control and HEV IgG positive sample wells. The plate reader was then calibrated with the blank well and the absorbance was read at 450nm. The cut-off value was calculated using the following formula: Cut-off value (COV) =NC+ 0.26; where NC is the mean absorbance value for three negative controls. If the Sample Optical Density (SOD) was less than the COV then the sample was considered IgG negative but if the SOD was more than the COV the sample was considered to be IgG positive.

The WANTAI HEV-IgM ELISA employed the exact same procedure (as that of WANTAI HEV-IgG ELISA) for the detection of anti-HEV IgM from the serum samples of the patients in this study except anti-human IgM antibodies (anti-IgM) conjugated to horseradish peroxidase (HRP-conjugate) was used in this procedure.

3.7.2 Anti-HAV IgM ELISA Procedure

WANTAI HAV-IgM ELISA is a solid-phase, two-step incubation antibody capture ELISA assay in which, polystyrene microwell strips are pre-coated with antibodies directed to human immunoglobin M proteins (anti-μ chain). At first, the wells were prepared by marking three wells for Negative control, two wells for Positive control and one well for Blank. The Specimen was diluted 1:1000 with normal saline. Then, 50μL of Positive and Negative Control and 100μL of the Specimen was added to their respective wells except the Blank and mixed by tapping the plate gently. The plate was covered and incubated for 20 minutes at 37°C. At the end of the incubation, the plate cover was removed and the each of the wells was washed 5 times with diluted Wash Buffer, allowing the microwells to soak for 30-60 seconds after every wash. After the final wash cycle the plate was turned down onto a paper towel and tapped to remove any remainders. Then, 100μL of HRP-Conjugate was added into each well except theBlank. The plate was covered and incubated for 40 minutes at 37°C. At the end of the incubation, the wells were again washed 5 times with Wash Buffer, allowing the microwells to soak for 30-60 seconds after every wash. At the end of the wash, the plate was turned down onto a paper towel and tapped to remove any remainders. For coloring, Chromagen A and Chromagen B solutions, 50μL each, was added into each well including the Blank. The plate was incubated at 37°C for 15 minutes, making sure that light was avoided. The enzymatic reaction between the Chromagen solutions and the HRP- Conjugate produced blue color in the positive control and in the HAV IgM positive sample wells. The reaction was stopped by adding 50μL of Stop Solution into each well. The solution turned yellow in Positive control and HEV IgG positive sample wells. The plate reader was then

calibrated with the Blank well and the absorbance was read at 450nm. The cut-off value was calculated using the following formula: Cut-off value (COV) =NC+ 0.16; where NC is the mean absorbance value for three negative controls. If the Sample Optical Density (SOD) was less than the COV then the sample was considered HAV IgM negative but if the SOD was more than the COV the sample was considered to be HAV IgM positive.

3.7.3 Anti-HCV IgG and IgM ELISA Procedure

"bioelisa HCV 4.0" was an immunoenzymatic method in which the wells of a microplate were coated with recombinant antigens representing epitopes of HCV: Core, NS3, NS4 and NS5. The number of strips was chosen as required for the test (Table 1). At first, 8 wells were reserved for blank and controls. The sample diluents were then added at 200µl of volume in rest of the wells and 10µl of each sample was added to the designated wells. If the antibodies specific for HCV were present in the samples, they formed stable complexes with the HCV antigens on the wells. Then, 200µl of negative control were transferred into each of 2 wells, 200µl of low positive control into each 3 wells and 200µl of positive control into each of 2 wells and one well was left empty for the substrate blank. The microplate was then covered with an adhesive seal and incubated for 1 hour at 37°C. After the incubation, the adhesive seal was removed and discarded. The contents of the wells were aspirated and were filled completely (approximately 350µl) with the washing solution. The process of aspiration and washing was repeated 5 more times allowing each column of wells to soak for at least 15 seconds before the next aspiration cycle. After the last washing, the microplate was blotted on a pad of absorbent tissue to remove any excess liquid from the wells. Then, 100µl of diluted conjugate (rabbit anti-human IgG conjugated with peroxidase) was transferred into each well except the one reserved for the substrate blank. The conjugate would bind to any antigen-antibody complexes formed. Any formation of bubbles was avoided upon addition of the diluted conjugate. The plate was againcovered with an adhesive seal and incubated for 30 minutes at 37°C. During the last 5-10 minutes of this incubation the substrate-chromogen solution was prepared. If the entire plate was used 280µl of chromogen (TMB) was added to the bottle containing the substrate buffer (14ml) and were mixed well. If the entire plate was not used, then (Table 1) was followed. The final solution was colourless. After the incubation, the adhesive seal was removed and discarded. The contents of the wells were aspirated and were filled completely (approximately 350µl) with the washing solution, repeating this process 5 more times, allowing each column of wells to soak for at least 15

seconds before the next aspiration cycle. Then, 100µl of the substrate-TMB solution was added to each well, including the blank. The plate was incubated for 30 minutes at room temperature (20-25°C). This solution would develop a blue colour if the sample was positive. The blue colour changed to yellow after blocking the reaction by adding 100µl of stop solution (sulphuric acid) in the same sequence and time intervals as for the substrate-TMB. The plate reader was then calibrated with the Blank well and the absorbance was read well within 30 minutes at 450nm. The intensity of colour was proportional to anti-HCV antibodies concentration in the sample. Wells containing negative samples remained colourless. The mean absorbance of the low positive control (LPCx) was calculated. The obtained value, multiplied by 0.9, was the cut-off value. Therefore, Cut-off = LPCx x 0.9; the absorbance of the sample was then divided by the cut-off value. If the samples were positive, the ratio absorbance/cut-off should be greater than or equal to1.0. If the samples were negative then the ratio absorbance/cut-off should be less than 0.9. If equivocal, then the ratio absorbance/cutoff should be greater than or equal to 0.9 but less than 1.0.

Table 1:Usage of specific volumes of conjugate diluents and concentrate conjugate according to the number of strips

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

3.7.4 HBsAg ELISA Procedure

"bioelisa HBsAg 3.0" is a direct immune-enzymatic method of the "sandwich" type in which guinea pig anti-HBs antibodies are coated to microplate wells and act as the capture antibody. The numbers of strips were chosen as required for the test (Table 1). At first, three wells were marked for Negative control, one well for Positive control and one well for Blank which was left empty. Then, 100µl of each control, positive and negative, were transferred to their assigned wells. Then 100µl of each of the samples to be analyzed were transferred to the corresponding wells. The plate was then covered with an adhesive seal and incubated for 1 hour at 37°C. If the sample contained HBsAg, the antigen would bind to the antibody on the

