Hepatitis E Virus: Epidemiology and Genotyping form Pakistan

MS Healthcare Biotechnology

By

Momina Ejaz

(00000329923)

Atta-ur-Rahman School of Applied Biosciences

National University of Sciences and Technology, Islamabad, Pakistan

2023

Hepatitis E Virus: Epidemiology and Genotyping form Pakistan

A thesis submitted in partial fulfilment of the requirement for the degree of Masters of Science in Healthcare Biotechnology

By

Momina Ejaz

(00000329923)

MS HCB

Supervisor: Dr. Aneela Javed

Atta-ur-Rahman School of Applied Biosciences

National University of Sciences and Technology, Islamabad, Pakistan

2023

FORM TH-4

National University of Sciences & Technology

MS THESIS WORK

We hereby recommend that the dissertation prepared under our supervision by: (Student Name & Regn No.) **Momina Ejaz and Reg No. 00000329923**
Titled: Hepatitis E Virus: Epidemiology and Genotyping form Pakistan be accepted in partial fulfillment of the requirements for the award of **MS Degree in Healthcare Biotechnology** degree with (A grade).

Examination Committee Members

1. Name: **Dr. Rumeza Hanif** Signature:

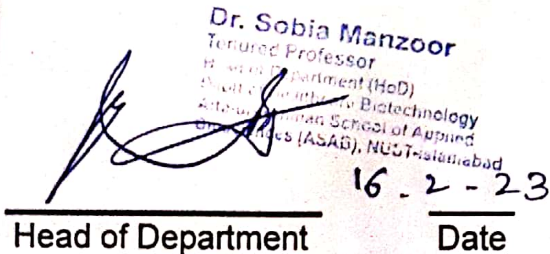
2. Name: **Dr. Amjad Ali** Signature:

3. Name: **Dr. Fouzia Parveen Malik** Signature:

Co-Supervisor's name: **Dr. Ali Zohaib** Signature:

Supervisor's name: **Dr. Aneela Javed** Signature:

Date: 9-2-2023

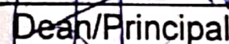


Head of Department — Date: 16.2.23

16.2.23
Date

COUNTERSINGED

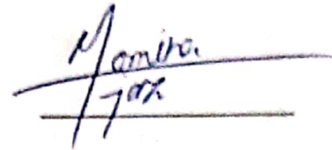
Date: __________



Dean/Principal

DECLARATION

I hereby declare that except where specific reference is made to the work of others, the content of this dissertation is original and have not been submitted in whole or in part for consideration for any other degree or qualification in this or any other University. This dissertation is the result of my own work and includes the outcome of the work done.



Signature of Student

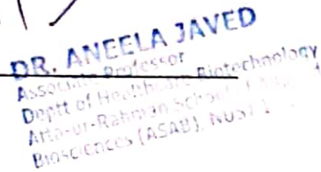
Momina Ejaz

00000329923

CamScanner

THESIS ACCEPTANCE CERTIFICATE

Certified that the contents and form of thesis entitled "**Hepatitis E Virus: Epidemiology and Genotyping from Pakistan**" submitted by **Momina Ejaz** have been found satisfactory for the requirement of the degree.



Supervisor: ______________________

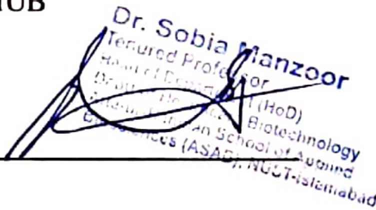
Prof. Dr. Aneela Javed

ASAB, NUST

Co-Supervisor: ______________________

Prof. Dr. Ali Zohaib

Department of Microbiology, IUB

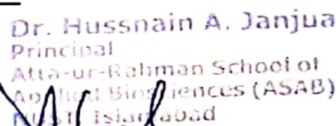


Head of the Department: ______________________

Prof. Dr. Sobia Manzoor

ASAB, NUST

Dated: 16.2-23



Principal: ______________________

Prof. Dr. Hussnain A. Janjua

ASAB, NUST

Dated: 20-2-23

CamScanner

CERTIFICATE FOR PLAGIARISM

It is certified that MS Thesis titled **"Hepatitis E Virus: Epidemiology and Genotyping from Pakistan"** submitted by **Momina Ejaz** has been examined,

I undertake the following:

a. The thesis has significant new work/knowledge as compared to already publish or are under consideration to be published work. No sentence, equation, diagram, table, paragraph or section has been copied verbatim from previous work unless it is placed under quotation marks and duly referenced.

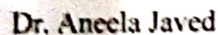
b. The work presented is original and own work of the author (i.e., there is no plagiarism). No ideas, processes, results, or words of others have been presented as the Author's own work.

c. There is no fabrication of data or results, which have been compiled/analyzed.

d. There is no falsification by manipulating research materials, equipment or processes, or changing or omitting data or results such that the research is not accurately represented in the research record.

e. The thesis has been checked using TURNITIN (copy of originality report attached) and found within the limits as per HEC plagiarism Policy and instructions issued from time to time.

Supervisor:



DR. ANEELA JAVED

Dr. Aneela Javed

ASAB, NUST.

This thesis is dedicated to all those who have hope and have ever dared to dream big

ACKNOWLEDGEMENTS

In the name of Allah, the Most Merciful, the Most Kind. All praises to Allah Almighty Who has bestowed His countless blessings upon me and without His mercy, I would not have been able to complete this thesis.

First of all, I would like to express my sincere gratitude to my supervisors **Dr. Aneela Javed** and **Dr. Ali Zohaib** for their guidance, attention, support, and cooperation throughout the research without whom this project would not have been completed. I would like to thank them for their encouragement and valuable input which helped me in learning the lab techniques. Their superior knowledge, experience, and prompt supervision have made this research project possible. I am grateful for their time, spiritual and moral guidance, patience, and encouragement.

I would like to thank Dr. Hussnain A. Janjua, Principal ASAB, and Dr. Sobia Manzoor, HOD Healthcare Biotechnology for providing the opportunity to carry out research work in a professional environment. My sincere regards and thanks to my GEC members Dr. Rumeza Hanif, Dr. Amjad Ali, and Dr. Fouzia Malik (SINES, NUST).

I would like to extend my gratitude to Muhammad Usman, Moazz Anwar, and Muhammad Ammar for their technical support and guidance, and extreme corporation in times of need.

I would also to thank my friends Sarah, Rida, Ariba, Ali, Sumaiya, and Iqra, for having my back in times of need, and my kind seniors Maaza Sana, Samreen, Haiqa, Kafila, Naila, Hamza Sher, Hafsa and Fareeha and juniors Saman and Iman for all the support. I would like to extend my special thanks to Aaima, Sabahat, and Anam for being reliable partners and sincere friends.

I am extremely grateful for my family as this journey would not have been possible without their extraordinary love, support, prayers, and encouragement.

Momina Ejaz

MS Healthcare Biotechnology

ASAB, NUST

Table of Contents

Table of Contents ix
List of Tables xiii
List of Abbreviations xiv
ABSTRACT xv
INTORDUCTION 1
1.1. Aim and Objectives: 5
2. Literature Review 6
2.1 Virus Discovery: 6
2.2 Genome Organization: 6
2.2.1 Two types of virions naturally exist during an HEV infection 7
2.3 Genomic Replication Strategy: 9
2.4 Genotypes: 11
2.5 Epidemiology: 12
2.6 Modes of Transmission: 13
2.6.1 Waterborne infection 13
2.6.2 Zoonotic transmission 14
2.6.3 Blood transfusion 15
2.6.4 Perinatal transmission 15
2.6.5 Transmission in breast milk 15
2.7 Clinical manifestations: 16
2.7.1 Acute Hepatitis E 16
2.7.2 Chronic hepatitis E 18
2.8 Diagnosis: 18
2.9 Special populations 20
Pregnant women 20
Pre-existing liver disease or malnutrition 21
Solid organ transplant recipients 21
HIV and other immunosuppressed hosts 21
2.10 Prevention 21
2.11 HEV in Pakistan 22
2.12 Genotyping of HEV form Pakistan 24
MATERIALS AND METHODS 27
3.1 Ethics Statement: 27
3.2 Amplification of Positive Control Plasmid DNA: 27

3.2.1 Competent BL21(DE3) is an E. coli B strain: 27
3.2.2 Transformation of competent BL21(DE3) cells: 28
3.2.3 Colony PCR 29
3.2.4 Gel Electrophoresis: 31
3.2.5 Miniprep: 31
3.2.6 Plasmid Nanodrop and PCR to confirm plasmid purity: 34
3.3 Optimizing the Hepevirus degenerate primers for detection of HEV virus using the transformed plasmid: 35
3.4 Study locals and Sampling: 38
3.5 Serodiagnosis of HEV 39
3.6 Molecular detection of HEV: 39
3.6.1.1 RNA Extraction: 39
3.6.2 cDNA Synthesis: 41
3.6.3 Nested Polymerase Chain Reaction: 41
3.6.4 PCR Products Analysis: 42
3.6.5 PCR Product Purification: 43
3.6.6 Cycle Sequencing: 44
3.6.7 Sequencing Cleanup: 45
3.6.8 Capillary Electrophoresis: 46
3.6.9 Sequence Editing 46
3.7 Alignment 47
3.8 Phylogenic Analysis 49
RESULTS 50
4.1 Amplification of Positive Control Plasmid DNA: 50
4.1.1 Transformed BL21 Cells: 50
4.1.2 Colony PCR 50
4.1.3 Miniprep 52
4.2 Optimizing the Hepevirus degenerate primers for detection of HEV virus using the transformed plasmid: 53
4.3 Epidemiological Characteristics of the Hepatitis E Cases 54
4.4 Positivity rate of HEV IgM positive samples 56
4.5 Detection and sequencing of HEV samples 57
4.6 Phylogenetic analysis of Genotype 1 strains 59
DISCUSSION 66
CCONCLUSIONS AND FUTURE PROSPECTS 70
REFRENCES 71

List of Figures

Figure 2.1: The genome organization of the Hepatitis E virus…………………………..…...7

Figure 2.2: Types of HEV found during an infection..……………………..………..9

Figure2.3: The genome replication strategy of the Hepatitis E virus……………….……10

Figure 2.4: Classification of the Hepatitis E virus…………………………………...…...11

Figure 2.5: Global distribution of the Hepatitis E virus…………………………...…..13

Figure 2.6: The verified and theorized HEV transmission pathways to humans………....16

Figure 2.7: HEV infection and hepatitis…….……………………………………….....19

Figure 2.8: Diagnostic algorithm of HEV….…………………………..…………..…20

Figure 3.1: PCR thermal cycling profile for PREDICT primers………..……………......30

Figure 3.2: Bacterial pellet of transformed bacterial cells ……………………….……....33

Figure 3.3: Touch down PCR program in the thermocycler. ……………………....…36

Figure 3.4: Touch down PCR thermal cycling temperature profile for Round 1……...….37

Figure 3.5: Touch down PCR thermal cycling temperature profile for Round 2……...…38

Figure 3.6: Map showing the sampling sites from Pakistan. ………….…...………......39

Figure 3.7: Ends trimming criteria to remove poor quality and ambiguous data ………...46

Figure 3.8: Sequence alignment of Hepatitis E sequences retrieved form NCBI……...…39

Figure 3.9: Analysis preferences for the construction of a phylogenetic tree…...……...…48

Figure 3.10: Distance column with the Indian sequence (AF093885.1) as a reference………………………………………….……………………………………49

Figure 4.1: Transformation of BL21 competent cells. ……………………………..…….50

Figure 4.2: Selection of colonies for colony PCR. ………………..……………..…….51

Figure 4.3: Representative Gel of colony PCR. ………………..……….……..…….51

Figure 4.4: Nanodrop concentration of the extracted plasmid. ……………..…….52

Figure 4.5: Gel representation of PCR product obtained after amplifying the PREDICT sequence in the UC 1 plasmid using a PCR. …………..…………….…………….53

Figure 4.6: Gel representation of optimization of HEV specific primers using the plasmid as a positive control.. …………..……………………..…………………..…….54

Figure 4.7: Gender distribution of patients. …………………………………..…….55

Figure 4.8: Age distribution of the patients. ………………………………..…….55

Figure 4.9: Status of seroconversion of patients during the hospital stay ………..….56

Figure 4.10: Number of IgM positive samples detected positive by PCR. …………...….57

Figure 4.11: Representative Gel of Round 1 PCR…………..…………...……………….58

Figure 4.12: Gel images of samples processed for detection of HEV in Round 1 PCR…..58

Figure 4.13: Gel images of all the positive samples processed for detection of HEV in Round 2 PCR……………………………………………………………………….59

Figure 4.14: A Phylogenetic tree of 4 representative sequences of Hepatitis E virus (HEV) and 30 sequences from the current study…………………………………………….60

Figure 4.15: A Phylogenetic tree of representative sequences of Hepatitis E virus (HEV) and 30 sequences from the current study. …………………………………………...61

Figure 4.16: A phylogenetic tree of representative sequences of Hepatitis E Virus (HEV) 6 sequences reported previously form Pakistan and 3 sequences form the current study

List of Tables

Table 2.1: Outbreaks of HEV in Pakistan……………………………………………….24

Table 2.2: History of HEV genotyping in Pakistan…………………..………………26

Table 3.1:Primers used for the detection of plasmid uptake by the competent BL21 cells……………………………………………………………………………………29

Table 4.2: Reagents preparation for miniprep ………………………………...……….32

Table 3.3: Degenerate primers used for the detection of HEV and product size…….……35

Table 5.4: Reagents and their quantities used for cDNA synthesis………………………41

Table 3.5: The volume of the HighPrep PCR reagent volume……………………………43

Table 3.6: Reagents and their quantities used for cycle sequencing……………………44

Table 3.7: The volume of the HighPrep DTR reagent……………………………………45

Table 4.1. Subtyping of the sequenced isolates against the reference sequence NC_001434…………………………………………………………….……………...64

List of Abbreviations

HEV	Hepatitis E virus
ALF	Acute liver failure
EM	Electron microscopy
NCR	Non-coding region
ORFs	Open reading frames
ESCRT	Endosomal sorting complex needed for transport
PSAP	Proline-serine-alanine-proline
MVB	Multivesicular bodies
ALT	Alanine aminotransferase
ELISA	Enzyme-Linked Immunosorbent Assay
HIV	Human Immunodeficiency virus
EIA	Enzyme immunoassays
PCR	Polymerase Chain Reaction
(RT)-PCR	Reverse transcription Polymerase Chain Reaction

ABSTRACT

Hepatitis E virus (HEV) is an emergent source of enterically transmitted viral hepatitis and an imminent public health concern. HEV has a substantial disease burden in areas afflicted with humanitarian emergencies such as flood-affected regions, externally or internally displaced populations, and densely packed areas where access to proper sanitation and safe drinking water is challenging. Although outbreaks and sporadic cases are linked to waterborne transmission, zoonosis has also been linked to the emergence of disease. It usually presents itself as a self-resolving aliment, but chronic HEV infections prove to be devastating in immunocompromised patients and pregnant females where the infection is associated with high maternal mortality and frequent fetal loss. HEV-related clinical complications are often undetected and not considered in the differential diagnosis. Pakistan is the fifth most populous nation in the world and is faced with several challenges including recent devastating floods, sub-optimal sanitary conditions, cluster accommodation, and unregulated cross-border movements. These drastic shifts in population dynamics make these territories vulnerable to proficient disease spread. The current study thus sought to analyze the epidemiological and viral genotype characteristics of the Hepatitis E virus, to assess the genetic variants circulating in the population. Consecutive patients presenting with symptoms associated with acute liver disease were sampled from three metropolitan cities including Lahore, Peshawar, and Karachi. HEV infection was confirmed by ELISA. ELISA-positive samples (75 samples) were further subjected to viral RNA extraction, followed by the amplification of the HEV Rdrp region using Nested PCR and degenerate primers. 40% of the samples (33 samples) were found to be positive for HEV RNA indicating an active infectious state. Sequencing and phylogenetic analysis revealed that HEV isolates cluster with genotype 1. Subtype 1g was

found to be the prevailing subtype as compared to the previously documented subtype 1a reported from Karachi. Thus, our results indicate the presence of a newly abundant circulating subtype and thus merit a thorough investigation to further evaluate the impacts of this shift on clinical manifestation and disease severity.

INTRODUCTION

Working its way through the world with a complex etiology and an understudied means of pathogenesis, the biphasic epidemiological profile of HEV has caused it to emerge as a global public health problem (Chauhan et al., 2019). Initially identified in the late 1970s as an epidemic non-A non-B hepatitis associated with an infectious water-borne illness, HEV has risen to be the leading cause of icteric hepatitis and acute liver failure (ALF) all over the world. (Donnelly et al., 2017a). Infection with HEV is associated with an annual incidence of 20 million infections, acute liver injury in 3.5 million, and 56,600 deaths (2800 per 1,000,000 infections) due to viral hepatitis (Stanaway et al., 2016; Thakur et al., 2020) and it is also estimated that nearly one-third of the world's population has been infected with HEV. Although the condition is self-limiting and has no chronic sequelae or carrier state in the normal population, it can develop into a chronic/carrier state in immune-impaired patients.

HEV is a small ssRNA virus approximately 7.2kb in length. It is non-enveloped in bile and feces but coated with a lipid envelope in the blood (quasi-enveloped) (Denner, 2019). It belongs to the family *Hepeviridae* and genus *Orthohepevirus* with the species *Orthohepevirus* A, B, C, and D. *Orthohepevirus* A contains eight genotypes that infect humans and multiple mammals. *Orthohepevirus* B circulates in chickens, *Orthohepevirus* C in rats and ferrets and *Orthohepevirus* D circulates in Bats. (Purdy et al., 2017). The global spread of the virus follows the socio-economic status of populations (Bihl & Negro, 2010). Five members of *Orthohepevirus* A are known to cause human infections. HEV genotypes 1 and 2 (HEV-1 and HEV-2) are human viruses and are found in the developing world. These genotypes are highly endemic in several parts of Asia, Africa, the Middle East, and Mexico. Contrarily, HEV genotypes 3 and 4 (HEV-3 and HEV-4) have zoonotic

origins and have been discovered in a variety of hosts, including humans, pigs, goats, cattle, donkeys, deer, camels, and yaks. (Zhou et al., 2019). The cross-species transmission is mainly attributed to direct contact with the infected animal or consumption of HEV-contaminated food products. Genotype 4 is mainly found in Asia and circulates between humans and swine, Genotype 3 is the major genotype responsible for autochthonous (locally acquired) transmission in the West (Donnelly et al., 2017a). Genotypes 5 and 6 have been reported in wild boars in Japan, Genotypes 7 and 8 were recently observed in dromedary (Lee et al., 2016) and Bactrian camels (Woo et al., 2016.) respectively. Many subtypes have also been described within the genotypes 1 to 4; 5 for HEV-1 (HEV-1a to HEV-1e), 2 for HEV-2 (HEV-2a and HEV-2b), 10 for HEV-3 (HEV-3a- HEV-3j) and 7 for HEV-4 (HEV-4a - HEV-4g) (Smith et al., 2016). Still, the actual host range of HEV is undetermined, leaving room for the discovery of novel HEV genotypes and strains. This fact further complicates the study of viral transmission and the risk of an HEV infection in humans Aside from the water and food-borne modes of transmission. HEV virus has been observed to spread in unique ways, including blood transfusion in organ transplant recipients. The prospect of the virus spreading from person to person has also been addressed. It has been claimed that pets such as cats, dogs, rabbits, and horses might serve as unintentional hosts for HEV and thereby spreading the disease to humans (Liang et al., 2014).

Initially identified in the late 1970s as an epidemic non-A non-B hepatitis associated with an infectious water-borne illness, HEV has been recognized to be the leading cause of icteric hepatitis and acute liver failure all over the world. The global spread of the virus follows the socio-economic status of populations. In low- and middle- income countries the virus majorly spreads as outbreaks, but sporadic cases are also seen. Poor sanitation and limited access to essential water, hygiene, and health services act as factors that actively

facilitate viral spread. Some of the outbreaks gravitate towards areas of conflict and humanitarian emergencies such as war zones, flood-ridden areas, refugee camps, or internally displaced populations, where proper sanitation and access to a safe water supply is especially challenging. Sporadic cases are also thought to be related to water contamination, albeit on a smaller scale. Even though developed nations with superior water supplies and cleanliness do not have a problem with the water-related transmission of HEV, HEV infections have nonetheless been documented. Initially, these cases were solely attributed to immigrants or individuals who had visited endemic regions, but over the past ten years, the identification and characterization of swine HEV in the United States, Europe, and many other countries, as well as their close association with human HEV discovered in the same geographic areas, have demonstrated that HEV is, in fact, a zoonotic virus and that domestic swine, wild deer, and boars are natural reservoirs of HEV. Infections in both cases are seconded to genotype 1 virus infection, with genotype 2 virus occurring much less frequently (Donnelly et al., 2017b). Genotype 1 is majorly localized in Asia and Africa; Genotype 2 is less common and is usually found in Africa and Mexico, but the true burden of the disease is not well known. These cases are usually associated with the genotype 3 virus and their origins stem form viruses originating in consuming undercooked animal meat (Including animal liver, particularly pork) in the form of common-source foodborne outbreaks.

HEV is typically diagnosed by detecting the presence of anti-HEV IgG or anti-HEV IgM antibodies, along with HEV RNA in the serum. HEV infections can cause anything from asymptomatic hepatitis to life-threatening hepatitis with acute liver failure (ALF). In endemic areas, an endemic disease caused by genotype 1 or 2 commonly targets young adults. Infections with genotypes 3 and 4 are seen to peak in older patients (>60 years of age) in advanced economies (Purcell & Emerson, 2008). After an incubation period of 3 to

6 weeks, acute infection often presents itself clinically in most individuals as a subclinical to a severe picture of cholestatic hepatitis with jaundice, anorexia, nausea, vomiting, and rarely fever, lasting 1 to 6 weeks. In both high- and low-endemicity regions, as many as one percent of cases may be classified as severe, life-threatening hepatitis with ALF. Unfortunately, pregnant women have the greatest incidence of serious illness and death. Before recently, it was assumed that HEV infection was self-limiting. In immune-compromised patients, however, persistent HEV infection accompanied by chronic liver disease and, in rare instances, progression to cirrhosis has been reported. Particularly, several cases of HEV infection in recipients of solid organ transplants (liver, kidney, or pancreas) have been documented with persistent detection of HEV RNA in stools or serum up to 15 months after the initial infection. In immunocompromised patients, persistent HEV infection followed by chronic, progressive liver damage that can develop into cirrhosis is a rare but clinically relevant event.

In developing countries such as Pakistan, sporadic infections and outbreaks associated with HEV infection have been reported as early as the 1950s and 1960s. Data from surveillance conducted by the Ministry of Health in 2011 indicated that HEV is responsible for nearly 12.2 % of cases of acute cases of hepatitis. This percentage can be considered an underestimation as systematic testing of non-A-C cases for Hepatitis E is not performed routinely in infectious disease units in the country. Moreover, there have been very few community-based studies on the incidence and prevalence of HEV in Pakistan, with much of the research concentrating on seroprevalence (R. U. Khan et al., 1995; Rashid et al., 2021; Siddiqui et al., 2005) or incidence in pregnant women (Nadeem et al., 2021).

Most examples of molecular detection and characterization in the literature are from case studies (Din et al., 2018) or conducted in an outbreak scenario The dominant genotype circulating in the population has been identified as genotype 1. However, the possibility of

novel HEV transmission to humans has so far gone unconsidered. As a result, the real clinical impact of hepatitis E in Pakistan must be determined. As a result, we investigated the epidemiological characteristics and genotype distribution of HEV infections using degenerate primers, in hospitalized patients to evaluate the incidence of Hepatitis E virus infections in settings other than an outbreak because the prevalence and outcome of HEV infection in an outbreak would differ from those of the general population, and to document the genotypes in current circulation in Pakistan.

1.1. Aim and Objectives:

The proposed research aims to investigate the epidemiological characteristics and genotype distribution of HEV infections in hospitalized patients to evaluate the incidence of Hepatitis E virus infections in densely populated areas of Pakistan.

The objective of this study includes:

- To investigate the circulating genotype of HEV in the major metropolitan cities of Pakistan
- To examine the evolutionary history of HEV in Pakistan and to foresee future consequences for preventive and treatment strategies.

LITERATURE REVIEW

2.1 Virus Discovery:

Dr. Mikhail Balayan, a Russian virologist, discovered HEV in 1983 by purposefully infecting himself with stool samples taken from HEV-infected Russian soldiers stationed in Afghanistan. Eventually, he acquired severe hepatitis. By utilizing electron microscopy (EM) to examine his feces, Dr. Balayan found the first morphological evidence of HEV virions, which appeared as unenveloped icosahedral particles with a "spiky" surface, measuring 27–30 nm in diameter. Due to its structural similarity to the calicivirus, HEV was first misclassified as a member of the family *Caliciviridae*. In 1990, HEV was cloned genetically and its whole genome was sequenced. Significant sequence differences between HEV and other known virus families led to its reassignment to the Hepeviridae family (Yin & Feng, 2019).

2.2 Genome Organization:

The Hepatitis E virus has a single-stranded RNA genome that is 7.2kb long and has a positive charge. The genome is mostly made up of a 26-bp-long 5' non-coding region (NCR), three open reading frames (ORFs 1-3), and a 3' non-coding region (NCR) (NCR). ORF3 overlaps with ORF2, whereas none of the open reading frames overlap with ORF1. (*Hepatitis E Virus Infection - UpToDate*, 2020).

ORF1 is a large, polyprotein that is made from the first two-thirds of the genome. It helps copy the genome. The remainder of the genome is made up of ORF2 and ORF3, which are both translated from sub-genomic RNA produced by the virus during its cycle of replication. HEV only has the ORF2 capsid protein. The ORF2 capsid protein of HEV is made up of 660 amino acids (a.a.) that are split into 3 structural domains: a shell (S) domain, a middle (M) domain, and a protrusion (P) domain. The ORF3 protein is only found in

HEV and helps the virus's particles leave the cell. Recently, ORF4 was found in the ORF1 coding area, but it seems to only be in genotype 1 HEV (Yin & Feng, 2019).

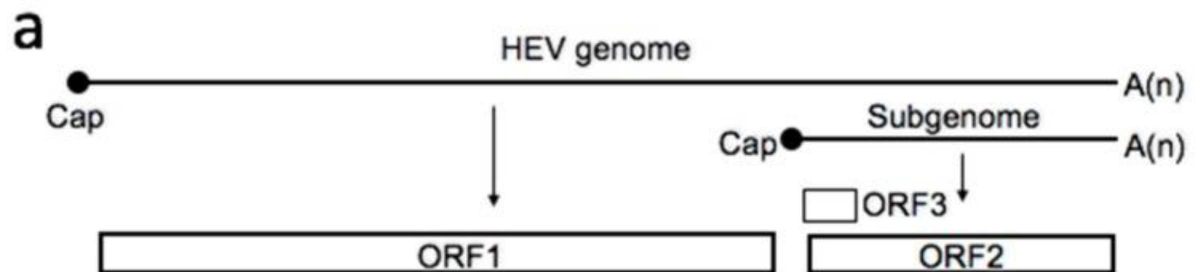


Figure 2.1: The genome organization of the Hepatitis E virus. The hepatitis E virus (HEV) genome contains a 5′ non-coding (5′NC) region and is capped with 7-methylguanosine (7mG), and a 3′NC region is polyadenylated (poly(A)). The ORF1 encodes nonstructural proteins.

2.2.1 Two types of virions naturally exist during an HEV infection

Enteric transmission, electron microscopy evidence of naked virions in the feces, and the fact that envelope proteins cannot be coded for all show that HEV is not an enveloped virus. Recent research has shown that the virus released from infected cells and circulating in the blood takes on a "quasienveloped" form called "eHEV" that is linked to a membrane. Under an electron microscope, these particles look like viruses with envelopes. HEV capsids are surrounded by membranes made by the host. eHEV particles have both the ORF2 and ORF3 proteins, while HEV particles do not. But both are hidden behind membranes made by the host and can only be seen by certain antibodies after detergents break down the membranes. eHEV is the only type of HEV that can be found in blood, which shows that it is the one that causes HEV to spread inside the host.

The most likely scenario is that the virus takes over the machinery of the endosomal sorting complex needed for transport (ESCRT). This makes a vesicle that looks like an exosome and carries the viral capsid and ORF3 protein. A common way for enveloped viruses to spread is by taking over ESCRT parts. This process can't happen without the HEV ORF3 protein. At the end of ORF3, there is a proline-serine-alanine-proline (PSAP) late domain

motif that interacts with the cell's TSG101 (tumor susceptibility gene-1) protein. This protein recruits complexes that help the HEV capsid break apart into multivesicular bodies (MVB). After the MVB fuses with the plasma membrane, a single eHEV particle that is wrapped in a membrane is released. As part of the process of making exosomes, eHEV membranes have tetraspannins like CD63, CD81, and CD9. During eHEV biogenesis, it seems likely that some host proteins are also packaged, but their identities and roles in the HEV life cycle are still mostly unknown. Most likely, the HEV particles in feces that don't have membranes came from eHEV that were released by the bile canaliculi, but their membranes were washed away by the bile. Because they don't have a lipid membrane, unwrapped viruses can last much longer in the environment, which makes it easier for them to spread to new hosts. The development of two types of the virion is a key part of how HEVs live. So, it's important to know how each type of virus contributes to the process of infection

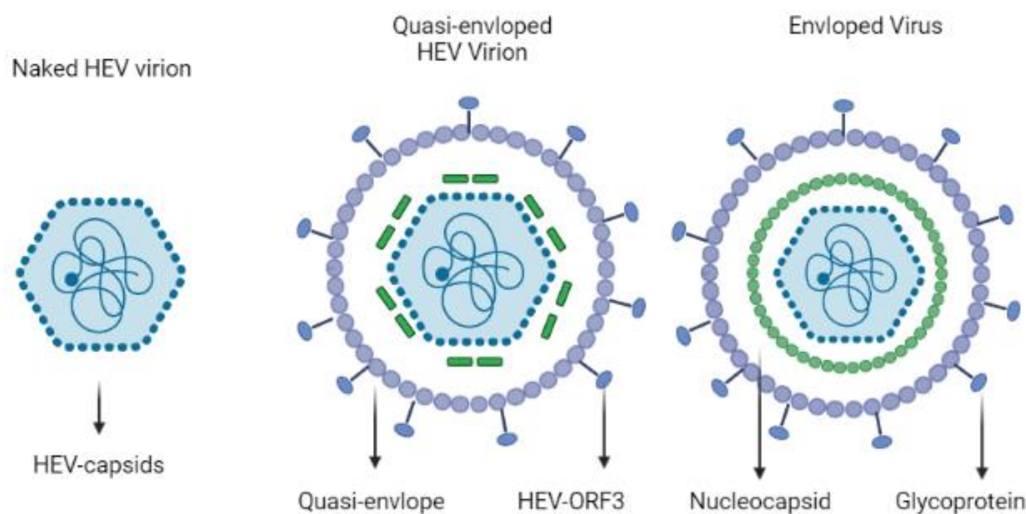


Figure 2.2: Types of HEV found during an infection. The presence of various virion structures is imperative to the unique infection process demonstrated by the Hepatitis E virus.

2.3 Genomic Replication Strategy:

After HEV enters the cell, the host ribosome translates the ORF1 gene. The ORF1 is capable of replicating and transcribing HEV genomic RNAs. Sub genomic RNAs simultaneously encode ORF2 and ORF3. ORF2 is a capsid protein that is translated from separate start codons into two forms, secretory and capsid. ORF2 can serve as a decoy to block neutralizing antibodies or exhaust the immune system. Capsid ORF2 can bind genomic RNA and create viral particles by oligomerizing. ORF3, an ion channel that may be phosphorylated and palmitoylated to form oligomers, is necessary for viral particle release from host cells. ORF3 can interact with both ORF2 and tumor susceptibility gene 101 (Tsg101), a component of the ESCRT machinery. Thus, the viral particle is carried via the exosomal route by multivesicular bodies (MVBs), which then fuse with the plasma membrane to release quasi-enveloped viruses from cells. The quasi-enveloped viruses released from the apical domain of hepatocytes reach the bile duct, where the lipid envelope is destroyed by detergents in the bile, whereas the quasi-enveloped viruses released from the basolateral domain enter the blood. Replication, assembly, and release of the hepatitis E virus (HEV) are depicted using a schematic (Ju & Ding, 2019).

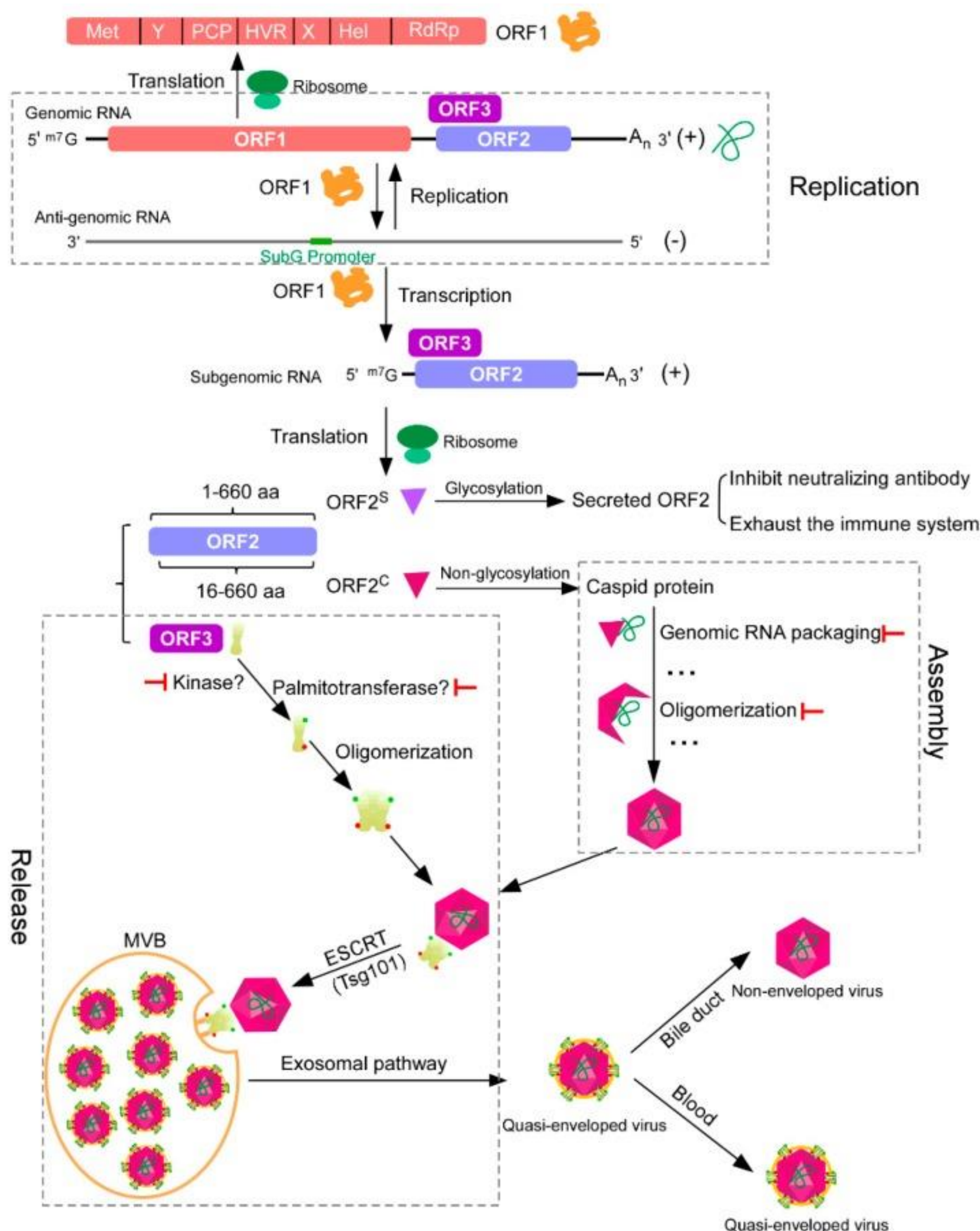


Figure 2.3: The genome replication strategy of the Hepatitis E virus. Host ribosomes translate ORF1 into a polyprotein that includes the RdRp after viral entrance and uncoating. A full-length (-)-sense genome is produced by the RdRp from the (+)-sense strand. Two (+)-sense RNAs are produced using the (-)-sense genome as a template: a full-length transcript and a subgenomic (sg) RNA that encodes ORFs 2 and 3. The subgenomic promoter is where the latter is transcribed from (SgP). The subgenomic RNA is converted into the HEV viroporin and capsid protein which is used for virus packaging and release.

2.4 Genotypes:

The Hepatitis E virus (HEV) belongs to the Hepeviridae family, which is further divided into two genera based on sequence divergence: **Orthohepevirus** and **Piscihepevirus**. The cutthroat trout virus is the only member of the genus Piscihepevirus. The genus Orthohepevirus contains avian and mammalian HEVs, which are further split into four separate species, Orthohepevirus A-D. The host range and sequence identities are used to classify these species.

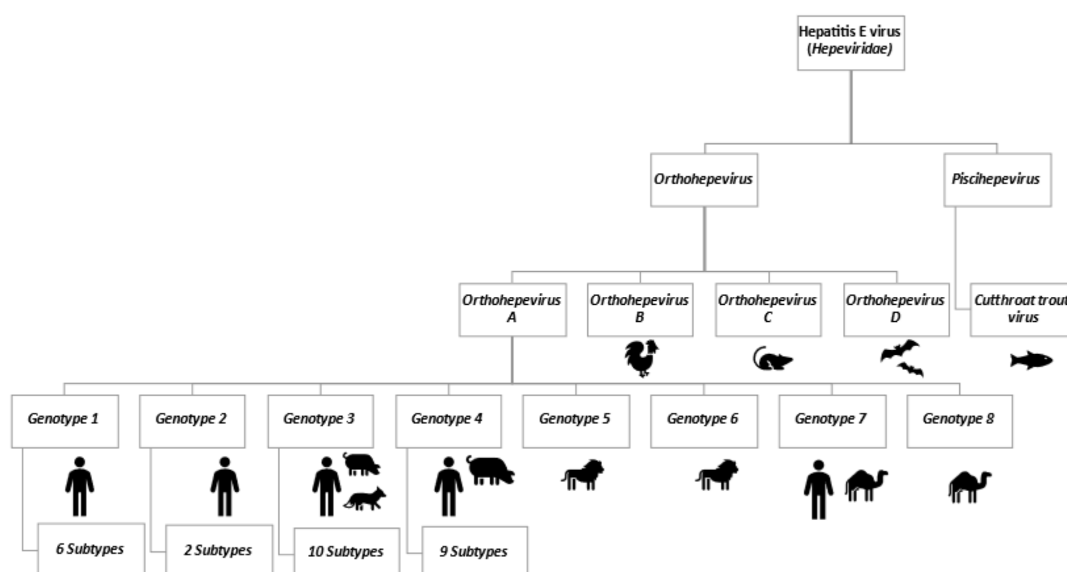


Figure 2.4: Classification of the Hepatitis E virus. The Hepeviridae family is further divided into two genera **Orthohepevirus and Piscihepevirus**. The Orthohepevirus is further divided into eight genotypes.