plate. After the incubation, the adhesive seal was removed and discarded. The contents of the wells were aspirated and were filled completely (approximately 350µl) with the washing solution. The process of aspiration and washing was repeated 3 more times allowing each column of wells to soak for at least 15 seconds before the next aspiration cycle. Then, 100µl of diluted conjugate (goat anti-HBs antibodies marked with peroxidase) was transferred into each well except the one reserved for the substrate blank. The plate was again covered with an adhesive seal and incubated for 30 minutes at 37°C. The conjugate would react with the antigen-antibody complex if any formed in the first incubation. During the last 5-10 minutes of this incubation the substrate-chromogen solution is prepared. If the entire plate was used 280µl of chromogen (TMB) was added to the bottle containing the substrate buffer (14ml) and mixed well. If the entire plate was not used, then Table 1 was followed. This working substrate solution was pink in colour and if changed to blue at this step then it was discarded. After the incubation, the adhesive seal was removed and discarded. The contents of the wells were aspirated and were filled completely (approximately 350µl) with the washing solution, repeating this process 3 more times, allowing each column of wells to soak for at least 15 seconds before the next aspiration cycle. Then, 100µl of the substrate TMB solution was added to each well, including the blank. The plate was incubated for 30 minutes at room temperature (20-25°C). This solution would develop a blue colour if the sample was positive for HBsAg. The blue colour changed to yellow after blocking the reaction by adding 100µl of stop solution (sulphuric acid) in the same sequence and time intervals as for the substrate-TMB. The plate reader was then calibrated with the Blank well and the absorbance was read well within 30 minutes at 450nm. The intensity of colour was proportional to the amount of HBsAg in the test specimens. The wells containing negative samples remained colourless. The mean absorbance of the negative control (NCx) was calculated. The obtained value must be less than 0.120, after subtracting the blank. The absorbance value obtained for the positive control (PC) should be equal to or higher than 0.700, after subtracting the blank. The presence or absence of HBsAg in the samples was then determined by relating the absorbance value of each sample to the cut-off value. The cut-off value was calculated by adding 0.040 to the mean absorbance of the negative control (i.e. Cut-off = NCx + 0.040). The absorbance of the sample was then divided by the cut-off value. If the samples were positive, the ratio absorbance/cut-off should be greater than or equal to1.0. If the samples were negative, then the ratio absorbance/cut-off should be less than 0.9. If equivocal, then the ratio absorbance/cut-off should be greater than or equal to 0.9 but less than 1.0.

3.8 Statistical analysis

Data from questionnaire and laboratory testing were entered, collated, coded and stored in Microsoft Excel spreadsheet version 2016. For statistical analysis, statistical software: STATA/IC 13 (StataCorp 4905, Lakeway Drive, College Station, Texas 77845, USA) was used.

3.8.1 Descriptive analysis

Prevalence of hepatitis E virus was calculated using positive samples divided by the total number of samples tested and the results were expressed as a percentage with 95% confidence interval (CI). Month wise prevalence of the microorganism was calculated to observe the outbreak. Prevalence of hepatitis e virus according to the sex within the month were presented by bar diagram.

3.8.2 Risk factor analysis

Univariable analysis were conducted for each of the independent variables (potential risk factors collected in the questionnaires) against the dependent variable (infection status of the patient). Chi square test was done to identify significant risk factors for the presence of hepatitis e virus. The variables: season, location, outbreak area, sex, source of water and boiling of water were treated using chi-square test for the prevalence of hepatitis e virus.

3.8.3 Logistic regression model

All variables ($p<0.2$) were forwarded to logistic regression model after chi square test. The variable: location was omitted due to collinearity. After adjusting the factor, season, outbreak area, sex, age, source of water, boiling of water were found as significant risk factor. Confounder was checked by considering the coefficient variation more than 10%. The validity of the model checked by Receiver operating characteristic curve (ROC) and goodness of fit test (lfit) (Dohoo et al., 2003). The result of multiple logistic regression model expressed by odds ratio (OR), 95% confidence interval and P value.

Chapter 4: Result

To accomplish the goal of the study the data were taken from ten different hospitals of Chattogram District over a period of 1 year. Hepatitis E virus patient and sample were selected according to the case definition. A total of 505 patients were selected over 1 year and serum samples were tested for Hepatitis E virus and 177 samples showed positive against HEV. The prevalence of Hepatitis E virus in Chattogram was 35.05% with (95% CI: 30.9-39.4).

Samples were tested both outbreak and non-outbreak area. The prevalence of hepatitis E virus from outbreak area was higher 73.4% (n=80) with (95% CI: 64.1-81.4) and the prevalence from non-outbreak area was 24.5% (n=97) with (95% CI: 20.3-29).

4.1 Prevalence of Hepatitis E virus in Chottagram

In the outbreak season (June, July and August) the prevalence was higher than the other month which create a epidemic curve. Among different months, the prevalence of HEV was highest in July 70.4% with (95% CI : 58.4-80.7) followed by June 51.7% with (95% CI : 38.4-64.8) and August 40% with (95% CI : 28-52.9). The prevalence of HEV in September and October was similar 28.6%. The lowest prevalence of HEV in 2018 was in January 14.3%. **(Graph 1)**

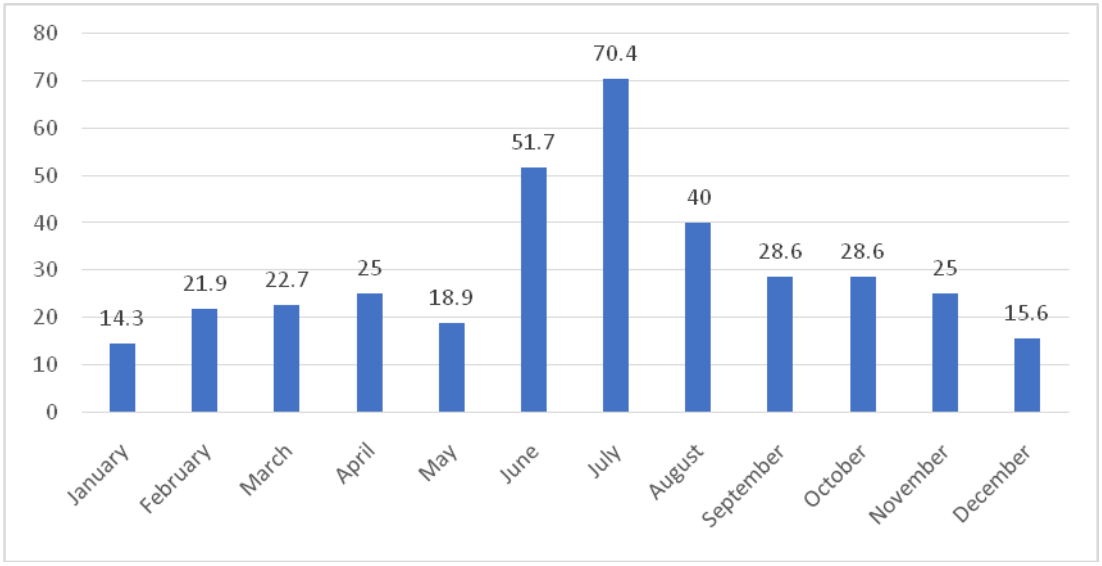

Figure 6: Prevalence of Hepatitis E virus by month

4.2 Prevalence of Hepatitis E virus month wise by Sex

The prevalence of Hepatitis E virus was found higher in male throughout the year. Highest infection found in July both in male and female as 39.44% and 30.99% respectively. In male, infection of HEV found higher in June, August, November and March as 35%, 27.69%, 25% and 22.86% respectively. The lowest was found as 9.38% in December . On the other hand, in the month of March and November, no infection of hepatitis E virus in female patients. Moderate infection was recorded in female in June (16.67%), August (12.31%), September (14.29%) and October (11.43%).(Figure: 7)