Orthohepevirus A is made up of at least seven HEV genotypes (HEV-1 to HEV-7) and can infect humans as well as a variety of other animal species such as pigs, boars, deer, mongooses, rabbits, and camels. Genotypes 1 through 4 infect people, with Genotype 1 and Genotype 2 being human-specific and Genotype 3 and Genotype 4 being zoonotic, infecting humans as well as various other animal species. So far, Genotype 5 and Genotype

6 have only been found in wild boars. Genotype 7, a camelid-borne virus, has recently been shown to infect humans and induce chronic illness in a liver transplant recipient. Orthohepevirus B, often known as avian HEV, predominantly infects hens and causes hepatitis-splenomegaly syndrome with reduced egg production. Members of the Orthohepevirus B family have recently been found in wild birds. Avian HEV appears to be incapable of infecting mammals. The Orthohepevirus C species, which includes isolates from rats, larger bandicoots, Asian musk shrews, ferrets, and minks, is not considered to infect humans. The Orthohepevirus D species is made up of bat isolates.

2.5 Epidemiology:

In endemic and non-endemic nations, HEV infection demonstrates two different epidemiological patterns. In the tropical and subtropical regions of Asia, Africa, the Middle East, and Central America, the illness is highly endemic and mostly spread by the fecal–oral route. The Spread of the virus from the mother to the child (Maternofoetal) and parenteral routes of infection are uncommon.

Infection with HEV results in both acute sporadic hepatitis and epidemics, with clinical manifestations ranging from self-limiting hepatitis to subacute and acute liver failure. The virus infects young adults most frequently and can cause severe illness in pregnant women and those with preexisting CLD. The majority of infections in endemic locations are caused by HEV genotypes 1 and 2. In contrast, in industrialized non-endemic countries such as the United States, Europe, and Japan, HEV infection generates uncommon sporadic cases affecting the elderly disproportionately. Previously, it was assumed that hepatitis E in industrialized nations was related to travel to underdeveloped nations; however, autochthonous infection is now a well-recognized reality.

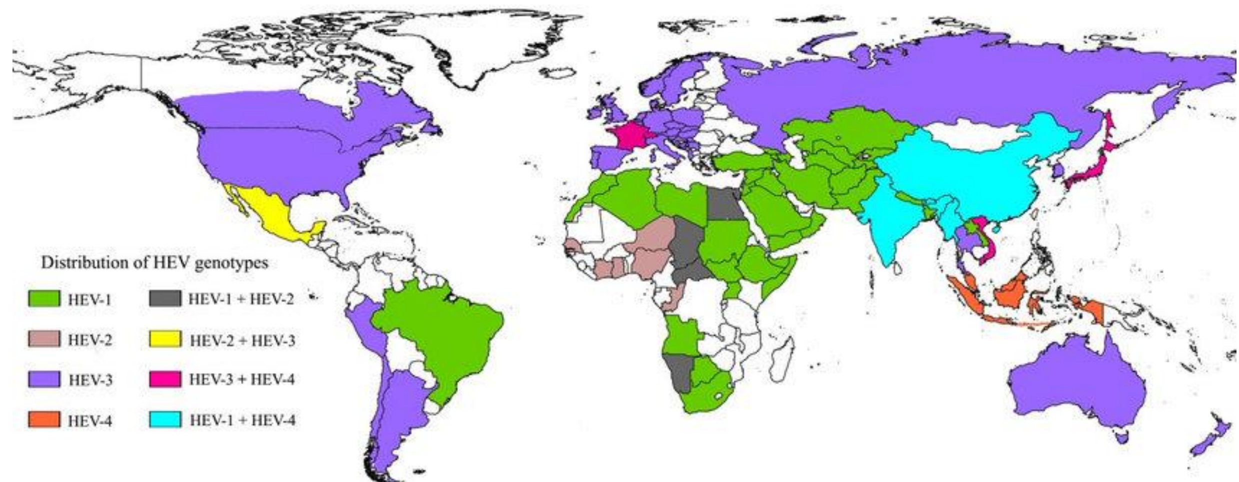


Figure 2.5: Global distribution of the Hepatitis E virus. The distribution of HEV genotypes (HEV-1 through -4) worldwide is shown on the map using various colors.

2.6 Modes of Transmission:

Several different methods exist for the spread of HEV, including contact with contaminated food and water, receiving a transfusion of blood, and maternal transmission. Patients are infectious during fecal shedding, notwithstanding the low risk of transmission between individuals. How a given genotype is passed on can vary greatly depending on the sort of organism it is. Above, we offered further detail about the different HEV genotypes.

2.6.1 Waterborne infection

In endemic locations, HEV genotype 1 and 2 infections are transmitted through fecally contaminated water. Thus, in resource-poor nations with insufficient sanitation and water purification facilities, there is a significant rate of lifetime HEV exposure associated with HEV genotypes 1 and 2 (and probably genotype 4) infection.

Acute hepatitis caused by an infection transmitted by water may manifest as an endemic condition with a high cumulative lifetime risk of exposure. This pattern is observed in the Nile River valley and is believed to be the result of tainted ground water from shallow wells. In other regions of the world (such as Africa, India, and Bangladesh), endemic

waterborne diseases persist at a baseline incidence but are interrupted by explosive outbreaks of acute HEV-associated hepatitis.

2.6.2 Zoonotic transmission

Infections caused by HEV genotypes 3 and 4 typically result from the ingestion of contaminated food. Most cases are sporadic and classified as acute viral hepatitis; nonetheless, occasional outbreaks have occurred, including an HEV epidemic linked to shellfish eating that impacted cruise passengers. The discovery of a significant anti-HEV seroprevalence in persons with occupational contact with animals also lends credence to the theory of zoonotic HEV transmission. The most common source of transmission is swine, followed by the eating of filter-feeding shellfish. Nonetheless, several animal species (including rats in certain places) have been recognized as the viral reservoir of illness. In Japan and parts of Europe, HEV transmission has been linked to the ingestion of undercooked deer meat, wild boar meat, pig liver sausage, and animal organs (e.g., Germany and France). Cow milk is significantly identified as a probable source of HEV genotype 4 in China, according to one study. In addition, HEV transmission to rhesus macaques has been shown following the administration of both raw and pasteurized milk. There are little data on the effectiveness of food preparation in reducing or eliminating HEV transmission. In one research, heating the liver at 191°F for five minutes or boiling the liver for five minutes inactivated HEV, therefore reducing the probability of transmission. It has been hypothesized that the lower transmission rates of genotype 3 HEV reported in the United States compared to Europe may be attributable to higher consumption of pre-processed/cooked commercial pig products, which are utilized less frequently in Western Europe.

2.6.3 Blood transfusion

Blood transfusions can spread HEV, particularly in endemic regions. In research evaluating the frequency and transmission of HEV in 225,000 blood donations, 79 HEV genotype 3 RNA-positive blood donations were identified. These contributions allowed for the preparation of 129 blood components, of which 62 were transfused. In 18 (42 percent) of 43 individuals who underwent follow-up testing, HEV infections were discovered. In a second investigation of blood samples from North American (United States and Canada) blood donors, HEV RNA was detected in 1 of 16,908 US donor samples, with greater levels in Canadian samples.

2.6.4 Perinatal transmission

Unfortunately, little is known about the vertical transmission of HEV from infected women to their infants. Transfer of HEV from mother to child causes significant instances of perinatal morbidity and mortality, yet this element of the disease's impact on the worldwide disease burden is often downplayed.

2.6.5 Transmission in breast milk

The potential for HEV transmission through breastfeeding is not established. However, until more information is available, nursing should be avoided by HEV-infected mothers. In one reported case, HEV was found in breast milk during the acute stage of HEV infection. Milk and serum HEV titters were comparable.

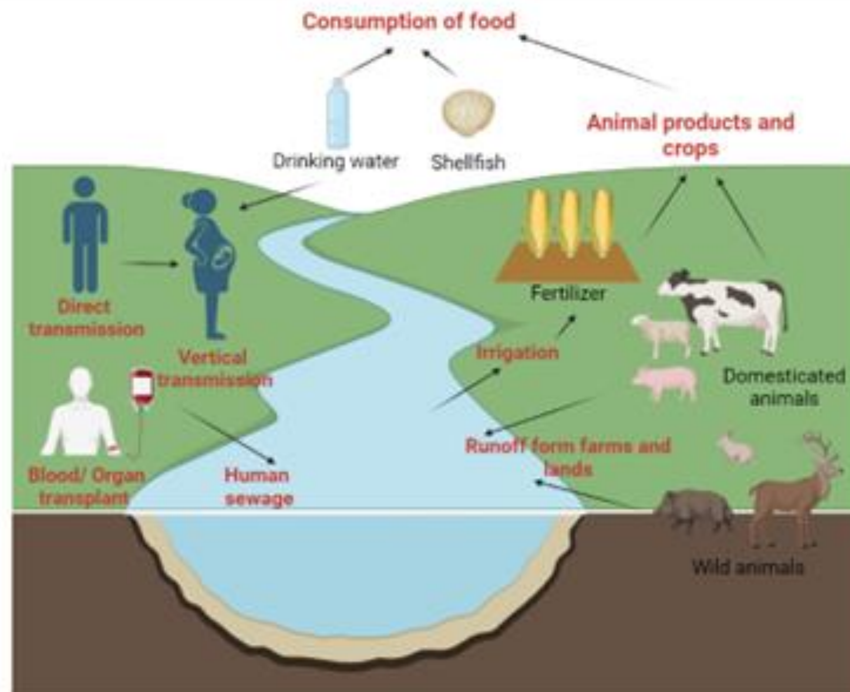


Figure 2.6: The verified and theorized HEV transmission pathways to humans. Theoretical modes of transmission include eating shellfish, sheep, and cows, as well as crops and drinking water, even though no verified outbreaks have occurred from these sources

2.7 Clinical manifestations:

2.7.1 Acute Hepatitis E

The majority of people who contract HEV will experience a self-limiting acute infection, although a severe form of liver failure called acute hepatic failure has been reported in a few cases.

- **Clinical features**

There is a wide variety of time frames for how long HEV infections can lay dormant, anywhere from 15 days to 60 days. Most people with acute HEV have no symptoms or rather mild ones. People of different ages and degrees of previous HEV exposure have different rates of developing clinical symptoms after an acute infection. Patients with jaundice symptoms often experience fatigue, loss of appetite, sickness, vomiting, stomach pain, a high body temperature, and enlargement of the liver. Diarrhea, arthralgia, pruritus,

and urticarial rash are additional symptoms that are seen less frequently. Additionally, patients may also exhibit extrahepatic manifestations on rare occasions.

- **Laboratory findings**

  Elevated levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are found in the blood. Serum ALT levels spike at the onset of symptoms and can climb into the hundreds before returning to normal during recovery. Within a week to six weeks following the onset of sickness, abnormal biochemical tests typically normalize on their own.

- **Complications**

  Most people who contract HEV will eventually be able to rid their bodies of it on their own. However, patients run the risk of developing consequences such as acute hepatic failure, cholestatic hepatitis, and chronic HEV.

- **Acute hepatic failure**

  Acute hepatic failure occurs in only a modest percentage (0.5 to 4 percent) of HEV-infected people. Pregnancy, malnutrition, and pre-existing liver disease all increase the risk of developing acute hepatic failure. Liver encephalopathy, increased aminotransferases (frequently accompanied by aberrant bilirubin and alkaline phosphatase levels), and reduced synthesis function (international normalized ratio 1.5) are all hallmarks of acute hepatic failure. Without access to intensive care and liver transplantation, patients with acute hepatic failure have a case fatality rate of between 0.5 and 3 percent.

- **Cholestatic hepatitis**

  Up to 60% of individuals with acute HEV have been reported to experience prolonged cholestasis, which is defined by a lengthy duration of jaundice (lasting >3 months). Itching is a common sign of cholestasis; however, patients may experience no other symptoms at all. Over the course of a few weeks to a few months, cholestatic hepatitis typically goes

away on its own. Clearance of the virus, rising IgG anti-HEV titters, and falling IgM anti-HEV levels are all signs of improvement and recovery.

2.7.2 Chronic hepatitis E

After six months of HEV RNA detection in serum or stool, the infection is considered chronic. Only those with compromised immune systems get chronic HEV (e.g., those with HIV infection, following solid organ or bone marrow transplantation). Although persistent HEV infection with genotype 4 has been observed in a transplant recipient, infection with genotype 3 is more common. Genotype 1 and 2 HEV infections that persist over time have not been documented. Until the progression to decompensated cirrhosis, patients with chronic viral hepatitis typically have only mild symptoms, such as fatigue and nonspecific observations. Patients with chronic HEV have aminotransferase levels that remain increased over time, detectable serum HEV RNA levels, and histologic abnormalities that are consistent with chronic viral hepatitis.

2.8 Diagnosis:

HEV can be difficult to diagnose since its clinical signs and symptoms often coincide with those of other causes of hepatitis. Direct detection of the virus, viral proteins, or nucleic acids by immune-electron microscopy or RT-PCR, or indirect diagnosis by identifying the presence of **anti-HEV IgG and/or anti-HEV IgM antibodies,** are the two basic classifications of diagnostic procedures.

Anti-HEV IgM antibodies are produced and are detectable 4 days after the onset of jaundice and last for up to 3 to 5 months. Detection of anti-HEV IgM antibodies in the patients' specimens is indicative of acute infection. Shortly after the appearance of IgM antibodies, **anti-HEV IgG antibodies** are produced by the immune system and reach a peak level at about 4 weeks after the onset of the symptoms. These antibodies persist in the body for up to 14 years hence diction of anti-HEV IgG antibodies can help point to previous

infection of the virus. The detection of **HEV RNA** form serum or stool samples from patients is considered the "gold standard" for confirmation of acute HEV infection. The RNA can be detected in the stool samples 1 week before for up to 6 weeks after the onset of clinical symptoms. The RNA can also be found in the serum for 3 to 4 weeks form the onset of illness. The time points of appearance of particular immune markers during the course of HEV infection can be seen in the figure below.

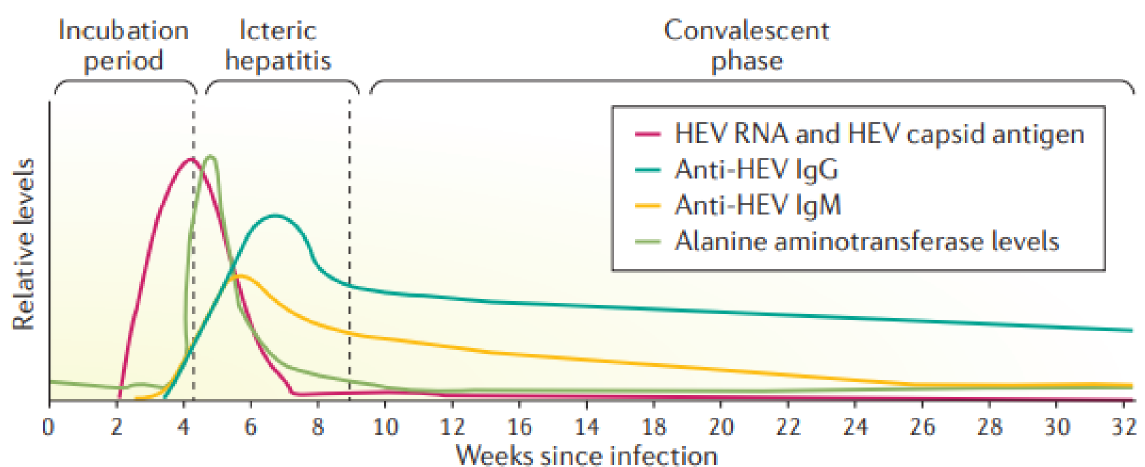


Figure 2.7: HEV infection and hepatitis E. Appearance of hepatitis E virus (HEV) RNA, capsid proteins, and antibodies during HEV infection. IgG, immunoglobulin G; IgM, immunoglobulin M.

When HEV infection is suspected based on clinical symptoms or an isolated rise in alanine aminotransferase, anti-HEV IgM is detected initially due to its high sensitivity and widespread availability. When the anti-HEV IgM is negative and alanine aminotransferase activity is elevated, when the HEV RNA in blood and stool persists for 3–6 months (to identify a chronic infection), and when a recent reduction in immunosuppression has been made or antiviral therapy has been initiated, immunocompromised patients should be tested for HEV RNA (to monitor chronic infection). Ribavirin treatment is used to eradicate HEV.

A negative HEV RNA concentration in the feces is required for viral clearance to be confirmed. HEV RNA testing can also be utilized in immunocompetent patients to confirm continued infection in the absence of anti-HEV IgM when a strong suspicion of infection exists or to undertake viral genotyping.

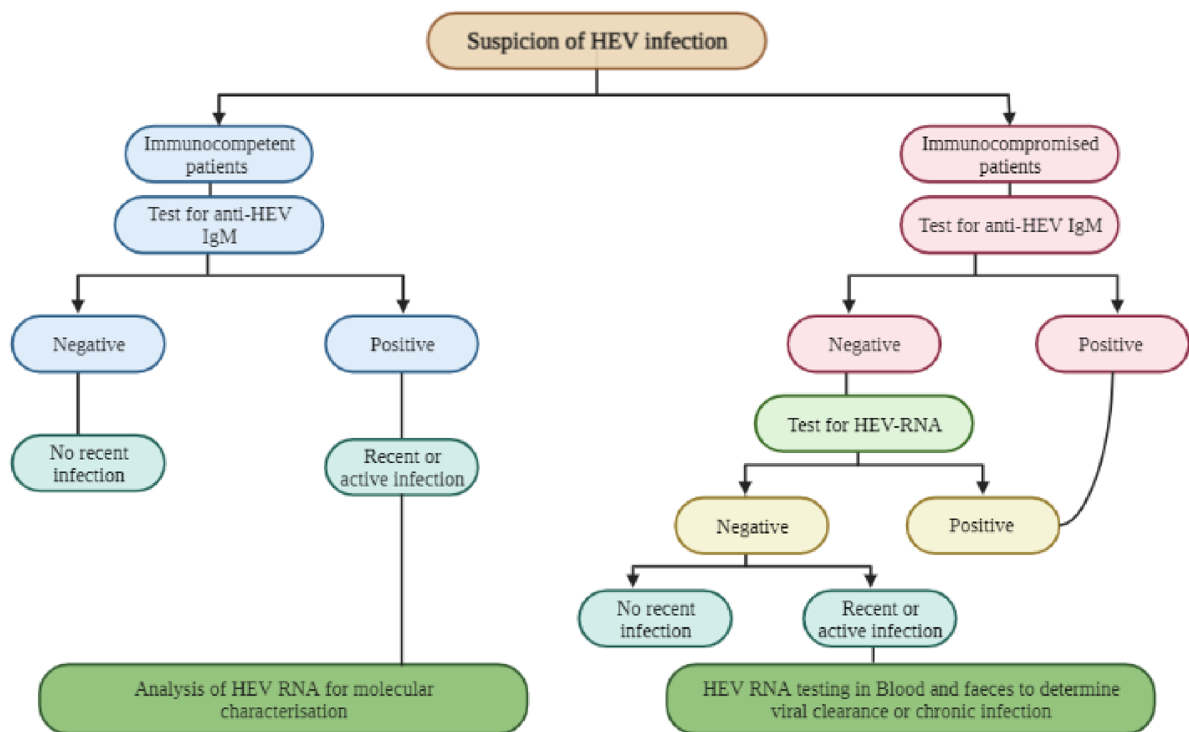


Figure 2.8: Diagnostic algorithm of HEV. The identification of anti-hepatitis E virus (HEV) immunoglobin IgM antibodies are used in the initial diagnosis of HEV, but further confirmation is performed by testing for viral RNA.

2.9 Special populations

Pregnant women

In regions where HEV infection is endemic, acute hepatic failure occurs more frequently when HEV occurs during pregnancy. Hepatic failure is more common during the third trimester. Acute HEV infection during pregnancy has been associated with a mortality rate of 15 to 25 percent. However, exposure to HEV early in life may reduce the risk of acute

liver failure in pregnant women who become reinfected later in life. Poor outcomes in pregnancy may also be due in part to poor nutritional status and lack of access to supportive medical care.

Pre-existing liver disease or malnutrition

Infection with HEV can lead to hepatic decompensation in patients with pre-existing liver disease (ie, acute to chronic liver disease) and in those who are malnourished.

Solid organ transplant recipients

A subset of patients who undergo solid organ transplantation (eg, kidney, liver, and kidney-pancreas) appear to develop chronic HEV infection.

HIV and other immunosuppressed hosts

Case reports have described chronic infection with HEV in patients with HIV infection and patients with non-Hodgkin lymphoma receiving rituximab, suggesting that immunosuppression predisposes patients to chronic infection outside of the transplant setting. Among HIV-infected patients, there appears to be a higher rate of HEV exposure as compared with the general population; however, overall rates of chronic HEV infection appear to be relatively low.

2.10 Prevention

- **General measures**

  It is recommended that travelers take standard precautions for preventing traveler diarrhea when visiting areas where HEV is endemic (e.g., Asia, Africa, the Middle East, and Central America). Avoiding raw or undercooked fish, beef, pork, and vegetables is also part of this advice, as does drinking water from unregulated sources and eating from street sellers. If you're visiting Europe, it's best to steer clear of any raw or undercooked pork/boar sausage or other wild animal foods (like a rabbit) that could be available.

- **Vaccines**

The effectiveness of recombinant vaccines against HEV has been proven. One Chinese study randomly allocated 112,604 healthy adults to receive either three doses of HEV recombinant vaccination or a hepatitis B vaccine as a control. Over 12 months following vaccination, the vaccine was 96% effective in protecting against HEV. The vaccine was 87% effective 4.5 years after the initial dose. China is the only country where you can legally purchase this vaccination. Even though in vitro action against nonhomologous genotypes (2–4) has been demonstrated, it is still unknown whether this vaccine will protect against all prevalent genotypes.

- **Immune globulin**

  The efficacy of pre- or postexposure immune globulin prophylaxis for the prevention of HEV has not been established.

2.11 HEV in Pakistan

Pakistan is hyperendemic for Hepatitis E virus infections. The disease presents itself both as outbreaks and sporadic cases of viral hepatitis. Pakistan has been at the epicenter of numerous outbreaks, most of which have been traced to the contamination of drinking water. In Pakistan, HEV is extremely endemic, affecting mostly the adult population, and instances of hepatitis E occur sporadically throughout the year. However, there have been reports of local outbreaks and epidemics of hepatitis E in Peshawar, Mardan, Abbottabad, Rawalpindi-Islamabad, Sargodha, Multan, Hyderabad, Quetta, and Karachi.

- Six hundred people with hepatitis E were admitted to a hospital in Lahore, Pakistan, in the early nineteen nineties. In 1972, an army regiment had an epidemic of acute viral hepatitis during field training. Over 250 cases of acute viral hepatitis were reported within 3 weeks, and all victims had used the same water source (untreated river water).
- Extremely rapid transmission of the HEV virus through water occurred in two locations in Islamabad, Pakistan, during December 1993 and the first three months of 1994. Out of a

total of 36,705 participants, 3,827 (10.4%) were found to have acute icteric hepatitis. The water supply analysis confirmed that the outbreak was spread through the water system. The case fatality rate was 11.4%, with all four deaths occurring in pregnant women in their third trimester. The other four deaths were in infants born to mothers who had acute icteric hepatitis. It is not compatible with a person-to-person transmission pattern of the disease given 83.7% of 1,463 alleged subsequent cases occurred within one month of the first incidence in the same household.

- • Tertiary care teaching hospitals Khyber Teaching Hospital and PGMI Lady Reading Hospital in Peshawar, Pakistan were the sites of a 2002 study. Of the 148 individuals diagnosed with acute hepatitis, 21 (or 14.1%) were found to have hepatitis E infection. Most people experienced the mild, self-limiting condition. The prevalence of hepatitis E antibodies in pregnant women was determined by testing 65 women. We found that 57% of pregnant women with jaundice tested positive for HEV, that HEV is endemic in Karachi, and that it is most common in the third trimester of pregnancy.
- • Human enterovirus (HEV) was linked to fecal pollution of a water supply in Abbottabad, Pakistan. The majority (104/109, 95%) of the 109 men hospitalized with hepatitis had acute hepatitis E, as determined by serologic testing. Anti-HEV antibodies were present in this population at a rate of 30% before the epidemic. Anti-HEV IgG levels were associated with decreased risk of developing clinical hepatitis and new HEV infection. An HEV isolate likely distinct from the one responsible for the Sargodha outbreak produced this. One HEV strain found in Sargodha's feces has been genetically linked to Chinese isolates that likely originated in Pakistan and crossed the Hindu Kush Mountains on route to China (emhj, 1996.)

Table 2.1: Outbreaks of HEV in Pakistan

Name of city	Year	Study group
CENTO region	1972	Army battalion
Islamabad	1993	General population
Lahore	1995	Military personnel
Karachi	2001	Pregnant with Jaundice
Peshawar	2002	Admitted to hospital
Abbottabad	2002	Hospitalized Military personnel

2.12 Genotyping of HEV form Pakistan

Keeping in mind the hyperendemic nature of the Hepatitis E virus in Pakistan, relatively few isolates have been reported. **Sar-55 (87-Pakistan -A)** was the first isolate, reported form Pakistan. It was sequenced form samples collected form an outbreak of Hepatitis E virus in Sargodha in 1987 (M. Iqbal et al., 1989). The isolate was proposed to belong to the central Asian cluster of genotypes 1. (Shahzad et al, 2001.)In **2006**, Using archived fecal samples from the 1987 outbreak, a new isolate **87-Pakistan-B** was obtained. Sequence analysis indicated a 99% sequence similarity to the initial isolate, Sar-55 (87-Pakistan -A), obtained from Sargodha. It was proposed that the 87-Pakistan-B might have originated as a variant form 87-Pakistan-A HEV that was endemic in Sargodha, Pakistan, in 1987.

In **2000,** fecal samples from an outbreak of HEV form Abbottabad in 1988 were phylogenetically analyzed to place the **Abb-2B(88-Pakistan-2B)** isolate into the South Asian cluster of genotype 1. These findings brought to light some interesting conclusions. It was believed that the **Abb-2B(88-Pakistan-2B)** isolate was an earlier phylogenetic representative of an HEV cluster, that spread successfully across South Asia. **Sar-55 (87-Pakistan -A)** on the other hand was a late derivative from the central Asian cluster,

ancestral isolates of which caused large outbreaks in China. So, it was speculated that **Sar-55 (87-Pakistan -A)** was an unusual introduction form China to the north and **Abb-2B(88-Pakistan-2B)** represents the true HEV strain that is endemic in Pakistan. Hence sequence analysis of further HEV isolates form both new and old sporadic cases of Hepatitis E was required to confirm these speculations (He, 2006).

A prospective study in the year **2007** was carried out to determine the molecular epidemiology of HEV during the two mini-outbreaks (March 2007; first mini-outbreak and December 2007; second mini-outbreak) form Lahore. Two HEV isolates namely, **L-7(FJ959399)** and **L-11 (FJ959398)** were obtained from the investigation. Both the isolates shared 88% and 90% sequence similarity to the **Sar-55 (87-Pakistan -A)** than to other Pakistani HEV isolates (T. Iqbal et al., 2011). Although these isolates were obtained from geographically distinct sites fecal-oral means of HEV genotype 1 transmission may be the leading cause of dissemination of the virus to far-off places in the country. Hence it was concluded that **Sar-55 (87-Pakistan -A)** was the main endemic HEV strain in regions of Pakistan.

Another investigation to further characterize HEV form Pakistan was conducted in Karachi form December **2007 to July 2008**. The data showed the isolates to cluster into two independent and phylogenetically distinct clusters into genotype1 namely, variant 1 (**AB513503-AB513530**) and variant 2 (**AB513496-AB513502**). The new strains were found to be more closely related to strains from Nepal, India, and Burma than to previously reported stains from Pakistan. This led to the theory that different epidemiological strains were circulating in different parts of the country. Another aspect of the genetic variability seen in HEV isolates that were proposed was that because HEV was constantly spreading in Pakistan, there was plenty of room for evolution in infected individuals. The lack of significant amino acid substitutions suggests that HEV genomic mutations may happen

naturally in infected people without much help from the host's immune system, and that the observed pattern of divergence may be caused by forces that don't let amino acid substitutions happen (A. Khan et al., 2011a).

To better understand the etiology of epidemic vs sporadic instances, as well as the connection of the many HEV isolates in the Pakistani region, additional study into the level of genetic diversity among isolates recovered from different epidemics and cases of sporadic acute hepatitis E, is needed.

Table 2.2: History of HEV genotyping in Pakistan

Year	HEV isolates/Strains form Pakistan	GenBank
1987	Pakistan- Sargodha- Sar-55 (**87-Pakistan -A**)	M80581
1987	Pakistan- Sargodha- **87-Pakistan-B**	AY160125
1988	Pakistan- Abbottabad- Abb-2B- **88-Pakistan-2B**	AF185822
2007	Pakistan-Lahore (L7 and L11)	FJ959399 and FJ959398
2007 to 2008	Pakistan-Karachi- South Pakistan	AB513503-AB513530 and AB513496-AB513502

MATERIALS AND METHODS

3.1 Ethics Statement:

The study was approved by the Internal Review Board (IRB) with IRB # 06-2021-01/03 of the Department of Microbiology, The Islamia University of Bahawalpur. Verbal and written informed consent was obtained from all participating individuals before sample collection. The samples were de-identified and analysis was performed anonymously.

3.2 Amplification of Positive Control Plasmid DNA:

3.2.1 Competent BL21(DE3) is an E. coli B strain:

Preparation of Reagents needed for the preparation of competent cells

- Autoclaved LB media (0.5g of Yeast Extract, 1g of Tryptone, and 1g of NaCl for 100ml of Media)
- Solution A (0.1M $CaCl_2$)
- Solution B (0.1M $CaCl_2$ + 15% glycerol)

Procedure

Competent cells were prepared following the following protocol

- A single colony of the BL21(DE3) strain of E. coli was picked from a cultured plate of cells using a sterile loop, and the colony was then inoculated in a flask containing 50ml LB media. After that, the flask was placed in a shaking incubator for overnight incubation at 37 degrees Celsius.
- The next day, the secondary inoculum was prepared by combining 2ml of primary inoculum with 50ml of LB media in another flask.
- It was incubated for 2 hours at 37 degrees in a shaking incubator.

- The optical density of this secondary inoculum was checked using a spectrophotometer. The optimal OD range is from 0.4 to 0.6 and these cells can be used for transformation. as it indicated the growth phase of the bacteria.
- The inoculum was then centrifuged at 4 degrees, 6000rpm for 10 minutes by transferring it from a flask into a chilled 50ml falcon tube, to obtain cells pellet.
- The supernatant was discarded, and the cells were resuspended in 10 ml of chilled Sol A (0.1M $CaCl_2$).
- The falcon was again centrifuged, and the pellet was now re-suspended in 1ml of Sol B (0.1M $CaCl_2$ + 15% glycerol).
- Aliquots of 100ul were made in separate microfuge tubes and stored at -80 degree centigrade to be used for plasmid transformation.

3.2.2 Transformation of competent BL21(DE3) cells:

Preparation of Reagents needed for the preparation of competent cells

- Autoclaved LB Agar (1.5 g Agar, 1g NaCl, 1g Tryptone and 0.5g Yeast extract for 100ml agar)
- Autoclaved LB Media (0.5g of Yeast Extract, 1g of Tryptone, and 1g of NaCl for 100ml of Media)
- Ampicillin

Method:

- BL21 competent cells were thawed on ice followed by the addition of UC1 plasmid DNA according to the following ration
  - 7 to 10ul for 100ul of BL21 competent cells
  - 5ul for 50ul of BL21 competent cells
- The components were gently mixed by using a pipette and incubated on ice for 30 minutes.

- Heat shock was provided to the cells by placing then in a water bath heated at 42 degrees for 2 minutes.
- The cells were immediately placed on ice for 5 minutes.
- 500ul of room temperature LB media was added to the cells and inverted once
- The cells were incubated for 1 hour and 15 minutes at 37 degrees in a shaking incubator.
- In the meantime, LB agar plates were made by adding 25ml of agar media having 25ul of ampicillin and was poured onto the Petri plate. The UC1 plasmid showed to grow well under high antibiotic concentrations.
- Then 100ul of transformed cells were spread on the ampicillin plate using a sterile spreader and were incubated overnight (about 16 hours) at 37 degrees.
- The next day, transformed colonies appeared after incubation.

3.2.3 Colony PCR

To confirm the uptake of the plasmid DNA, colony PCR was carried out using a primer set targeting a 412bp region in the plasmid. The details of the primers are given in table 3.1.

Table 3.1: Primers used for the detection of plasmid uptake by the competent BL21 cells

Primer name	**Primer Sequence (5' to 3'**	**Annealing temperature**	**Amplicon size**
PREDICT-Fwd	GGGCCTAGAGAAGATATTTGTACT	55°C	412bp
PREDICT-Rvs	CGCCATTGACATCCTCGAAG		

Method:

- Then transformed colonies were picked and suspended in PCR tubes containing 10ul of NF water.
- The PCR tubes were vortex to lyse the colonies.
- The PCR tubes were then incubated in the water bath for 10 minutes at 95 degrees for further lysis using heat.
- The PCR reaction was carried out using the DFS-Taq DNA polymerase (Cat. No Rec14102016_EN). The PCR reaction mixture was composed of 5 µl 10x Reaction Buffer, 1 µl 10mM dNTPs, 3 µl MgCl2, 1.5 ul of 10uM PREDICT-F and PREDICT-R each, 0.5µl Taq polymerase enzyme and 2 µl of plasmid as a template with the total volume of 50µl using PCR water. The reaction was run on SimpliAmp™ Thermal Cycler (A24812). The PCR temperature profile is shown in the figure.

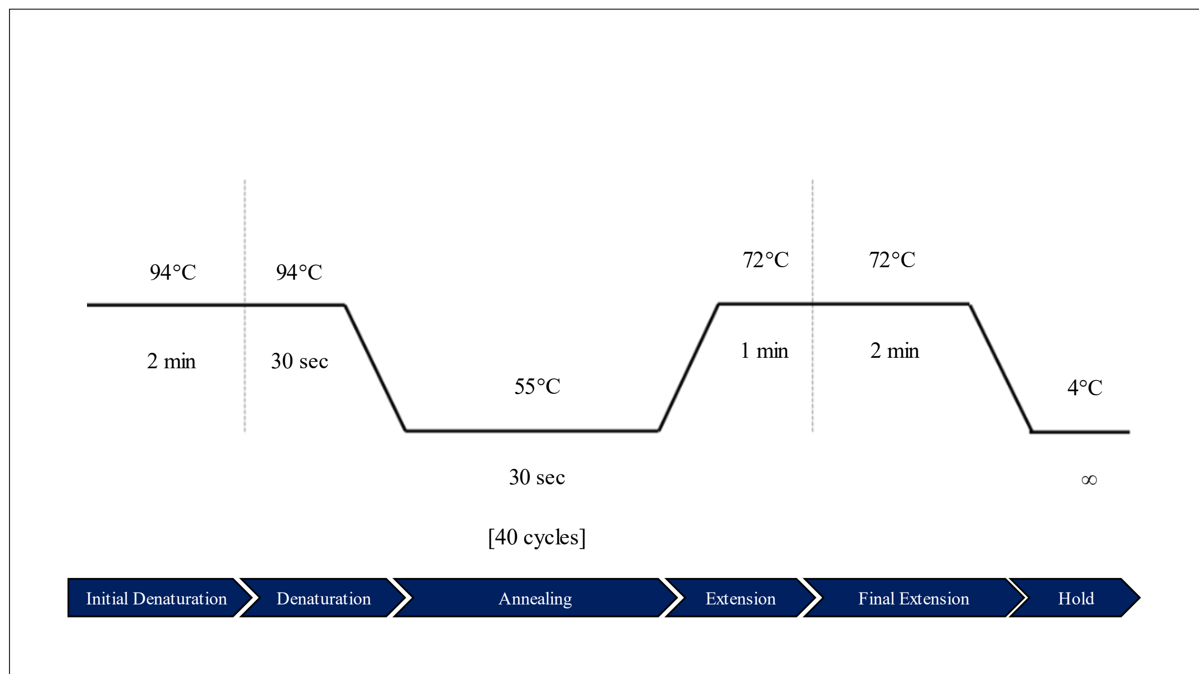


Figure 3.1: PCR thermal cycling temperature profile for PREDICT primers.

3.2.4 Gel Electrophoresis:

PCR product was confirmed using the Gel electrophoresis technique.

Materials:

- 1X Tris-Acetate-EDTA (TAE) Buffer
- Biological grade Agarose (bioWorld)
- Ethidium Bromide

Method:

PCR products were analyzed through agarose gel electrophoresis. 2% agarose gel was prepared in 1X TAE buffer. For this, 2 g agarose was dissolved in 100ml 1X TAE buffer via heating in a microwave. The mixture was allowed to cool to about 60 C and 10µl ethidium bromide was added. The gel was then poured into the casting tray and allowed to polymerize for about 30 mins. After polymerization, the PCR products were loaded along with 1X loading dye in the wells. The gel was run in 1X TAE running buffer at a constant voltage of 90 V for 47 mins. The gels were then visualized using ChemiDoc (ChemiDoc™ MP System #1708280, BioRad, USA).

3.2.5 Miniprep:

Preparation of Reagents needed for miniprep

Materials:

- Alkaline lysis solution I
- Alkaline lysis solution II
- Alkaline lysis solution III
- Transformed colonies
- Ethanol

Table 3.2: Reagents preparation for miniprep

Reagent	Composition
Alkaline Lysis Solution I (Autoclaved and stored at 4°C)	• 50mM glucose • 25mM Tris-Cl (pH= 8) • 10mM EDTA (pH= 8)
Alkaline Lysis Solution II (Freshly prepared for every miniprep and stored at room temperature)	• 5N NaOH = 80ul • 10% SDS= 200ul • dH2O=1120
Alkaline Lysis Solution III (Autoclaved and stored at 4°C)	• 5M Potassium acetate= 1.2ml • GAA= 230ul • dH2O=570ul

Method:

- Inoculate a single colony of transformed bacteria with the appropriate antibiotic in 5 mL of LB-rich media.
- Shake vigorously while incubating the culture at 37°C for the entire night.
- 1.5 mL of the culture should be added to a microcentrifuge tube. Centrifuge for 3 minutes at room temperature at maximum speed to create a cell pallet as shown in figure 3.2. The original culture's leftovers should be kept at 4 °C.

Figure 3.2: Bacterial pellet of transformed bacterial cells. The pellet contains bacterial cells containing the plasmid of interest. Further lysis and purification procedures extract the desired plasmid form other components including but not limited to proteins and bacterial DNA.

- Aspirate off the medium when centrifugation is complete, leaving the bacterial pellet dry. By vigorously vortexing, resuspend the bacterial pellet in 150uL of ice-cold Alkaline lysis solution I.
- Let it sit for five minutes at room temperature.
- Pour 200uL of freshly made Alkaline lysis solution II into each suspension of bacteria. Close the tube tightly, then quickly invert it five times to mix the contents. Avoid vortexing! Keep the tube chilled.
- Proceed by adding 250µL of ice-cold Alkaline lysis solution III. Close the tube and disperse Alkaline lysis solution III through the viscous bacterial lysate by inverting the tube several times. Store the tube for 3–5 min on ice.