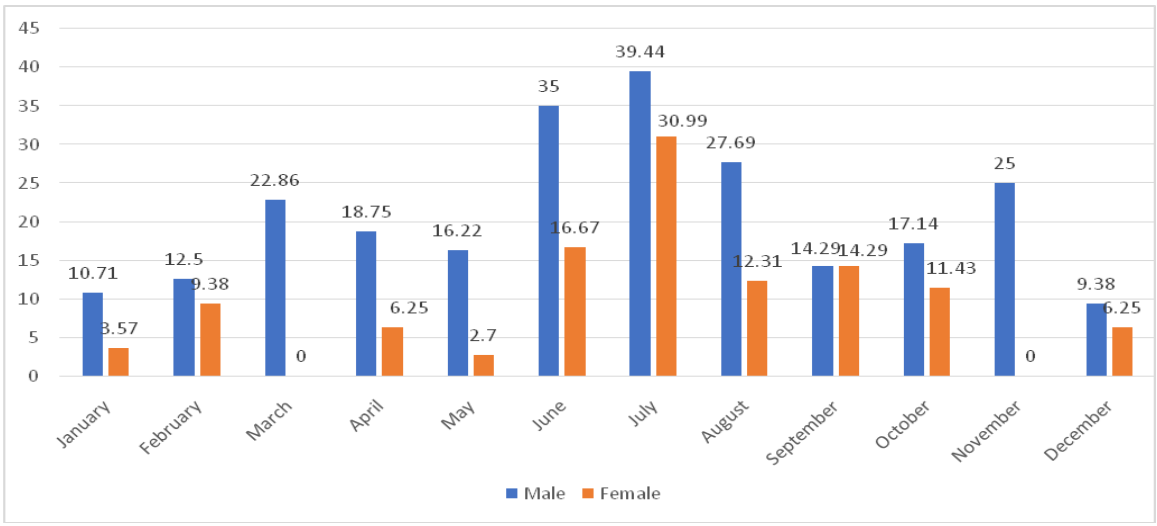

Figure 7: Prevalence of Hepatitis E virus month wise by sex

4.3 Risk factors associated with Hepatitis E infection in Chattogram

To know the association of different variables with the presence of Hepatitis E virus, first univariate analysis was performed by chi square test. P value <0.05 was considered as significant.

Season

The prevalence of Hepatitis E virus was found significantly higher (p=0.000) in rainy season (46%; 95% CI: 29.2-52.9) than the summer (32.9%; 95% CI: 25.8-40.7) and winter season (19.5%; 95% CI: 13.1-27.4). (Table 2)

Location

In case of location the seroprevalence of Hepatitis E virus was significantly higher (P=0.000) in Halishahar (73.4%; 95% CI: 64.1-81.4) region where outbreak occurred whereas other parts of Chattogram city corporation and outside city corporation was 34.9% and 16.3% respectively.

Table 2: Univariate analysis of Hepatis E virus in Chattogram district

Variables	Categories	Positive number	Prevalence (%)	95% CI	P value
Season	Summer (164)	54	32.9	25.8-40.7	0.000
	Rainy (213)	98	46	39.2-52.9	
	Winter (128)	25	19.5	13.1-27.4	
Location	CTG city corporation (175)	61	34.9	27.8-42.4	0.000
	Halishahar (109)	80	73.4	64.1-81.4	
	Outside city corporation (221)	36	16.3	11.7-21.8	
Outbreak area	Yes (109)	80	73.4	64.1-81.4	0.000
	No (396)	97	24.5	20.3-29	
Sex	Male (287)	118	41.1	35.4-47	0.001
	Female (218)	59	27.1	21.3-33.5	
Age	1-20 years (86)	27	31.4	21.8-42.3	0.189
	21-40 years (280)	98	35	29.4-40.9	
	41-60 years (111)	46	41.4	32.2-51.1	
	61-older (28)	6	21.4	8.3-40.9	
Source of water	Deep tubewell (101)	27	26.7	18.4-36.5	0.000
	Shallow tubewell (217)	36	16.6	11.8-22.2	
	Supplied water (WASA) (187)	114	60.9	53.6-67.9	
Boiling of water	Yes (85)	5	5.9	1.9-13.2	0.000
	No (420)	172	40.9	36.2-45.8	

Outbreak area

The investigation shows the prevalence was higher in outbreak area (73.4%) and lower in non-outbreak area (24.5%). The findings documented a significant association (p=0.000) between the outbreak area and hepatitis E virus infection.

Sex

The findings of the study revealed a highly significant association (p=0.001) between the male patient and Hepatitis E infection. Male was found highly susceptible (41.1%; 95% CI: 35.4-47) than the female (27.1%; 95% CI: 21.3-33.5)

Age

There was no significant differences found in different age group for Hepatitis E virus. However age group of 41 to 60 years found to be highly infected (41.4%; 95% CI: 32.2-51.1) than the other age group.

Source of water

The present findings documented a significant ($p<0.05$) association between the source of the water and hepatitis E infection. People drinking supply water from WASA have got higher rate of infection of Hepatitis E (60.9%; 95% CI: 53.6-67.9) followed by shallow tube well and deep tube well.

Boiling of water

In case of water treatment, people drinking water with no treatment (40.9%, 95% CI: 36.2-45.8) was significantly (p=0.000) higher infected than the people having water after treating with boiling (5.9 %, 95 % CI: 1.9-13.2).

Table 3: Multivariate analysis of Hepatitis E virus infection in Chattogram

Variables	Categories	prevalence	Odds ratio	95% CI	P value
Season	Winter (128)	19.5	1		
	Summer (164)	32.9	1.1	0.5-2.2	0.79
	Rainy (213)	46	2.2	1.1-4.1	0.02
Outbreak area	No (396)	24.5	1		
	Yes (109)	73.4	2.8	1.4-5.6	0.005
Sex	Female (218)	27.1	1		
	Male (287)	41.1	2.5	1.5-4.1	0.001
Age	1-20 years (86)	31.4	1		
	21-40 years (280)	35	1.4	0.7-2.8	0.405
	41-60 years (111)	41.4	3.3	1.5-7.4	0.003
	61-older (28)	21.4	0.5	0.1-1.7	0.266
Source of water	Shallow tube well (217)	16.6	1		
	Deep tube well (101)	26.7	1.9	1.0-3.7	0.03
	Supplied water (WASA) (187)	60.9	13.5	6.8-26.7	0.000
Boiling of water	Yes (85)	5.9	1		
	No (420)	40.9	54.6	18.5-161.6	0.000

Result of the full reduced (only including factors identified in univariate analysis $p<0.2$) model of multivariate regression analysis is presented in Table 3. The prevalence of hepatitis E virus was 2.2 time higher (OR=2.2, 95% CI: 1.1-4.1) in rainy and 1.1 time higher (OR=1.1; 95% CI: 0.5-2.2) in summer than the winter season. In case of outbreak area, the prevalence of HEV was 2.8 time higher (OR=2.8, 95%CI: 1.4-5.6) than the non-outbreak area. In case of age category, the odds of occurring infection with hepatitis E virus in the age group 41-60 years was 3.3 times higher than 1-20 years age group. The odds of HEV was significantly higher in supplied water of WASA (OR=13.5, 95%CI: 6.8-26.7) than the shallow tube well.