- Centrifuge the bacterial lysate at maximum speed for 15 min at 4°C in a microcentrifuge. Transfer the supernatant to a fresh tube ensuring not to move any white debris.
- Precipitate the nucleic acids from the supernatant by adding 500ul of ethanol at room temperature. Mix the solution by vortexing, and then allow it to stand for 2 min at room temperature.
- Centrifuge at maximum speed for 15 min at 4°C in a microcentrifuge to collect the precipitated nucleic acids.
- Remove the supernatant by gentle aspiration.
- Add 100uL of 80% ethanol to the pellet and invert the closed tube several times.
- Recover the plasmid DNA by centrifugation at maximum speed for 10 min at 4°C in a microcentrifuge and remove all the supernatant by gentle aspiration
- Store the open tube at room temperature until the ethanol has evaporated and no fluid is visible in the tube (5–10 min).
- Dissolve the nucleic acids in 30 μL of TE (pH 8.0)
- Vortex the solution gently for a few seconds. Store the DNA solution at −20°C.

3.2.6 Plasmid Nanodrop and PCR to confirm plasmid purity:

After the plasmid purification, the extracted plasmids were tested for purity and concentration by first quantifying using a nanodrop and then another PCR to amplify the PREDICT sequence in the plasmid. This step was used to confirm the integrity of the extracted plasmid during the purification stage along with the purity to access the optimal working of the PREDICT plasmid in a PCR reaction before using the plasmid as a positive control for further viral detection.

3.3 Optimizing the Hepevirus degenerate primers for detection of HEV virus using the transformed plasmid:

The Hepeviruses family-specific primers as described by (Rasche et al., 2016) and (Drexler et al., 2012) for the detection of HEV virus in bats and camels respectively, were optimized for the detection of HEV-causing infection in humans. The details of the primers are given in table 3.3.

Table 3.3: Degenerate primers used for the detection of HEV and product size

Virus	Primer name	Primer Sequence (5' to 3')	Region	Annealing temperature	Amplicon size
Hepevirus	DE-F4228	ACYTTYTGTGCYYTITTTGGTCCITGGTT	Rdrp	52°C	Round 1 371bp
	DE-R4598	CCGGGTTCRCCIGAGTGTTTCTTCCA			
	DE-R4565	GCCATGTTCCAGAYGGTGTTCCA		52°C	Round 2 338bp

The first step in the optimization was the confirmation of the workability of the primers by testing them against a positive control with which a positive result can be obtained and subsequent detection of the virus form the patient samples can be justified. For this reason, the primers were tested using the UC1 plasmid as a positive control as it had Hepevirus-specific sequence inserts in its structure. Both sets of primers for the heminested PCR were tested first for round 1 and then round 2 as described below.

Round 1

The PCR reaction was carried out using the DFS-Taq DNA polymerase (Cat. No Rec14102016_EN). The round 1 PCR reaction mixture was composed of 2.5 µl 10x Reaction Buffer, 0.5 µl 10mM dNTPs, 2 µl MgCl2, 1ul of 10uM DE-F4228 and DE-R4565 each, 0.5µl Taq polymerase enzyme and 1µl of plasmid DNA template with the total volume of 25µl using PCR water. The reaction was run on SimpliAmp™ Thermal Cycler (A24812).

The PCR temperature profile is shown in figure 3.3

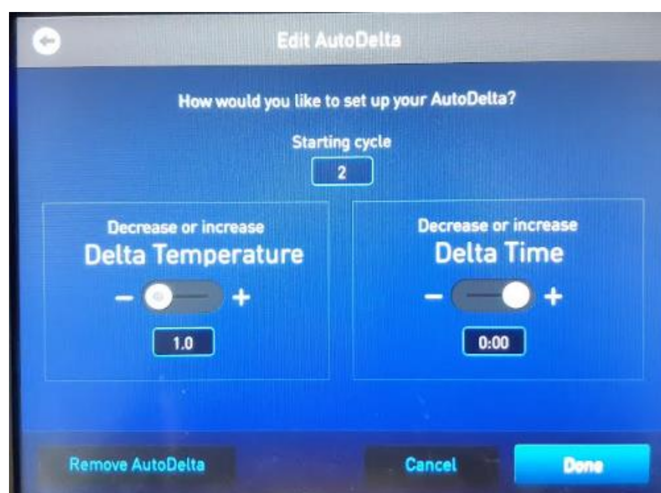


Figure 3.3: Touch down PCR program in the thermocycler. The thermocycler was set to perform a touchdown PCR by enabling the auto-delta mode and programming the ramp rate to decrease by 1-degree centigrade.

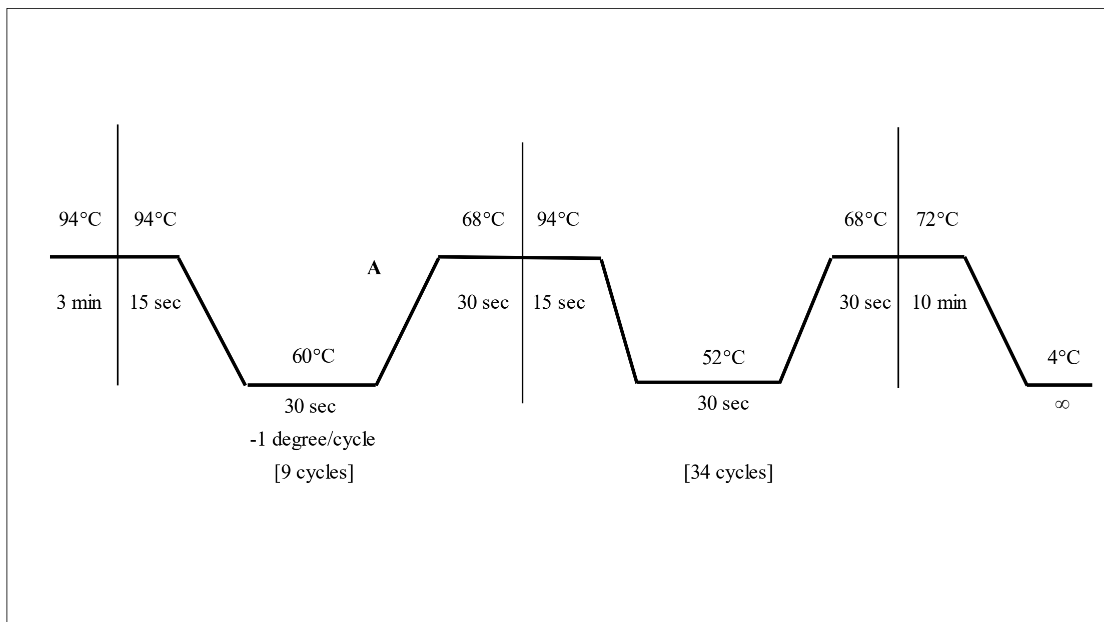


Figure 3.4: Touch down PCR thermal cycling temperature profile for Round 1

Round 2

The PCR reaction was carried out using the DFS-Taq DNA polymerase (Cat. No Rec14102016_EN). The round 1 PCR reaction mixture was composed of 2.5 µl 10x Reaction Buffer, 0.5 µl 10mM dNTPs, 2 µl MgCl2, 1ul of 10uM DE-F4228 and DE-R4565 each, 0.5µl Taq polymerase enzyme and 1µl of plasmid DNA template with the total

volume of 25µl using PCR water. The reaction was run on SimpliAmp™ Thermal (A24812). The PCR thermal profile for Round 2 PCR is given in Figure 3.5.

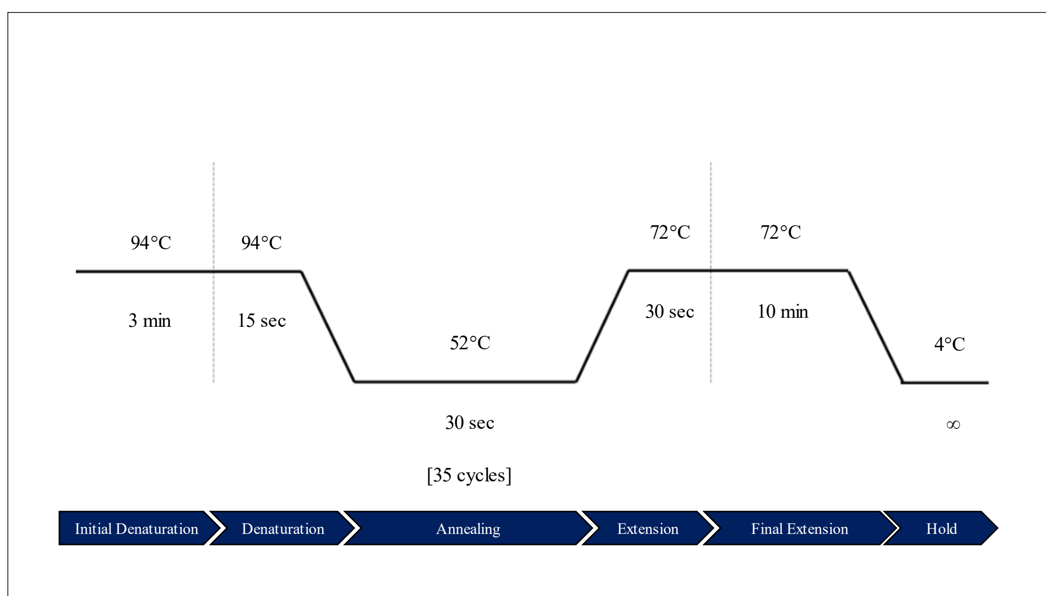


Figure 3.5: Touch down PCR thermal cycling temperature profile for Round 2

3.4 Study locals and Sampling:

The present survey of HEV was carried out in 3 metropolitan cities of Pakistan i.e., Lahore, Karachi, and Peshawar. The sampling sites provide major healthcare services in the region. The study period spanned over 6 months form June 2022 to November 2022. A total of 75 samples, 32 from Karachi, 32 from Peshawar, and 11 from Lahore were collected. At the sampling sites patients experiencing signs of acute liver illness, such as tiredness, weakness, nausea, lack of appetite, infrequently bleeding, ascites, and hepatic encephalopathy, provided blood samples at the sampling locations.

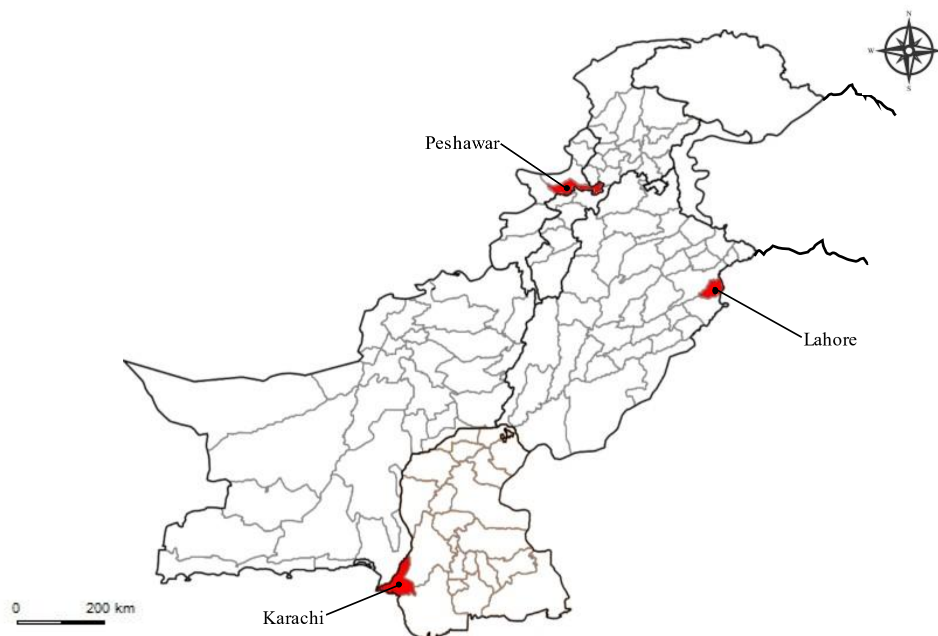


Figure 3.6: Map showing the sampling sites from Pakistan. Samples were collected based on convenience sampling form Peshawar, Lahore and Karachi. Three major metropolitan cities of Pakistan.

3.5 Serodiagnosis of HEV

Once tested negative for other means of liver pathology, enzyme immunoassays (EIA) utilizing commercially available ELISA kits were utilized to test the samples for the presence of antibodies against HEV (Wantai Biopharmaceutical, Beijing, China). Samples that showed positive for IgM or IgG antibodies were processed for further testing.

3.6 Molecular detection of HEV:

3.6.1.1 RNA Extraction:

Viral RNA was extracted from the serum samples using the Qiagen viral RNA extraction kit (catalog no. 52904). The components of the kit were reconstituted according to the manufacturer's protocol. Carrier RNA, reconstituted in Buffer AVE was added to buffer AVL. Buffer AW1 and AW2 were prepared by adding 25 and 30 ml of 100% ethanol, respectively.

Both the serum and the AVE buffer were brought to room temperature before extraction. A sterile 1.5 ml microfuge tube was filled with 560ul of prepared buffer AVL. After that, we added 140ul of a serum sample to the buffer and thoroughly mixed it by pulse vortexing for 15 seconds to create a homogeneous solution. Incubating the sample for 10 minutes at room temperature resulted in the lysis of viral particles. To get rid of moisture that had accumulated within the tube caps, they were centrifuged for a few seconds. To ensure effective binding of the homogeneous solution, 560ul of 100% ethanol was added to the sample and stirred by pulse vortexing for further 15 seconds. To get rid of any remaining liquid in the tubes' caps, they were centrifuged for a few seconds. With the sample in a 2ml collection tube, 630ul was injected into the QIamp Mini column and centrifuged at 6000xg (8000 rpm) for 1 minute. We discarded the filtrate and transferred the QIamp Mini column to a fresh 2 ml collection tube. The centrifugation of the remaining material was performed using the same column. The mixture was then centrifuged at 6000xg (8000 rpm) for 1 minute after adding 500ul of buffer AW1. The spin column was transferred to a fresh collecting tube, and the filtrate was discarded. After adding 500ul of buffer AW2, we spun for 3 minutes at maximum speed (20,000 xg; 14,000 rpm). After discarding the filtrate, another centrifugation at maximum speed for 1 minute was conducted. Once the QIamp Mini column was in the 1.5ml microfuge tube, 60ul of buffer AVE was added. The RNA was eluted from the column by centrifugation at 6000xg (8000 rpm) for 1 minute, after a minute incubation at room temperature. To avoid the RNA from being repeatedly frozen and thawed, 11ul of the isolated RNA was aliquoted into sterile PCR tubes and kept at -80°C.

.

3.6.2 cDNA Synthesis:

cDNA was synthesized using Thermoscientific RevertAid First Strand cDNA Synthesis kit. Reverse transcription was performed on 11ul of isolated RNA using 1ul (100pmol) of random hexamers. A sterile, RNA-free PCR tube was filled with the ingredients and placed on ice.

Table 3.4: Reagents and their quantities used for cDNA synthesis

<table>
<tr><th>Component</th><th>Volume</th></tr>
<tr><td>RNA</td><td>11ul</td></tr>
<tr><td>Random Hexamers</td><td>1ul</td></tr>
<tr><td>Total volume</td><td>12ul</td></tr>
<tr><td colspan="2">This mixture was micro-centrifuged for 15 sec and heated at 65°C for 5 minutes in a PCR machine</td></tr>
<tr><td>5X RT Reaction Buffer</td><td>4ul</td></tr>
<tr><td>10mM dntps</td><td>2ul</td></tr>
<tr><td>Ribolock</td><td>1ul</td></tr>
<tr><td>Revertaid enzyme</td><td>1ul</td></tr>
</table>

The total volume of 20ul made was then micro centrifuged for 15sec and then placed in PCR for incubation at 25°C for 5 minutes, then 42°C for 60 minutes. Then reverse transcriptase enzyme was deactivated by heating the mixture to 70°C for 5 minutes. The cDNA was saved at -20°C until further processing.

3.6.3 Nested Polymerase Chain Reaction:

The Hepeviruses were screened busing a heminested reverse transcription (RT)-PCR as described in (Rasche et al., 2016) and (Drexler et al., 2012). The details of the primers are given in table 3.3.

Targeting the viral RNA-dependent RNA polymerase (RdRp) region of the genome using broadly reactive oligonucleotides. The assay was developed to specifically target and amplify all Hepeviridae family members included in GenBank, as was previously published. The assay was shown to be sensitive to changes in copy number of about 10 per response. HEV genotype 3 transcripts was used to determine the sensitivity in vitro

Round 1

The PCR reaction was carried out using the DFS-Taq DNA polymerase (Cat. No Rec14102016_EN). The round 1 PCR reaction mixture was composed of 2.5 µl 10x Reaction Buffer, 0.5 µl 10mM dNTPs, 2 µl MgCl2, 1ul of 10uM DE-F4228 and DE-R4565 each, 0.5µl Taq polymerase enzyme and 7 µl of cDNA template with the total volume of 25µl using PCR water. The reaction was run on SimpliAmp™ Thermal Cycler (A24812) using the same thermal profile given in Figure 3.5.

Round 2

The PCR reaction was carried out using the DFS-Taq DNA polymerase (Cat. No Rec14102016_EN). The round 1 PCR reaction mixture was composed of 5 µl 10x Reaction Buffer, 1 µl 10mM dNTPs, 3 µl MgCl2, 1.5 ul of 10uM DE-F4228 and DE-R4565 each, 0.5µl Taq polymerase enzyme and 2 µl of round 1 PCR product as a template with the total volume of 50µl using PCR water. The reaction was run on SimpliAmp™ Thermal Cycler (A24812). using the same thermal profile given in Figure 3.6.

3.6.4 PCR Products Analysis:

Materials:

- 1X Tris-Acetate-EDTA (TAE) Buffer
- Biological grade Agarose (bioWorld)
- Ethidium Bromide

Method:

Agarose gel electrophoresis was used to examine first- and second-round PCR results. The 2% agarose gel was made in 1X TAE buffer. To do this, we microwave-heated 100 ml of 1X TAE buffer until 2 g of agarose was completely dissolved. After cooling the liquid to about 60 degrees Celsius, 10 l of ethidium bromide was added. After waiting 30 minutes for the gel to polymerize, it was placed into the casting tray. The polymerized PCR products were injected into the wells along with 1X loading dye. The gel was subjected to a continuous voltage of 90 V in a 1X TAE running buffer for 47 minutes. Next, we used ChemiDoc (ChemiDocTM MP System #1708280, BioRad, USA) to inspect the gels.

3.6.5 PCR Product Purification:

The positive PCR products were purified using the High prep PCR clean-up system (Cat. No AC-60005). The HighPrep-PCR reagents were shaken thoroughly to resuspend the magnetic beads. The PCR reaction was then transferred to the 96-well plate. The volume of the HighPrep PCR reagent volume was calculated according to the following proportions

Table 3.5: The volume of the HighPrep PCR reagent volume

PCR Reaction Volume (ul)	HighPrep PCR Volume at 1.8X(ul)
10	18
20	36
50	90

A PCR sample and HighPrep PCR reagent were combined by pipetting up and down six to eight times. After stirring, the liquid was let to sit for 5 minutes at room temperature. For 3 minutes, the sample plate was positioned on each of the 96 magnetic separators. When the solution becomes clearer as a result of the beads being drawn to the edge of the well, the

incubation period may be considered complete. The sample plate was left on the magnet while the supernatant was thrown away. Aspirating the supernatant was done carefully so as not to disturb the beads. Each well had 200ul of 80% ethanol poured into it and let sit at room temperature for 30 seconds. The supernatant was removed, and two further washes in 80% ethanol were performed. After 10 to 15 minutes of incubation at room temperature, the beads were completely dry. At that point, the sample plate was taken out of the magnetizing apparatus. After adding 40ul of elution buffer to each well, we pipetted up and down five times to thoroughly combine the solutions. The sample plate was incubated for 2 minutes and then returned to the magnetic separator. The eluate was poured into labeled microfuge tubes once the magnetic beads had settled out of the solution.

3.6.6 Cycle Sequencing:

The Purified PCR products were then subjected to cycle sequencing with the BigDye^{TM} Terminator Cycle Sequencing Kit (Applied Biosystems, Cat No.4337455). The recipe is given in the table

Table 3.6: Reagents and their quantities used for cycle sequencing

Component	Volume
Big dye	4ul
Buffer	2ul
Primer	1ul
Template	1ul
dH2O	8ul
Total volume	12ul

3.6.7 Sequencing Cleanup:

The sequencing products were cleaned using the HighPrep DTR kit (Cat. No DT-70005). Initially, the HighPrep DTR was brought to room temperature. The reagents were shaken thoroughly to fully resuspend the magnetic beads. 10ul of HighPrep DTR reagent was added to each sample. Freshly prepared 85% ethanol was added according to the following proportions

Table 3.7: The volume of the HighPrep DTR reagent

Reaction volume	85% Ethanol (ul)
5	30
10	40
15	50
20	60

Through 7–10 rounds of pipetting up and down, we were able to fully combine the DTR reagent with the material. The 96 magnetic separation instrument held the sample plate for 4-5 minutes. The supernatant was thrown away once the solution settled and the beads were drained. 100ul of 85% ethanol was added to each well while the sample plate was still on the magnet. After the magnetic beads had settled into their original positions, the mixture was let to rest for 1-2 minutes. The supernatant was discarded once again, and two further washes were performed in ethanol (85%). After incubating the beads for 10 minutes at room temperature, they were dry. After removing the sample plate from the magnet, 40ul of elution buffer was pipetted into each well and well mixed 20 times. A sample plate was incubated for 5 minutes at room temperature before being returned to the magnetic separation apparatus, where the magnetic beads were separated from the solution within 5 to 7 minutes. The sequencing process was then applied to the elute.

3.6.8 Capillary Electrophoresis:

Sequencing was carried out in 3130 Genetic Analyzer utilizing performance optimization polymer 6 as a separation matrix. The resultant data was generated in the form of ABI format and exported in a USB for further analysis.

3.6.9 Sequence Editing

In general, automated DNA sequencers generate low-quality reads. Particularly at the sequencing primer sites and at the end of longer sequence runs, the sequence data is of poor quality. Failure to exclude these sequences distorts the sequence assembly and, consequently, the subsequent sequence analysis. The **sequencher software** was used to trim the poor quality or ambiguous data. The ends trimming criteria can be seen in the figure below. After pruning the data, the sequences were exported in FASTA format for analysis.

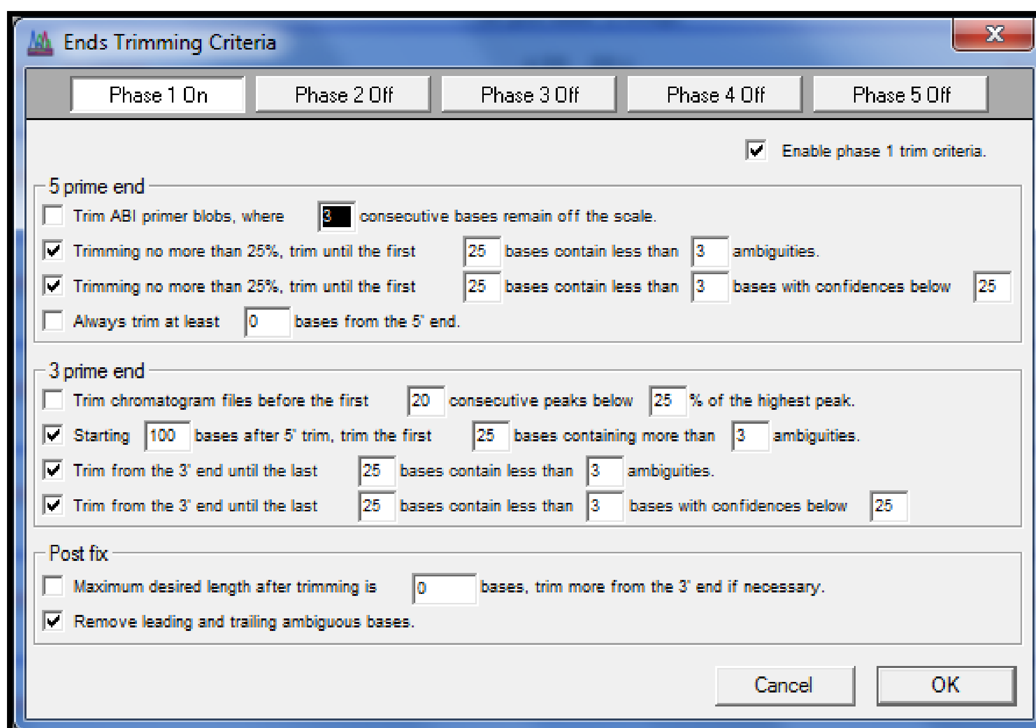


Figure 3.7: Ends trimming criteria to remove poor quality and ambiguous data. The following parameters were set in sequencher to trim the noisy ends of the sequence reads and get a clean sequence for further analysis.

3.7 Alignment

To analyze the phylogenetic history of the sequenced isolates, sequences of other HEV genotypes and subtypes were downloaded from NCBI in the FASTA format. These included a representative **Genotype 2** sequence from **Mexico** (M74506.1), six representative **Genotype** 3 sequences from **Kirgizstan** (AF455784.1), **Japan** (AB 091394.1 and AB074920.3), **USA** (AF082843.1, AF060668.1, and AF060669.1) and four representative **Genotype 4** sequences from **Japan** (AB074915.3 and AB080575) and **China** (AB108537).

The data of subtypes included sequences of **subtype 1a** from **India** (AF459438, AF076239.3, AF398914.1, AF093885.1, and X99441.1), **Burma** (M73218.1) and **Nepal** (AF051830.1), four representative sequences of **subtype 1b** from **China** (D11092.1, M94177.1, and D11093.1) and **Pakistan** (M80581.1), one representative strain of **subtype 1c, 1d,1e** and **1f** each from **India** (X98292.1), **Morocco** (AY230202.1), **Chad** (AY204877.1) and **India** (JF443721.1) respectively, and eleven representative sequences of **subtype 1g** form **Mongolia** (LC225387.1), **India** (KY436505.1), **Japan** (LC314156.1, LC314157.1, LC314158.1, and LC314155.1), **UK** (MH504155.1, MH504158.1, and MH504163.1) and **France** (MN401238.1).

The sequences were imported into MEGA X and aligned using Muscle. The resulting alignment can be seen in the figure.

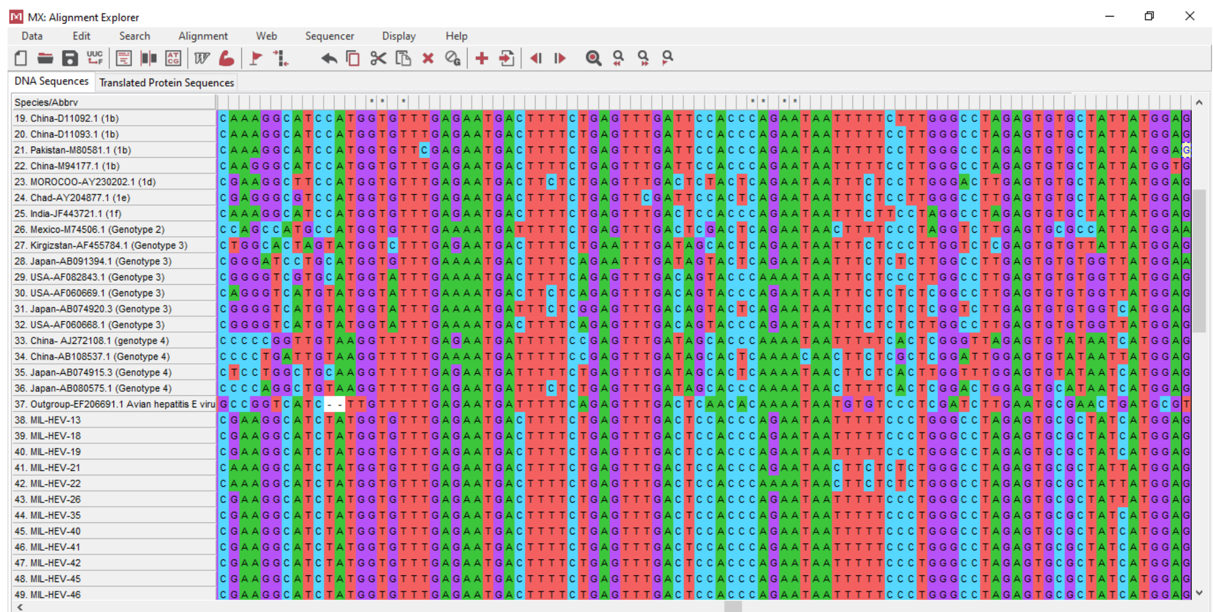


Figure 3.8: Sequence alignment of Hepatitis E sequences retrieved from NCBI

The resulting alignment was exported in the MEGA format for the construction of the phylogenetic tree. A maximum likelihood tree was constructed using Kimura 2-parameter model. The analysis preferences can be seen in figure 3.9.

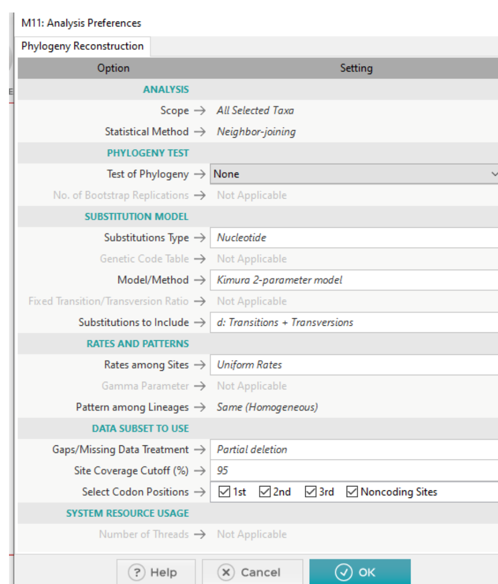


Figure 3.9: Analysis preferences for the construction of a phylogenetic tree. The phylogenetic tree was constructed using the Maximum likelihood method and a Bootstrap value of 1000 for the accurate estimation of phylogeny.

3.8 Phylogenic Analysis

For the estimation of the isolates with other reference sequences, the sequences were aligned using muscle in Uniport UGENE. After the alignment, a distance column was generated using the similarity algorithm indicated in percentages.

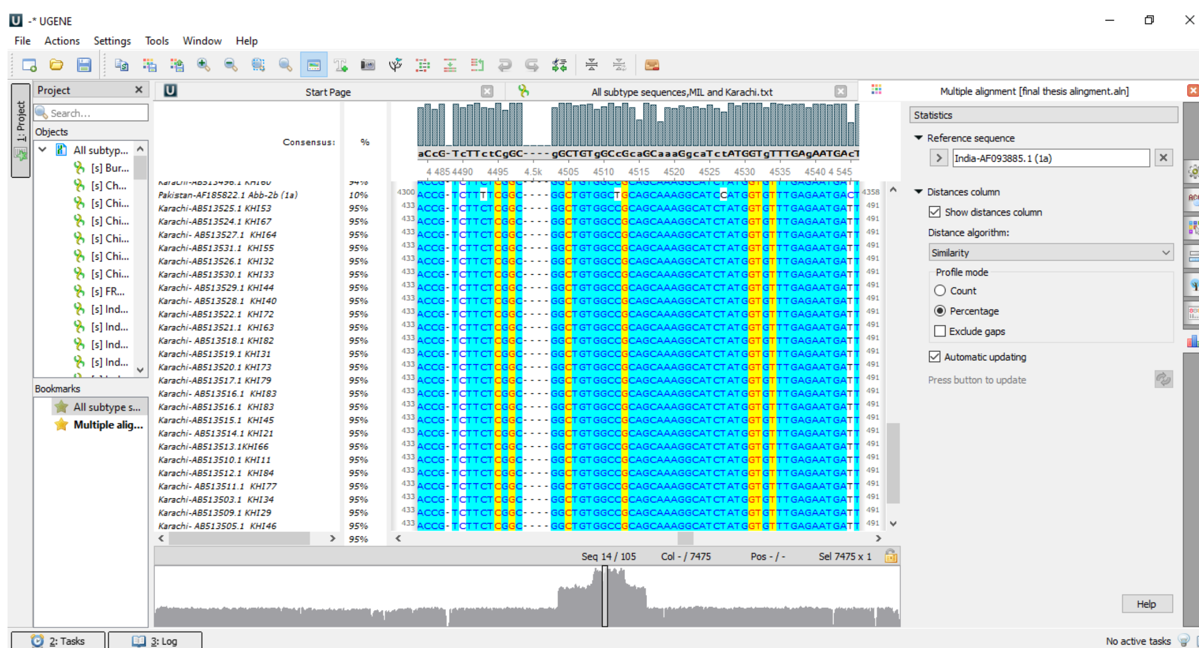


Figure 3.10: Distance column with the Indian sequence (AF093885.1) as a reference. The sequence similarity was used to indicate the similarity between the sequences obtained in the current study to sequences reported from elsewhere in the world. As a result of this particular analysis a high similarity of the current sequences was seen to sequences obtained from Pakistan in 2011; which themselves have a high similarity to the genotype 1a sequence (AF093885.1) reported from India.

RESULTS

4.1 Amplification of Positive Control Plasmid DNA:

4.1.1 Transformed BL21 Cells:

Transformed BL21 colonies appeared on the Ampicillin Agar plate. Their presence showed that transformation was successful and competent cells took up the plasmid. This indicates that the UC1 plasmid containing ampicillin-resistant gene caused the colonies to grow on ampicillin. Cells that were unable to take the plasmid did not survive on an antibiotic plate.

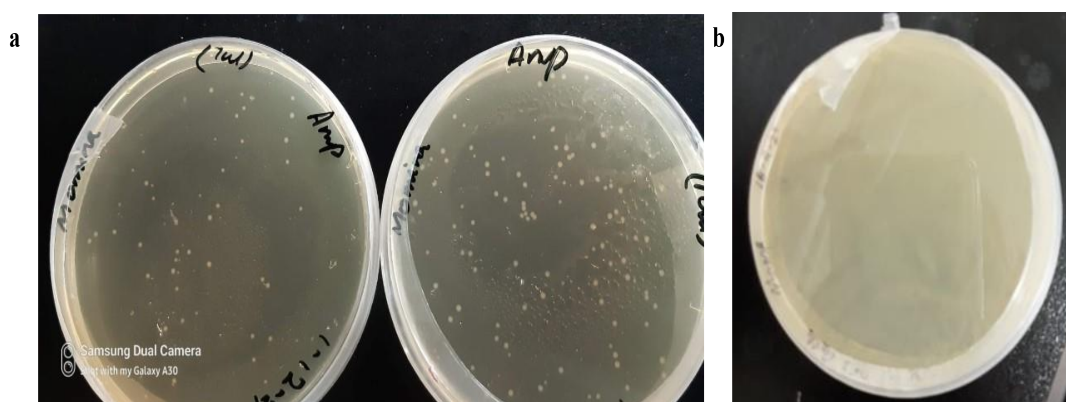


Figure 4.1: Transformation of BL21 competent cells. a) Growth of transformed BL21 cells on ampicillin media with 7 and 10ul of plasmid used for initial transformation of 100ul of competent cells. b) Control plate showing no growth.

4.1.2 Colony PCR

To confirm the presence of the UC1 plasmid in the colonies, colony PCR was carried out by directly lysing by both searing the bacterial cell walls by vigorous vortexing and heat by subjecting the bacteria to a high temperature of 95 C for 10 minutes. Two isolated colonies indicated by red circles in Figure 4.2 were chosen for testing. The colonies after lysis and PCR amplification were run on a 2% agarose gel and showed a band of 412bp as seen in Figure 4.3. the band size correlated to the size of the PREDICT sequence inserted into the plasmid.

Figure 4.2: Selection of colonies for colony PCR. Two isolated colonies, indicated by red circles, form separate ends of the ampicillin plate and were picked for testing the presence of the plasmid.

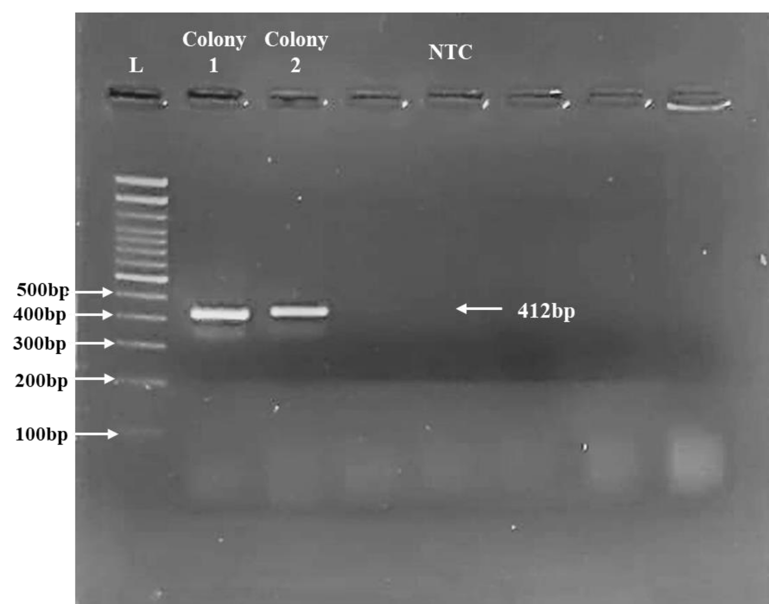


Figure 4.3: Representative Gel of colony PCR. Lanes 2 and 3 represent 412 bp bands of the PCR carried out by targeting the PREDICT sequence in the UC1 control plasmid. The main aim was to confirm the successful transformation of the plasmid. Lane 4 contains the no template control and Lane 1 contains a 100bp DNA ladder for size comparison.

4.1.3 Miniprep

After confirmation of the presence of the UC1 plasmid in the colonies, other colonies from the same plate were grown in an ampicillin media. This step was followed by the extraction of the plasmid DNA from the transformed cells by a procedure discussed in section 3.2.5. the purified plasmid was checked for purity and concentration using a Nanodrop spectrophotometer. Figure 4.4 represents the concentration and purity of the extracted plasmid by manual plasmid miniprep.

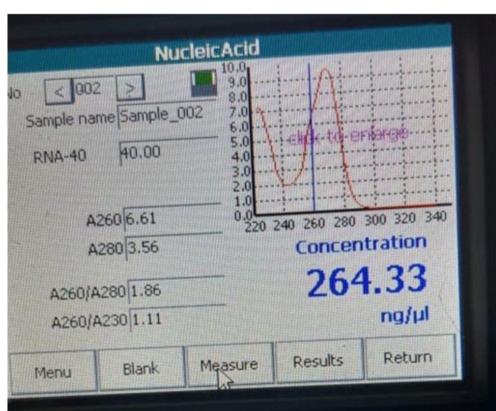


Figure 4.4: Nanodrop concentration of the extracted plasmid. The nanodrop shows the presence of 264.33 ng/ul of DNA in the sample with a purity of 1.86 which makes the extracted DNA of good concentration and quality.