In case of treatment of water, non-boiled water (OR=54.6, 95%CI: 18.5-161.6) was significantly higher on HEV in Chattogram than the boiled water.

Chapter 5: Discussion

Bangladesh is one of the densely populated countries with considerable poverty and poor hygienic conditions, thereby providing all the favorable conditions for developing a wide range of infections, among which Hepatitis E virus (HEV) infection is of extensive burden.

The clinical characteristics of HEV infection range to acute, subacute and chronic hepatitis with crucial complications such as jaundice, liver failure, neurological disorders and chronic hepatic syndromes (Kamar et al., 2012). Urban residents are often exposed to HEV infection due to the clogged places for the living of heavy population density and poor quality of drinking water supplied by municipal or city corporations. Numerous human fatality cases were detected from urban areas of Bangladesh by HEV causing the outbreak of jaundice. Largely are confirmed pregnant women, their neonates or women of reproductive age which pregnancy was not confirmed (Gurley et al., 2014). Acute hepatitis E infection in Bangladesh was first recorded in a patient of Netherland who was traveled in Bangladesh and got infection through contaminated food (Zaaijer et al., 1993). Some Bangladeshi peacekeepers were diagnosed with acute hepatitis E infection during the peace mission in Haiti and presented great attention of HEV in Bangladesh (Drabick et al., 1997). There are some other history of infection with HEV with the patients of Japan, Spain, and Italy who are travelled in Bangladesh (Sanayama et al., 2008; Fogeda et al., 2009; La Rosa et al., 2011; Romanò et al., 2011)

The current study provides some substantial insights into the sero-prevalence of hepatitis E virus and some risk factors associated with it in Chattogram. The study revealed the prevalence of Hepatitis E virus in Chattogram was 35.05% which is somewhat higher than the previous study from southern part of Bangladesh with 22.5% prevalence (Labrique et al., 2009) and on the other hand comparatively lower than a previous study with 63.6% of patients was detected with Anti-HEV immunoglobulin M (Sheikh et al., 2002). In comparison to the countries, present study not correspond with the result of other neighboring countries where children of Chennai, India showed fluctuated positivity between 5.3 to 16.7%, higher prevalence found in Egypt (84.3%), however lower in Pakistan (12.2%) (Mohanavalli et al., 2003; Stoszek et al., 2006; Control and Prevention, 2011). High

prevalence suggests extensive exposures of the community and horizontally transmitted throughout the areas.

The study was conducted in the Hepatitis E outbreak area (Halishahar, Chattogram) and found consistent result as before where they reported as 77% IgM antibodies to HEV in urban community in Dhaka, Bangladesh with jaundice and maternal fatality (Gurley et al., 2014). The high population density along with other risk factors including contaminated drinking water supply through municipal, poor hygienic management might be a greater risk of contagion who worked outside.

Month-wise report indicates the epidemic curve of HEV which express the outbreak of the virus, most proportion of patients were come into the hospital with sign of jaundice and diagnosed as HEV positive in the three-progressive month June, July and August. Similar outbreak was appeared in India by the month of June-July in 2010 due to sewage contamination of drinking water (Majumdar et al., 2013).

During the study, the prevalence of HEV was higher in male than the female which is correspond to the previous long-term prospective study finding from Italy (Romanò et al., 2011) and contrast to the epidemiological study of hepatitis E in China where infection rate and fatality was significantly higher in female (Zhuang et al., 1991). This may be due to more activity of male person who work outside and movement a lot in different parts of the city. They might expose to the infection source during food intake through contaminated drinking water.

Seasonality plays a significant role in the transmission of hepatitis E virus too. Present study revealed that, the higher prevalence in rainy season (46%) than that of other seasons, because the main source of transmission of HEV is water and the possibility of water stagnation become high in rainy season in urban Chattogram. Moreover during rainy season, the source of drinking water can contaminate easily and spread hepatitis E, (Shrestha et al., 2003)

Previous study suggest that, acute hepatitis in different age group were somewhat same prevalence in Nepal (Shrestha et al., 2003) but a typical scenario has been found in Egyptian pregnant women where sero-prevalence of hepatitis E was higher in more than 30 years of age group (Stoszek et al., 2006). Egyptian results supported the present study with increases

of HEV IgG infection with the age but totally different result found in (Clayson et al., 1997). The increases of HEV IgG infection with the advance of age might be interpreted due to the decrease of immunity level against the virus. In addition further workload and stress aggravates the level of infection.

One of the factors in Bangladesh especially in the big cities (such as Dhaka and Chattogram) are contaminated water linked with poor domestic and personal hygiene. The finding suggested that water from shallow and deep tube well was safer than from the supplied water or taps water from WASA. Contamination of HEV occurred in different section of the supply chain, originating pump and at spigots in households are responsible for the transmission of HEV. Previously reported hepatitis E outbreaks have always interconnected disease transmission to fecal contamination of the municipal water system and spread microorganisms to a great extent of the population. So, HEV infection was significantly associated with supplied water for drinking by the municipal.

This outbreak of Hepatitis E can be traced to contamination of drinking water supplies with human fecal matter since the virus is known to spread enterically via the fecal-oral route. The contaminated drinking water may include water that has not been boiled or filtered, and it has been observed from our study that only 16.8% of the study participants consumed boiled or filtered water while the rest consumed the municipal-supplied water or water from the tube well which are known to be sources of hepatitis infection, also confirmed in a recent study where HEV infection was significantly associated with drinking water from the above mentioned drinking water sources (Gurley et al., 2014). The contamination of these drinking water sources was either due to poorer water treatment or placement of the tube wells at insufficient distance from latrines or sewage-contaminated ponds or tanks (Islam et al., 2001). Hygiene awareness and knowledge of the links between poor hygiene and disease are lowest among the typically poorly educated people. Most of the people lived in slum of the urban area had either education until the primary level or no education at all. Enteric infection may be increased and transmitted into the children due to fewer sanitation practices.

Chapter 6: Conclusion

Hepatitis E virus infection is a global concern and one of the most important public health threats mainly by drinking water and unhygienic condition. The current study represents the circulating of Hepatitis E virus in Chattogram District of Bangladesh. This appears to be one of the first few studies to evaluate the sero-prevalence of HEV from Chattogram, Bangladesh. The study provided the result of 35.05% sero-prevalence of HEV in the year of 2018 and surprisingly higher prevalence 73.4% in outbreak area. Some variables are attributable to the presence of hepatitis E virus such as rainy seasons play a vital role for contamination of the source of drinking water. The geographical features and climate conditions of this country leads the infection rate. Drinking water from municipal supply source is the important factor for transmission of HEV and those who boiled water prior to consumption became safe from it. Quarantine is an effective measure of prevention but in case of HEV it is impossible due to high rate of subclinical infection. However, public health measures such as the provision of safe drinking water, improvement of sanitary and sewerage systems and development of mass awareness to the prevailing situation are absolutely necessary.