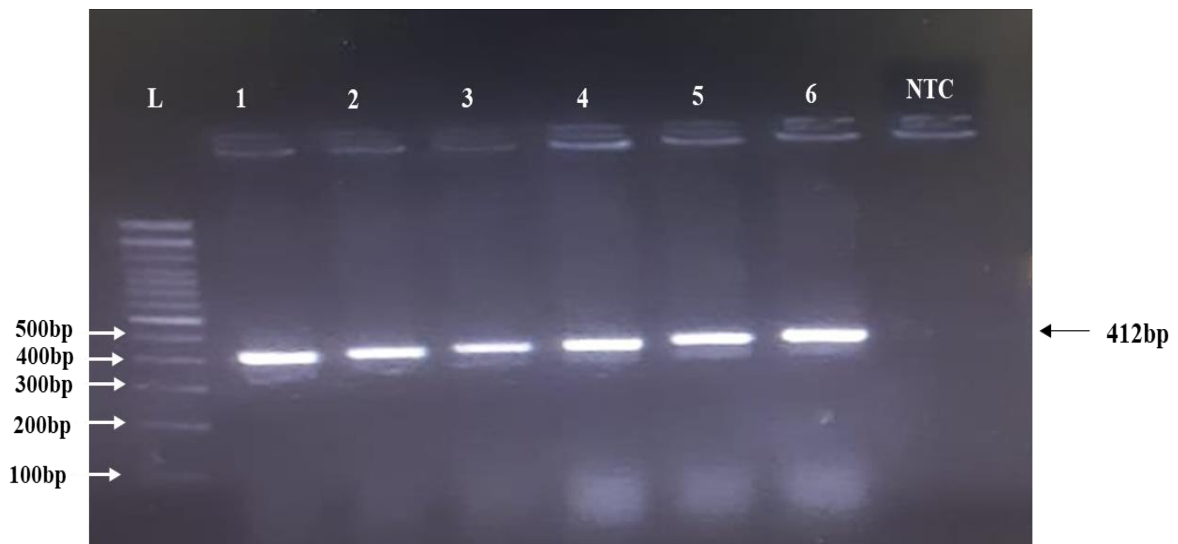


Figure 4.5: Gel representation of PCR product obtained after amplifying the PREDICT sequence in the UC 1 plasmid using a PCR. Lanes 2 to 7 represent 412 bp bands of the PCR carried out by targeting the PREDICT sequence in the UC1 control plasmid. The main aim was to confirm the successful purification of the plasmid by miniprep. Lane 8 contains the no template control and Lane 1 contains a 100bp DNA ladder for size comparison.

4.2 Optimizing the Hepevirus degenerate primers for detection of HEV virus using the transformed plasmid:

After the verification of the purity of the UC1 plasmid, it was used to optimize the working of the Hepevirus-specific degenerate primers to amplify the Hepevirus sequence. For this, the PCR was carried out using the protocols already defined. Resultantly we obtained a 371bp and 338bp PCR product after using the extracted plasmid as a template for round one amplification. The round 1 product was used as a template for round 2 amplification. The desired bands can be seen in Figure 4.6. This confirmed that the plasmid can be used as a positive control for the subsequent testing of HEV- positive samples.

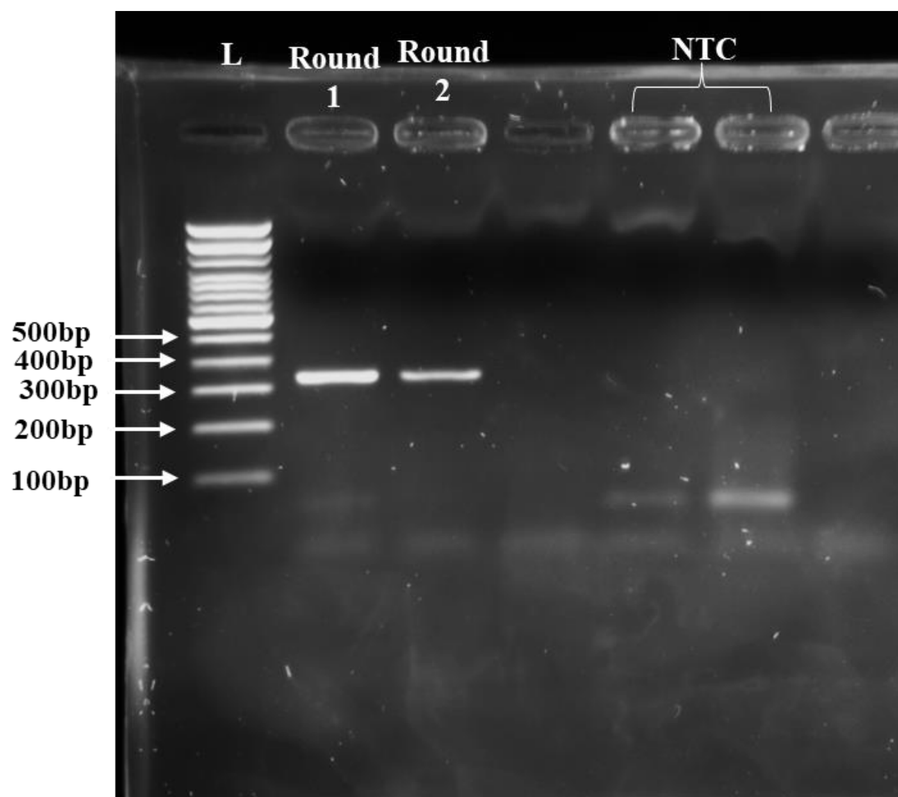


Figure 4.6: Gel representation of optimization of HEV-specific primers using the plasmid as a positive control. Lane 2 a 371bp product obtained after round 1 of the heminested PCR. Lane 3 represents a 338bp product obtained after round 2 of the heminested PCR using the round 1 product as a template. Lanes 5 and 6 contain round 1 and round 2 with no template controls respectively. Lane 1 contains a 100bp DNA ladder for size comparison.

4.3 Epidemiological Characteristics of the Hepatitis E Cases

Between June 2022 to November 2022, a total of 75 cases of infectious sickness were recorded; 32 were located in Karachi, 32 in Peshawar, and 11 in Lahore. Each incident occurred sporadically and no correlation between the patients could be established. Out of the 75 diagnosed patients, 49 (65.3%) were male and 26 (34.6%) were female. The patients lay between the ages of 12 and 68 (median 47 years). None of the patients had traveled abroad or outside of their resident province two months before testing.

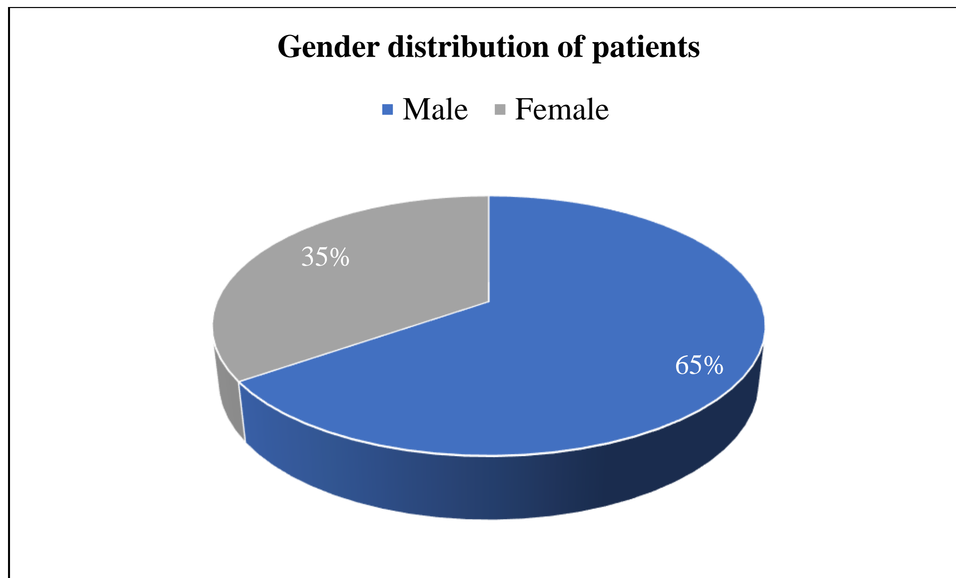


Figure 4.7: Gender distribution of patients. Out of the 75 diagnosed patients, 49 (65.3%) were male and 26 (34.6%) were female.

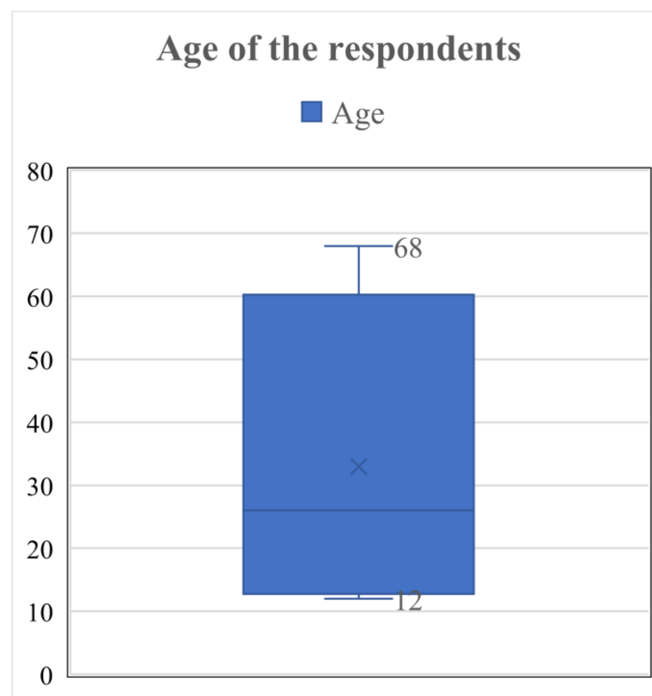


Figure 4.8: Age distribution of the patients. The patients lay between the ages of 12 and 68 (median 47 years).

4.4 The positivity rate of HEV IgM positive samples

All 75 diagnosed patients were anti-HEV IgM positive with a few (24%, 18/75) who had converted to anti-HEV IgG positive within the timespan they exited the hospital.

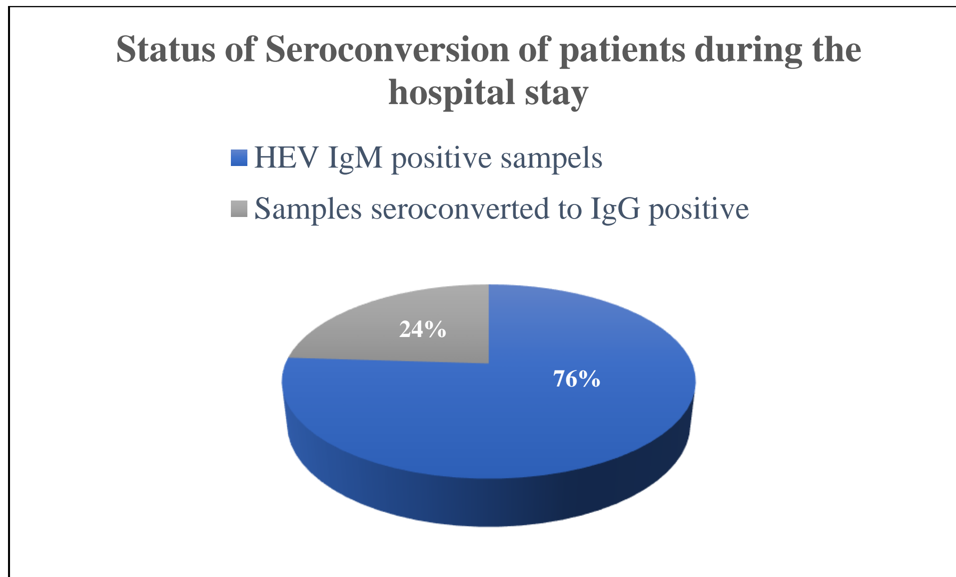


Figure 4.9: Status of seroconversion of patients during the hospital stay. Few (24%, 18/75) had converted to anti-HEV IgG positive within the timespan they exited the hospital.

4.5 Detection and sequencing of HEV samples

HEV RNA was detected in 33 (44%) of the samples that initially tested positive for HEV IgM antibodies.

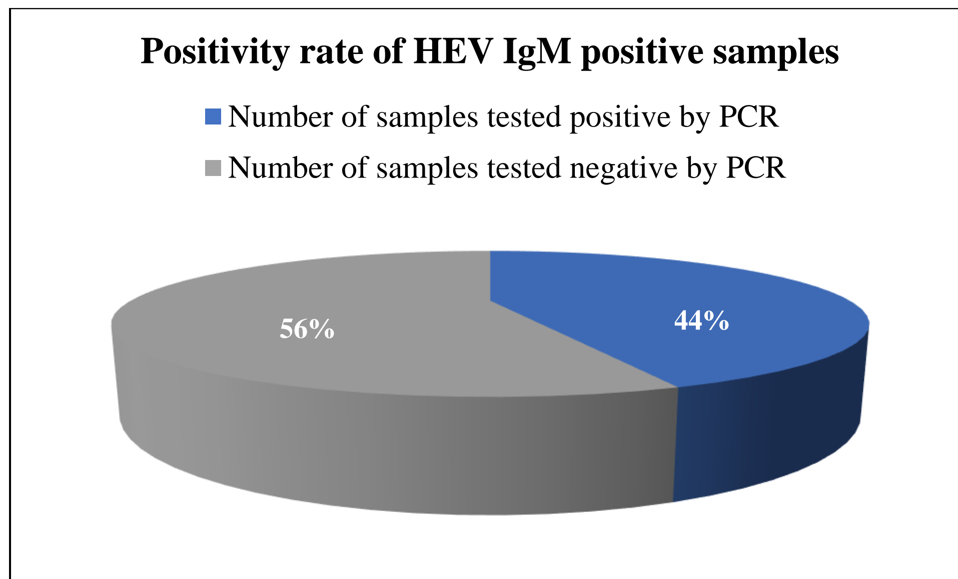


Figure 4.10: Number of IgM-positive samples detected positive by PCR. Out of the total 75 samples collected from 3 metropolitan cities only 33 (44%) samples tested positive by PCR.

The positive samples proceeded for sequencing and the obtained sequences of the RNA-dependent RNA polymerase (RdRp) region of ORF1 were deposited in GenBank (OP946453- OP946483). The representative gel images of all the samples can be seen in Figure 4.11.

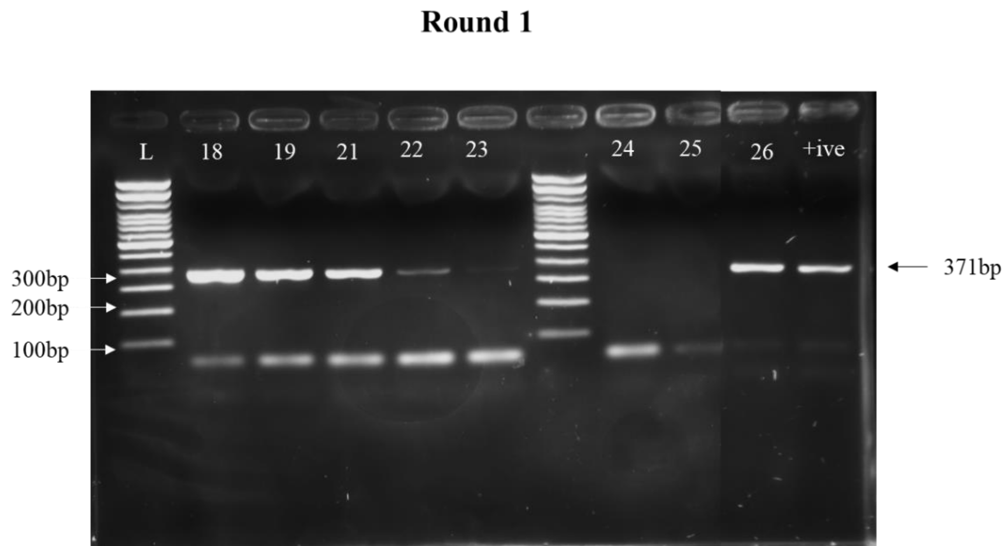


Figure 4.11: Representative Gel of Round 1 PCR. Lanes 2-5 and 6-8 represent patient samples. Lanes 1 and 6 contain a 100bp DNA ladder for size comparison.

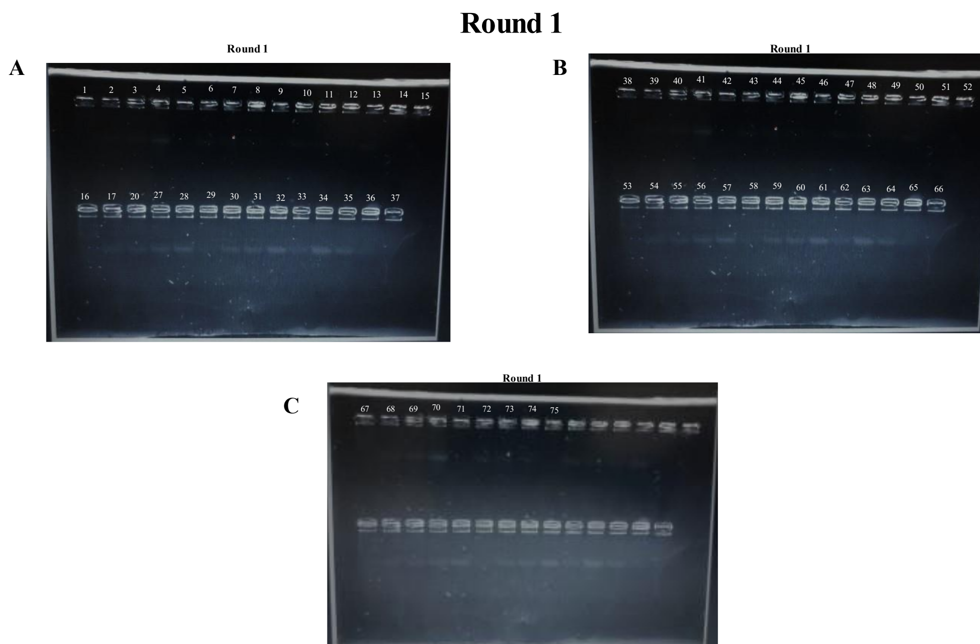


Figure 4.12: Gel images of samples processed for detection of HEV in Round 1 PCR.

Round 2

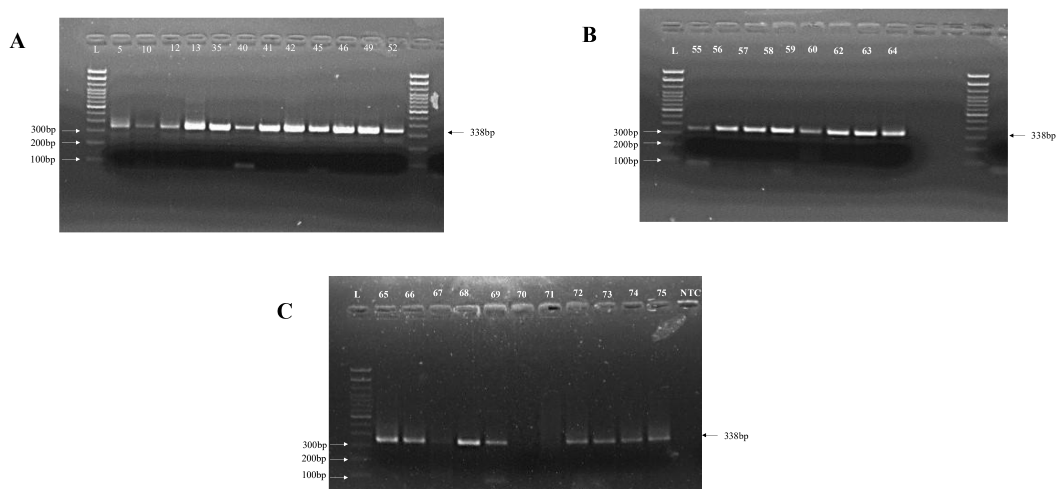


Figure 4.13.: Gel images of all the positive samples processed for detection of HEV in Round 2 PCR.

4.6 Phylogenetic analysis of Genotype 1 strains

To analyze the phylogenetic history of the sequenced isolates, sequences of other HEV genotypes and subtypes were downloaded from NCBI in the FASTA format and were used for the construction of phylogenetic trees. Initially to determine the genotype of the sequences form the current study a phylogenetic tree was constructed using representative sequences of **Genotype 1** form Mongolia (LC225387.1), **Genotype 2** sequences from **Mexico** (M74506.1), **Genotype** 3 sequence from **Kirgizstan** and **Genotype 4** sequences from **Japan** (AB074915.3). All the sequences were seen to cluster around the representative genotype 1 sequences form Mongolia indicating the sequences form the current study belong to Genotype 1. The phylogenetic tree constructed using the Maximum -likelihood method can be seen in Figure 4.14.