.

Chapter 7: Limitation

The study has several potential limitations. The study was conducted only with ten selective hospital and hospitalized patients, so there is ultimate chance to miss hepatitis E infected patients those are diagnosed other hospital or clinics or some patients might not come to the hospital. Due to taking only data from hospital, the result not represents the actual prevalence of HEV. Though all the data were collected from hospital record books there are missing of important variables those might me associate with the infection and outbreak. In case of testing procedure, only ELISA was used but in modern age polymerase chain reaction (PCR) is more authentic. Budget and time were the main constraint.

Chapter 8: Recommendations

Hepatitis E virus remains a substantial cause of morbidity and mortality worldwide, predominantly in the developing countries. Densely populated areas are prone to be infected and outbreak site due to lots of interaction and unhygienic conditions. Infected person should be quarantine and referred to the hospital for proper treatment. Hepatitis E virus spread through the contaminated water so, people should be aware with drinking water. Supplied water from municipal should be boiled with proper heat or filtered prior to drink. Sanitation system of developing country is much poorer than the developing world. To get rid from the viruses there is no other option than the proper sanitation and hygiene management. Vaccination might be another way of prevention and control of hepatitis virus.

References

Aggarwal, R., 2010. Hepatitis E virus and person-to-person transmission. Clinical Infectious Diseases 51, 477-478.

Aggarwal, R., Naik, S., 2009. Epidemiology of hepatitis E: current status. Journal of Gastroenterology and Hepatology 24, 1484-1493.

Aggarwal, R., Naik, S.R., 1994. Hepatitis E: intrafamilial transmission versus waterborne spread. Journal of Hepatology 21, 718-723.

Albinana-Gimenez, N., Clemente-Casares, P., Bofill-Mas, S., Hundesa, A., Ribas, F., Girones, R., 2006. Distribution of human polyoma-viruses, adenoviruses, and hepatitis E virus in the environment and in a drinking-water treatment plant. Environmental SDcience and Technology 40, 7416-7422.

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

Batts, W., Yun, S., Hedrick, R., Winton, J., 2011. A novel member of the family Hepeviridae from cutthroat trout (Oncorhynchus clarkii). Virus Research 158, 116-123.

Berto, A., Grierson, S., Hakze-van der Honing, R., Martelli, F., Johne, R., Reetz, J., Ulrich, R.G., Pavio, N., Van der Poel, W.H., Banks, M., 2013. Hepatitis E virus in pork liver sausage, France. Emerging Infectious Diseases 19, 264.

Berto, A., Martelli, F., Grierson, S., Banks, M., 2012. Hepatitis E virus in pork food chain, United Kingdom, 2009–2010. Emerging Infectious Diseases 18, 1358.

Bihl, F., Negro, F., 2009. Chronic hepatitis E in the immunosuppressed: a new source of trouble? Journal of Hepatology 50, 435-437.

Boccia, D., Guthmann, J.-P., Klovstad, H., Hamid, N., Tatay, M., Ciglenecki, I., Nizou, J.-Y., Nicand, E., Guerin, P.J., 2006. High mortality associated with an outbreak of hepatitis E among displaced persons in Darfur, Sudan. Clinical Infectious Diseases 42, 1679-1684.

Borkakoti, J., Hazam, R.K., Mohammad, A., Kumar, A., 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., Tessé, S., Lunazzi, A., Eloit, M., Rose, N., Nicand, E., Pavio, N., 2011. Close similarity between sequences of hepatitis e virus recovered from humans and swine, France, 2008− 2009. Emerging Infectious Diseases 17, 2018.

Bouwknegt, M., Lodder-Verschoor, F., Van Der Poel, W.H., Rutjes, S.A., De Roda Husman, A.M., 2007. Hepatitis E virus RNA in commercial porcine livers in The Netherlands. Journal of Food Protection 70, 2889-2895.

Boxall, E., Herborn, A., Kochethu, G., Pratt, G., Adams, D., Ijaz, S., Teo, C.G., 2006. Transfusion-transmitted hepatitis E in a 'nonhyperendemic'country. Transfusion Medicine 16, 79-83.

Brassard, J., Gagné, M.-J., Généreux, M., Côté, C., 2012. Detection of human food-borne and zoonotic viruses on irrigated, field-grown strawberries. Applied Environary Microbiology 78, 3763-3766.

Cao, D., Meng, X.-J., 2012. Molecular biology and replication of hepatitis E virus. Emerging Microbes and Infections 1, e17.

Chibber, R.M., Usmani, M.A., Al-Sibai, M.H., 2004. Should HEV infected mothers breast feed? Archives of Gynecology and Obstetrics 270, 15-20.

Christensen, P.B., Engle, R.E., Hjort, C., Homburg, K.M., Vach, W., Georgsen, J., Purcell, R.H., 2008. Time trend of the prevalence of hepatitis E antibodies among farmers and blood donors: a potential zoonosis in Denmark. Clinical Infectious Diseases 47, 1026-1031.

Clayson, E.T., Shrestha, M.P., Vaughn, D.W., Snitbhan, R., Shrestha, K.B., Longer, C.F., Innis, B.L., 1997. Rates of hepatitis E virus infection and disease among adolescents and adults in Kathmandu, Nepal. Journal of Infectious Diseases 176, 763-766.

Colson, P., Borentain, P., Queyriaux, B., Kaba, M., Moal, V., Gallian, P., Heyries, L., Raoult, D., Gerolami, R., 2010. Pig liver sausage as a source of hepatitis E virus transmission to humans. The Journal of Infectious Diseases 202, 825-834.

Colson, P., Coze, C., Gallian, P., Henry, M., De Micco, P., Tamalet, C., 2007. Transfusion-associated hepatitis E, France. Emerging Infectious Diseases 13, 648.

Control, C.f.D., Prevention, 2011. Establishment of a viral hepatitis surveillance system--Pakistan, 2009-2011. Morbidity and Mortality Weekly Report 60, 1385.

Córdoba, L., Huang, Y.-W., Opriessnig, T., Harral, K.K., Beach, N.M., Finkielstein, C.V., Emerson, S.U., 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, 5338-5349.

Cossaboom, C.M., Heffron, C.L., Cao, D., Yugo, D.M., Houk-Miles, A.E., Lindsay, D.S., Zajac, A.M., Bertke, A.S., Elvinger, F., Meng, X.J., 2016. Risk factors and sources of foodborne hepatitis E virus infection in the United States. Journal of Medical Virology 88, 1641-1645.

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

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

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. New England Journal of Medicine 361, 1025-1027.

Dawson, G.J., Chau, K.H., Cabal, C.M., Yarbough, P.O., Reyes, G.R., Mushahwar, I.K., 1992. Solid-phase enzyme-linked immunosorbent assay for hepatitis E virus IgG and IgM antibodies utilizing recombinant antigens and synthetic peptides. Journal of Virological Methods 38, 175-186.

Debing, Y., Moradpour, D., Neyts, J., Gouttenoire, J., 2016. Update on hepatitis E virology: Implications for clinical practice. Journal of Hepatology 65, 200-212.

Di Bartolo, I., Diez-Valcarce, M., Vasickova, P., Kralik, P., Hernandez, M., Angeloni, G., Ostanello, F., Bouwknegt, M., Rodríguez-Lázaro, D., Pavlik, I., 2012. Hepatitis E virus in pork production chain in Czech Republic, Italy, and Spain, 2010. Emerging Infectious Diseases 18, 1282.

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

Dohoo, I.R., Martin, W., Stryhn, H., 2003. Veterinary epidemiologic research. AVC Incorporated Charlottetown, Canada.

Drabick, J.J., Gambel, J.M., Gouvea, V.S., Caudill, J.D., Sun, W., Hoke Jr, C.H., Innis, B.L., 1997. A cluster of acute hepatitis E infection in United Nations Bangladeshi peacekeepers in Haiti. The American Journal of Tropical Medicine and Hygiene 57, 449-454.

Favorov, M.O., Kosoy, M.Y., Tsarev, S.A., Childs, J.E., Margolis, H.S., 2000. Prevalence of antibody to hepatitis E virus among rodents in the United States. The Journal of Infectious Diseases 181, 449-455.

Feagins, A., Opriessnig, T., Guenette, D., Halbur, P., Meng, X., 2008. Inactivation of infectious hepatitis E virus present in commercial pig livers sold in local grocery stores in the United States. International Journal of Food Microbiology 123, 32-37.

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

Forgách, P., Nowotny, N., Erdélyi, K., Boncz, A., Zentai, J., Szűcs, G., Reuter, G., Bakonyi, T., 2010. Detection of hepatitis E virus in samples of animal origin collected in Hungary. Veterinary Microbiology 143, 106-116.

Geng, Y., Zhang, H., Huang, W., Harrison, T.J., Geng, K., Li, Z., Wang, Y., 2014. Persistent hepatitis e virus genotype 4 infection in a child with acute lymphoblastic leukemia. Hepatitis Monthly 14.

Girones, R., Carratalà, A., Calgua, B., Calvo, M., Rodriguez-Manzano, J., Emerson, S., 2014. Chlorine inactivation of hepatitis E virus and human adenovirus 2 in water. Journal of Water and Health 12, 436-442.

Gurley, E.S., Hossain, M.J., Paul, R.C., Sazzad, H.M., Islam, M.S., Parveen, S., Faruque, L.I., Husain, M., Ara, K., Jahan, Y., 2014. Outbreak of hepatitis E in urban Bangladesh resulting in maternal and perinatal mortality. Clinical Infectious Diseases 59, 658-665.

Hazam, R.K., Singla, R., Kishore, J., Singh, S., Gupta, R.K., Kar, P., 2010. Surveillance of hepatitis E virus in sewage and drinking water in a resettlement colony of Delhi: what has been the experience? Archives of Virology 155, 1227-1233.

Hewitt, P.E., Ijaz, S., Brailsford, S.R., Brett, R., Dicks, S., Haywood, B., Kennedy, I.T., Kitchen, A., Patel, P., Poh, J., 2014. Hepatitis E virus in blood components: a prevalence and transmission study in southeast England. The Lancet 384, 1766-1773.

Howard, C.M., Handzel, T., Hill, V.R., Grytdal, S.P., Blanton, C., Kamili, S., Drobeniuc, J., Hu, D., Teshale, E., 2010. Novel risk factors associated with hepatitis E virus infection in a large outbreak in northern Uganda: results from a case-control study and environmental analysis. The American Journal of Tropical Medicine and Hygiene 83, 1170-1173.

Inagaki, Y., Oshiro, Y., Hasegawa, N., Fukuda, K., Abei, M., Nishi, M., Okamoto, H., Ohkohchi, N., 2015. Clinical features of hepatitis E virus infection in Ibaraki, Japan: autochthonous hepatitis E and acute-on-chronic liver failure. The Tohoku Journal of Experimental Medicine 235, 275-282.

Islam, M., Siddika, A., Khan, M., Goldar, M., Sadique, M.A., Kabir, A., Huq, A., Colwell, R., 2001. Microbiological analysis of tube-well water in a rural area of Bangladesh. Applied Environary Microbiology 67, 3328-3330.

Izopet, J., Labrique, A.B., Basnyat, B., Dalton, H.R., Kmush, B., Heaney, C.D., Nelson, K.E., Ahmed, Z.B., Zaman, K., Mansuy, J.-M., 2015. Hepatitis E virus seroprevalence in three hyperendemic areas: Nepal, Bangladesh and southwest France. Journal of Clinical Virology 70, 39-42.

Johne, R., Heckel, G., Plenge-Bönig, A., Kindler, E., Maresch, C., Reetz, J., Schielke, A., Ulrich, R.G., 2010. Novel hepatitis E virus genotype in Norway rats, Germany. Emerging Infectious Diseases 16, 1452.

Joshi, D.M., Kumar, A., Agrawal, N., 2009. Studies on physicochemical parameters to assess the water quality of river Ganga for drinking purpose in Haridwar district. Rasayan Journal of Chemistry 2, 195-203.

Kamar, N., Bendall, R., Legrand-Abravanel, F., Xia, N.-S., Ijaz, S., Izopet, J., Dalton, H.R., 2012. Hepatitis e. The Lancet 379, 2477-2488.

Kamar, N., Izopet, J., Cintas, P., Garrouste, C., Uro-Coste, E., Cointault, O., Rostaing, L., 2010. Hepatitis E virus-induced neurological symptoms in a kidney-transplant patient with chronic hepatitis. American Journal of Transplantation 10, 1321-1324.

Khudyakov, Y., Kamili, S., 2011. Serological diagnostics of hepatitis E virus infection. Virus Research 161, 84-92.

Khuroo, M., Kamili, S., Khuroo, M., 2009. Clinical course and duration of viremia in vertically transmitted hepatitis E virus (HEV) infection in babies born to HEV-infected mothers. Journal of Viral Hepatitis 16, 519-523.

Khuroo, M.S., Duermeyer, W., Zargar, S.A., Ahanger, M.A., Shah, M.A., 1983. Acute sporadic non-A, non-B hepatitis in India. American Journal of Epidemiology 118, 360-364.

Kulkarni, M., Arankalle, V., 2008. The detection and characterization of hepatitis E virus in pig livers from retail markets of India. Journal of Medical Virology 80, 1387-1390.

Kuniholm, M.H., Purcell, R.H., McQuillan, G.M., Engle, R.E., Wasley, A., Nelson, K.E., 2009. Epidemiology of hepatitis E virus in the United States: results from the Third National Health and Nutrition Examination Survey, 1988–1994. The Journal of Infectious Diseases 200, 48-56.

Kuno, A., Ido, K., Isoda, N., Satoh, Y., Ono, K., Satoh, S., Inamori, H., Sugano, K., Kanai, N., Nishizawa, T., 2003. Sporadic acute hepatitis E of a 47-year-old man whose pet cat was positive for antibody to hepatitis E virus. Hepatology Research 26, 237-242.

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

Labrique, A.B., Zaman, K., Hossain, Z., Saha, P., Yunus, M., Hossain, A., Ticehurst, J., Nelson, K.E., 2009. Population seroprevalence of hepatitis E virus antibodies in rural Bangladesh. The American Journal of Tropical Medicine and Hygiene 81, 875-881.

Labrique, A.B., Zaman, K., Hossain, Z., Saha, P., Yunus, M., Hossain, A., Ticehurst, J.R., Nelson, K.E., 2010. Epidemiology and risk factors of incident hepatitis E virus infections in rural Bangladesh. American Journal of Epidemiology 172, 952-961.

Lam, W.-Y., Chan, R.C., Sung, J.J., Chan, P.K., 2009. Genotype distribution and sequence variation of hepatitis E virus, Hong Kong. Emerging Infectious Diseases 15, 792.

Leblanc, D., Poitras, E., Gagné, M.-J., Ward, P., Houde, A., 2010. Hepatitis E virus load in swine organs and tissues at slaughterhouse determined by real-time RT-PCR. International Journal of Food Microbiology 139, 206-209.

Lee, J.-T., Shao, P.-L., Chang, L.-Y., Xia, N.-S., Chen, P.-J., Lu, C.-Y., Huang, L.-M., 2013. Seroprevalence of hepatitis E virus infection among swine farmers and the general population in rural Taiwan. PLoS One 8, e67180.

Lin, C.-C., Wu, J.-C., Chang, T.-T., Chang, W.-Y., Yu, M.-L., Tam, A.W., Wang, S.-C., Huang, Y.-H., Chang, F.-Y., Lee, S.-D., 2000. Diagnostic value of immunoglobulin G (IgG) and IgM anti-hepatitis E virus (HEV) tests based on HEV RNA in an area where hepatitis E is not endemic. Journal of Clinical Microbiology 38, 3915-3918.

Lin, J., Norder, H., Uhlhorn, H., Belák, S., Widen, F., 2014. Novel hepatitis E like virus found in Swedish moose. The Journal of General Virology 95, 557.

Majumdar, M., Singh, M.P., Pujhari, S.K., Bhatia, D., Chawla, Y., Ratho, R., 2013. Hepatitis E virus antigen detection as an early diagnostic marker: report from India. Journal of Medical Virology 85, 823-827.

Mamun-Al-Mahtab, S.R., Khan, M., Karim, F., 2009. HEV infection as an aetiologic factor for acute hepatitis: experience from a tertiary hospital in Bangladesh. Journal of Health, Population, and Nutrition 27, 14.

Mansuy, J.-M., Huynh, A., Abravanel, F., Recher, C., Peron, J.M., Izopet, J., 2009. Molecular evidence of patient-to-patient transmission of hepatitis E virus in a hematology ward. Clinical Infectious Diseases 48, 373-374.

Matsubayashi, K., Kang, J.H., Sakata, H., Takahashi, K., Shindo, M., Kato, M., Sato, S., Kato, T., Nishimori, H., Tsuji, K., 2008. A case of transfusion-transmitted hepatitis E caused by blood from a donor infected with hepatitis E virus via zoonotic food-borne route. Transfusion 48, 1368-1375.

Matsuda, H., Okada, K., Takahashi, K., Mishiro, S., 2003. Severe hepatitis E virus infection after ingestion of uncooked liver from a wild boar. The Journal of Infectious Diseases 188, 944-944.

Meng, X., 2010. Hepatitis E virus: animal reservoirs and zoonotic risk. Veterinary Microbiology 140, 256-265.

Meng, X.J., Dea, S., Engle, R.E., Friendship, R., Lyoo, Y.S., Sirinarumitr, T., Urairong, K., Wang, D., Wong, D., Yoo, D., 1999. Prevalence of antibodies to the hepatitis E virus in pigs from countries where hepatitis E is common or is rare in the human population. Journal of Medical Virology 59, 297-302.

Mohanavalli, B., Dhevahi, E., Menon, T., Malathi, S., Thyagarajan, S., 2003. Prevalence of antibodies to hepatitis A and hepatitis E virus in urban school children in Chennai. Indian Pediatrics 40, 328-331.

Mushahwar, I.K., 2008. Hepatitis E virus: molecular virology, clinical features, diagnosis, transmission, epidemiology, and prevention. Journal of Medical Virology 80, 646-658.

Myint, K.S.A., Endy, T.P., Gibbons, R.V., Laras, K., Mammen, M.P., Sedyaningsih, E.R., Seriwatana, J., Glass, J.S., Narupiti, S., Corwin, A.L., 2006. Evaluation of diagnostic assays for hepatitis E virus in outbreak settings. Journal of Clinical Microbiology 44, 1581-1583.

Navaneethan, U., Al Mohajer, M., Shata, M.T., 2008. Hepatitis E and pregnancy: understanding the pathogenesis. Liver International 28, 1190-1199.

Okamoto, H., 2007. Genetic variability and evolution of hepatitis E virus. Virus Research 127, 216-228.

Okamoto, H., Takahashi, M., Nishizawa, T., Usui, R., Kobayashi, E., 2004. Presence of antibodies to hepatitis E virus in Japanese pet cats. Infection 32, 57-58.

Pérez-Gracia, M.T., Mateos, M.L., Galiana, C., Fernández-Barredo, S., García, A., Gómez, M.T., Moreira, V., 2007. Autochthonous hepatitis E infection in a slaughterhouse worker. The American Journal of Tropical Medicine and Hygiene 77, 893-896.

Perttilä, J., Spuul, P., Ahola, T., 2013. Early secretory pathway localization and lack of processing for hepatitis E virus replication protein pORF1. Journal of General Virology 94, 807-816.

Pudupakam, R., Huang, Y., Opriessnig, T., Halbur, P., Pierson, F., Meng, X., 2009. Deletions of the hypervariable region (HVR) in open reading frame 1 of hepatitis E virus do not abolish virus infectivity: evidence for attenuation of HVR deletion mutants in vivo. Journal of Virology 83, 384-395.

Raj, V.S., Smits, S.L., Pas, S.D., Provacia, L.B., Moorman-Roest, H., Osterhaus, A.D., Haagmans, B.L., 2012. Novel hepatitis E virus in ferrets, the Netherlands. Emerging Infectious Diseases 18, 1369.

Rein, D.B., Stevens, G.A., Theaker, J., Wittenborn, J.S., Wiersma, S.T., 2012. The global burden of hepatitis E virus genotypes 1 and 2 in 2005. Hepatology 55, 988-997.

Reuter, G., Fodor, D., Forgách, P., Kátai, A., Szűcs, G., 2009. Characterization and zoonotic potential of endemic hepatitis E virus (HEV) strains in humans and animals in Hungary. Journal of Clinical Virology 44, 277-281.

Romanò, L., Paladini, S., Tagliacarne, C., Canuti, M., Bianchi, S., Zanetti, A.R., 2011. Hepatitis E in Italy: a long-term prospective study. Journal of Hepatology 54, 34-40.

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

Schlauder, G.G., Dawson, G.J., Erker, J.C., Kwo, P.Y., Knigge, M.F., Smalley, D.L., Rosenblatt, J.E., Desai, S.M., Mushahwar, I.K., 1998. The sequence and phylogenetic analysis of a novel hepatitis E virus isolated from a patient with acute hepatitis reported in the United States. Journal of General Virology 79, 447-456.

Sheikh, A., Sugitani, M., Kinukawa, N., Moriyama, M., Arakawa, Y., Komiyama, K., Li, T.-C., Takeda, N., Ishaque, S.M., Hasan, M., 2002. Hepatitis e virus infection in fulminant hepatitis patients and an apparently healthy population in Bangladesh. The American Journal of Tropical Medicine and Hygiene 66, 721-724.

Shrestha, S.M., Shrestha, S., Tsuda, F., Nishizawa, T., Gotanda, Y., Takeda, N., Okamoto, H., 2003. Molecular investigation of hepatitis E virus infection in patients with acute hepatitis in Kathmandu, Nepal. Journal of Medical Virology 69, 207-214.

Sonoda, H., Abe, M., Sugimoto, T., Sato, Y., Bando, M., Fukui, E., Mizuo, H., Takahashi, M., Nishizawa, T., Okamoto, H., 2004. Prevalence of hepatitis E virus (HEV) Infection in wild boars and deer and genetic identification of a genotype 3 HEV from a boar in Japan. Journal of Clinical Microbiology 42, 5371-5374.

Stoszek, S.K., Abdel-Hamid, M., Saleh, D.a.A., Kafrawy, S.E., Narooz, S., Hawash, Y., Shebl, F.M., El Daly, M., Said, A., Kassem, E., 2006. High prevalence of hepatitis E antibodies in pregnant Egyptian women. Transactions of the Royal Society of Tropical Medicine and Hygiene 100, 95-101.

Takahashi, M., Kusakai, S., Mizuo, H., Suzuki, K., Fujimura, K., Masuko, K., Sugai, Y., Aikawa, T., Nishizawa, T., Okamoto, H., 2005. Simultaneous detection of immunoglobulin A (IgA) and IgM antibodies against hepatitis E virus (HEV) is highly specific for diagnosis of acute HEV infection. Journal of Clinical Microbiology 43, 49-56.

Takahashi, M., Tanaka, T., Azuma, M., Kusano, E., Aikawa, T., Shibayama, T., Yazaki, Y., Mizuo, H., Inoue, J., 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, 3671-3679.

Tam, A.W., Smith, M.M., Guerra, M.E., Huang, C.-C., Bradley, D.W., Fry, K.E., Reyes, G.R., 1991. Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length viral genome. Virology 185, 120-131.

Tei, S., Kitajima, N., Takahashi, K., Mishiro, S., 2003. Zoonotic transmission of hepatitis E virus from deer to human beings. The Lancet 362, 371-373.

Teshale, E.H., Grytdal, S.P., Howard, C., Barry, V., Kamili, S., Drobeniuc, J., Hill, V.R., Okware, S., Hu, D.J., Holmberg, S.D., 2010a. Evidence of person-to-person transmission of hepatitis E virus during a large outbreak in Northern Uganda. Clinical Infectious Diseases 50, 1006-1010.

Teshale, E.H., Howard, C.M., Grytdal, S.P., Handzel, T.R., Barry, V., Kamili, S., Drobeniuc, J., Okware, S., Downing, R., Tappero, J.W., 2010b. Hepatitis E epidemic, Uganda. Emerging Infectious Diseases 16, 123.

van Tong, H., Hoan, N.X., Wang, B., Wedemeyer, H., Bock, C.-T., Velavan, T.P., 2016. Hepatitis E virus mutations: functional and clinical relevance. EBioMedicine 11, 31-42.

Verma, V., Arankalle, V., 2010. Hepatitis E virus-based evaluation of a virion concentration method and detection of enteric viruses in environmental samples by multiplex nested RT-PCR. Journal of Applied Microbiology 108, 1630-1641.

Wedemeyer, H., Pischke, S., Manns, M.P., 2012. Pathogenesis and treatment of hepatitis E virus infection. Gastroenterology 142, 1388-1397. e1381.

Wenzel, J.J., Preiß, J., Schemmerer, M., Huber, B., Plentz, A., Jilg, W., 2011. Detection of hepatitis E virus (HEV) from porcine livers in Southeastern Germany and high sequence homology to human HEV isolates. Journal of Clinical Virology 52, 50-54.

Widdowson, M.-A., Jaspers, W.J., van der Poel, W.H., Verschoor, F., de Roda Husman, A.M., Winter, H., Zaaijer, H.L., Koopmans, M., 2003. Cluster of cases of acute hepatitis associated with hepatitis E virus infection acquired in the Netherlands. Clinical Infectious Diseases 36, 29-33.

Woo, P.C., Lau, S.K., Teng, J.L., Tsang, A.K., Joseph, M., Wong, E.Y., Tang, Y., Sivakumar, S., Xie, J., Bai, R., 2014. New hepatitis E virus genotype in camels, the Middle East. Emerging Infectious Diseases 20, 1044.

Xu, B., Zhi, N., Hu, G., Wan, Z., Zheng, X., Liu, X., Wong, S., Kajigaya, S., Zhao, K., Mao, Q., 2013. Hybrid DNA virus in Chinese patients with seronegative hepatitis discovered by deep sequencing. Proceedings of the National Academy of Sciences 110, 10264-10269.

Zaaijer, H., Kok, M., Lelie, P., Timmerman, R., Chau, K., Van der Pal, H., 1993. Hepatitis E in The Netherlands: imported and endemic. The Lancet 341, 826.

Zhang, H., Dai, X., Shan, X., Meng, J., 2008. The Leu477 and Leu613 of ORF2-encoded protein are critical in forming neutralization antigenic epitope of hepatitis E virus genotype 4. Cellular and Molecular Immunology 5, 447.

Zhang, J., Zhang, X.-F., Huang, S.-J., Wu, T., Hu, Y.-M., Wang, Z.-Z., Wang, H., Jiang, H.-M., Wang, Y.-J., Yan, Q., 2015. Long-term efficacy of a hepatitis E vaccine. New England Journal of Medicine 372, 914-922.

Zhao, C., Ma, Z., Harrison, T.J., Feng, R., Zhang, C., Qiao, Z., Fan, J., Ma, H., Li, M., Song, A., 2009. A novel genotype of hepatitis E virus prevalent among farmed rabbits in China. Journal of Medical Virology 81, 1371-1379.

Zhuang, H., Cao, X.-Y., Liu, C.-B., Wang, G.-M., 1991. Epidemiology of hepatitis E in China. Gastroenterologia Japonica 26, 135-138.

Zuthi, M., Biswas, M., Bahar, M., 2009. Assessment of supply water quality in the Chittagong city of Bangladesh. ARPN Journal of Engineering and Applied Sciences 4, 73-80.