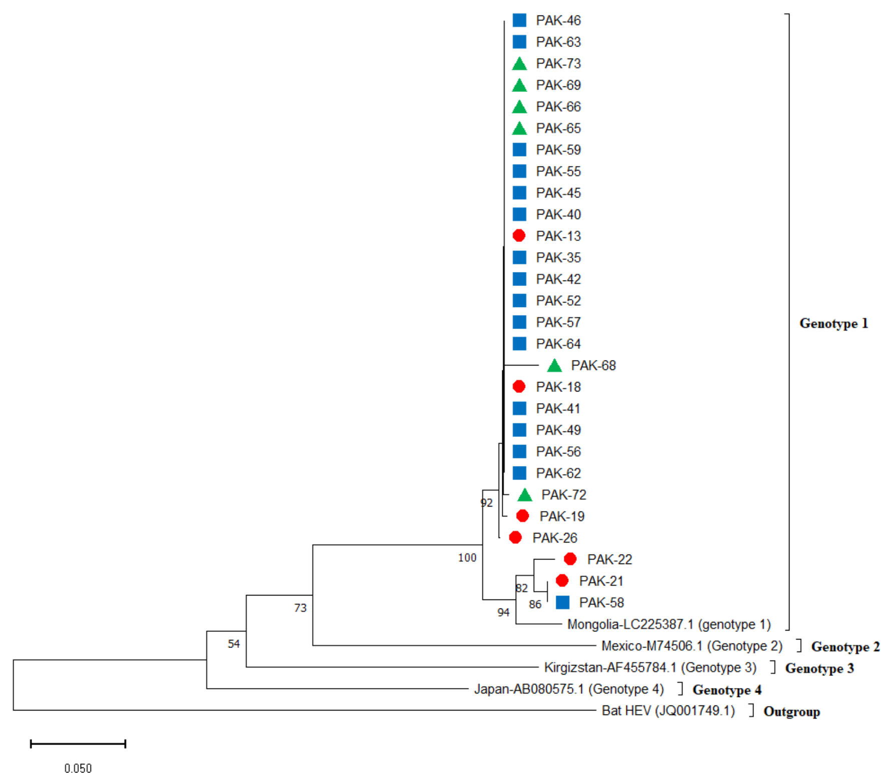


Figure 4.14: **A Phylogenetic tree of 4 representative sequences of Hepatitis E virus (HEV) and 30 sequences from the current study.** This tree was generated using Maximum -likelihood method in MEGA11. Each genetic distance was calculated using the Kimura 2 parameter model. A Bat Hepatitis E virus was taken as an outgroup. The distance scale represents the number of differences between the sequences.

Many subtypes, HEV-1a to HEV-1g have been described within genotype 1. Subtype analysis was performed in two ways. First, a phylogenetic tree was constructed using representative strains from each of the subtypes including seven representative sequences of subtype 1a from India (AF459438, AF076239.3, AF398914.1, AF093885.1, and X99441.1), Burma (M73218.1) and Nepal (AF051830.1), four representative sequences of subtype 1b from China (D11092.1, M94177.1, and D11093.1) and Pakistan (M80581.1), one representative strain of subtype 1c, 1d,1e and 1f each from India (X98292.1), Morocco (AY230202.1), Chad (AY204877.1) and India (JF443721.1) respectively, and eleven representative sequences of subtype 1g form Mongolia (LC225387.1), India (KY436505.1), Japan (LC314156.1, LC314157.1, LC314158.1, and LC314155.1), UK

(MH504155.1, MH504158.1, and MH504163.1) and France (MN401238.1). All the isolates in the current study clustered around the representative sequences of subtype 1g, especially with subtype 1g sequences previously reported from an outbreak within Karachi in 2011.

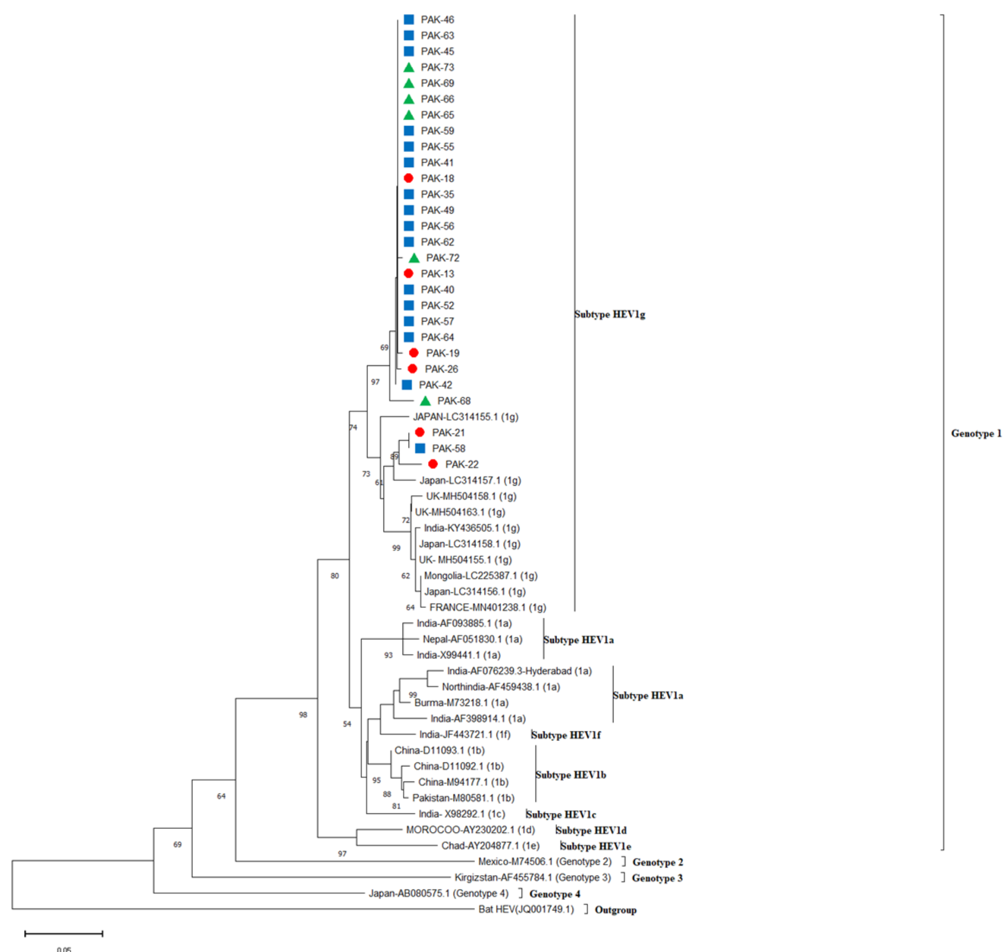


Figure 4.15: **A Phylogenetic tree of representative sequences of Hepatitis E virus (HEV) and 30 sequences from the current study.** This tree was generated using Maximum -likelihood method in MEGA11. Each genetic distance was calculated using the Kimura 2 parameter model. A Bat Hepatitis E virus was taken as an outgroup. The distance scale represents the number of differences between the sequences.

The Hepatitis E Virus Genotyping tool version 0.1 was used to confirm the grouping of the isolates into subtype 1g. The software accesses the robustness of the phylogenetic assignment using bootstrap values. A neighbor-joining tree is constructed for the given sequence and a genotype or variant is allocated if the bootstrap value is greater than 70.

According to phylogenetic and phylogenetic subgroup analyses, validated by bootstrap values, all the present isolates belong to Genotype 1 subtype 1g as shown in Table 4.1.

Table 4.1. Subtyping of the sequenced isolates against the reference sequence NC_001434

Sr. no	Name	Blast Result	Blast score	Reference	Phylo-major result	Phylo-major support	Phylo-minor result	Phylo-minor support
1	PAK-12	Hepeviridae Orthohepevirus A	92.6045	NC_001434	HEV1	100.0	HEV1g	78.0
2	PAK-13	Hepeviridae Orthohepevirus A	92.53247	NC_001434	HEV1	100.0	HEV1g	79.0
3	PAK-18	Hepeviridae Orthohepevirus A	91.71429	NC_001434	HEV1	100.0	HEV1g	78.0
4	PAK-19	Hepeviridae Orthohepevirus A	91.78886	NC_001434	HEV1	100.0	HEV1g	70.0
5	PAK-21	Hepeviridae Orthohepevirus A	91.42857	NC_001434	HEV1	100.0	HEV1g	100.0
6	PAK-22	Hepeviridae Orthohepevirus A	90.39548	NC_001434	HEV1	100.0	HEV1g	96.0
7	PAK-26	Hepeviridae Orthohepevirus A	91.374664	NC_001434	HEV1	100.0	Could not assign	
8	PAK-35	Hepeviridae Orthohepevirus A	92.50814	NC_001434	HEV1	100.0	HEV1g	86.0
9	PAK-40	Hepeviridae Orthohepevirus A	93.37748	NC_001434	HEV1	100.0	HEV1g	83.0
10	PAK-41	Hepeviridae Orthohepevirus A	92.6045	NC_001434	HEV1	100.0	HEV1g	82.0

11	PAK-42	Hepeviridae Orthohepevirus A	92.68293	NC_001434	HEV1	100.0	HEV1g	84.0
12	PAK-45	Hepeviridae Orthohepevirus A	93.220345	NC_001434	HEV1	100.0	HEV1g	85.0
13	PAK-46	Hepeviridae Orthohepevirus A	93.26599	NC_001434	HEV1	100.0	HEV1g	77.0
14	PAK-49	Hepeviridae Orthohepevirus A	92.810455	NC_001434	HEV1	100.0	HEV1g	89.0
15	PAK-52	Hepeviridae Orthohepevirus A	93.00699	NC_001434	HEV1	100.0	HEV1g	79.0
16	PAK-55	Hepeviridae Orthohepevirus A	92.65176	NC_001434	HEV1	100.0	HEV1g	87.0
17	PAK-56	Hepeviridae Orthohepevirus A	92.53247	NC_001434	HEV1	100.0	HEV1g	84.0
18	PAK-57	Hepeviridae Orthohepevirus A	92.85714	NC_001434	HEV1	100.0	HEV1g	86.0
19	PAK-58	Hepeviridae Orthohepevirus A	92.43421	NC_001434	HEV1	100.0	HEV1g	100.0
20	PAK-59	Hepeviridae Orthohepevirus A	92.58064	NC_001434	HEV1	100.0	HEV1g	84.0
21	PAK-62	Hepeviridae Orthohepevirus A	92.58064	NC_001434	HEV1	100.0	HEV1g	77.0
22	PAK-63	Hepeviridae Orthohepevirus A	92.607	NC_001434	HEV1	100.0	HEV1g	80.0
23	PAK-64	Hepeviridae Orthohepevirus A	92.58064	NC_001434	HEV1	100.0	HEV1g	90.0
24	PAK-65	Hepeviridae Orthohepevirus A	93.26599	NC_001434	HEV1	100.0	HEV1g	74.0

25	PAK-66	Hepeviridae Orthohepevirus A	92.76315	NC_001434	HEV1	100.0	HEV1g	85.0
26	PAK-68	Hepeviridae Orthohepevirus A	92.73927	NC_001434	HEV1	100.0	Could not assign	
27	PAK-69	Hepeviridae Orthohepevirus A	92.53247	NC_001434	HEV1	100.0	HEV1g	84.0
28	PAK-72	Hepeviridae Orthohepevirus A	91.147545	NC_001434	HEV1	100.0	HEV1g	90.0
29	PAK-73	Hepeviridae Orthohepevirus A	92.6045	NC_001434	HEV1	100.0	HEV1g	77.0
30	PAK-74	Hepeviridae Orthohepevirus A	92.59259	NC_001434	HEV1	100.0	HEV1g	75.0
31	PAK-75	Hepeviridae Orthohepevirus A	92.59259	NC_001434	HEV1	100.0	HEV1g	80.0

To evaluate the phylogenetic history of Hepatitis E virus in Pakistan. A phylogenetic tree was constructed with the sequences of HEV isolates collected at different intervals form Pakistan along with the representative sequences of Genotype 2, 3 and 4. Representative sequences form the three metropolitan cites clustered with the subtype HEV1g sequences previously collected form an outbreak form South Karachi which was itself distinguished form other genotype 1 sequences previously reported form Pakistan. This indicates the presence of a newly prevalent genotype form Pakistan,

Figure 4.16: **A phylogenetic tree of representative sequences of Hepatitis E Virus (HEV) 6 sequences reported previously form Pakistan and 3 sequences form the current study.** The tree was generated by Neighbor joining method in MEGA11 with genetic distance calculated using Kimura 2 parameter model. A Bat HEV was taken as an outgroup. The distance scale represents the number of differences between the sequences

DISCUSSION

In this study, 75 individuals were diagnosed with HEV using anti-HEV IgM and IgG antibodies. Only 44% of the anti-HEV IgM-positive individuals tested positive for HEV-RNA. This outcome can be explained by HEV's transient viremia, which occurs during the first two weeks of infection and reduces one week following jaundice symptoms. Detecting HEV viral RNA and anti-HEV IgM antibodies through ELISA may improve acute HEV infection identification, especially within endemic countries. It is noted that PCR-based methods are reliable and sensitive for identifying the Hepatitis E virus. RNA can be discovered a week after the symptoms emerge, despite negative serological testing hence why PCR predicts acute HEV infection better than ELISA (Chandra et al., 2012).

About the link between gender and HEV susceptibility, it was demonstrated that gender dependence was consistent with research undertaken in other developing nations. More males than females were discovered to be infected with the virus (Conte et al., 2021).

Hepatitis E was seen to be most common between the ages of 12 to 68 years. This leads us to believe that the current epidemiology of HEV in Pakistan follows a similar pattern of an increase in age-specific incidence as reported in other South Asian nations, which is likewise driven by increasing viral exposure coincident with adulthood (Labrique et al., 2010).

In Pakistan, several isolates have been reported from different geographical regions. Sar-55 (87-Pakistan -A) was reported from Sargodha in a 1987 epidemic, Abb-2B(88-Pakistan-2B) was reported from Abbottabad in an epidemic in 1998, 87-Pakistan-B form archival material was collected during an outbreak investigation in Sargodha in 1987. Two distinct variants, variant 1 (AB513503-AB513530) and variant 2 (AB513496-AB513502) were reported from Karachi from 2007 to 2008. All these strains belonged to the HEV-1

genotype. Hence, Genotype 1 is the dominant cause of hepatitis E in Pakistan (A. Khan et al., 2011b). Sequences were aligned using Uniprot UGENE to investigate the evolutionary relationship between the HEV isolates sequenced in this current study, and other Pakistani HEV genotype 1 isolates as well as those isolated from various nearby parts of the world. A phylogenetic tree was generated using MEGAX. The HEV genotyping program version 0.1 was used for the subtyping. ORF2 or the RdRp region of ORF1 is examined to assess genetic diversity and evolutionary links among HEV strains. Compared to trees restricted to full genomes, those demonstrating the evolution of an organism based on the RdRp region appear to correlate more strongly (A. Khan et al., 2011b). Hence the Rdrp region in the ORF1 was targeted in the present study.

The Pakistani HEV isolates OP946453- OP946483, share 92-99% sequence identity to variants reported from Karachi. All the reported sequences showed a significantly high similarity to the Indian Strains AF093885.1 and AF398914.1 (94-98%) but only 7-9% sequence similarity with 87 Pakistan -A and Abb-2B. The phylogenetic analysis showed that all the isolates in the current study clustered with other HEV Pakistan genotype 1 strains from a subgroup. Though the isolated variants come from distinct geographical regions of Pakistan, they still share 94-100% nucleotide homology, suggesting they may come from a common source where they emerged from a single isolate. The water-borne introduction of the virus into Pakistan and the following population mutations and adaptations that led to subtype 1g becoming the predominant circulating subtype, may be responsible for the great resemblance to Indian subtype 1a isolates.

Both endemic and sporadic forms of the disease, as found in many underdeveloped nations, are mostly brought on by infection with Genotype 1 through tainted water supplies. Whereas proper sanitation and access to a safe water supply are particularly difficult to achieve, outbreaks are seen to favor areas of conflict and humanitarian catastrophes like

war zones, flood-prone regions, refugee camps, or populations that are internally displaced (*Hepatitis E*, n.d.). Regionally distinct water contamination techniques include the use of river water for sewage disposal. As a sanitary disposal method, open defecation in backyards and open fields can lead to the contamination of open drinking water sources such as rivers, streams, and unprotected wells. Floods and monsoons flush fecally contaminated water into catchment areas such as backyards, open fields, and groundwater into open waterways, causing an outbreak of water-borne diseases. Quite often, Pakistani cities are supplied with piped drinking water, with the standard practice being to place the pipes next to sewage drains or even across sewage channels. During intermittent water delivery schedules, sewage penetrates deteriorated pipes, polluting the piped water with feces. Multiple outbreaks of hepatitis E in Pakistan have been related to this phenomenon (Bryan et al., 1994; Rab et al., 1997) even worse is when extreme rains and flooding reroute the city's sewage drains into the city's water source, contaminating the city's drinking water. The contamination of municipal water sources by sewage drains alone has been reported in hundreds of thousands of cases.

The research indicates that while all the examined isolates are notably distinct from previously known isolates from Pakistan, they are all closely related to subtype 1g strain HE-JA14-2173 which was also found to be spread by water contamination (A. Khan et al., 2011b). The most prevalent genotype in the country was previously believed to be subtype 1a, Sar-55 (87-Pakistan -A), but evidence from this study reveals that subtype 1g has become the most prevalent and is now the leading cause of Hepatitis E virus infection in the nation. The data gathered from this study can be used as a guide for policymakers to develop strategies for disease prevention and control, identify the target audience for awareness campaigns, educate high-risk populations, create efficient vaccination and treatment schedules, and support the development of more specialized and sensitive

diagnostic assays (Labrique et al., 2010). In the absence of strong evidence for a long-term sequel of HEV genotype 1 infection, the fatalities associated with viral infection should not be overlooked in developing countries such as Pakistan. This current study sheds light on the population burden of HEV from the major metropolitan cities of Pakistan and highlighted the circulation of a new prevalent subtype associated with HEV-induced pathology. Further studies can be conducted to establish the role of sub-genotypic variation in cohort studies. Such investigations will help understand the demographic as well as the immunological characteristics of the disease to successfully reduce the fatalities caused by the emergent threat.

CONCLUSION AND FUTURE PROSPECTS

According to the findings of the research, even though each of the examined isolates is noticeably distinct from previously known isolates from Pakistan, they are all closely related to the subtype 1g strain **HE-JA14-2173**, which was also discovered to be spread by water contamination. This is even though each of the examined isolates is noticeably distinct from previously known isolates from Pakistan. In the past, it was thought that the genotype with the highest prevalence in the country was subtype 1a, **Sar-55 (87-Pakistan-A).** However, the findings of this study indicate that **subtype 1g has surpassed subtype 1a** as the genotype with the highest prevalence and is now the leading cause of Hepatitis E virus infection in the country. The data that was gathered from this study can be used as a guide for policymakers to develop strategies for the prevention and control of disease, identify the target audience for awareness campaigns, educate high-risk populations, create efficient vaccination and treatment schedules, and support the development of diagnostic assays that are more specialized and sensitive. Even if there isn't a lot of data to suggest that HEV genotype 1 infection have long-term consequence, the fact that viral infections are a leading cause of death in underdeveloped nations like Pakistan isn't something that should be ignored. This recent study provides light on the population burden of HEV from the main metropolitan cities of Pakistan and highlights the circulation of a novel prevalent subtype linked with HEV-induced disease. The study was carried out in Pakistan. To determine the significance of sub-genotypic variation in cohort studies, more research could be carried out. This research will assist understand the demographic as well as the immunological aspects of the disease, which is necessary to successfully limit the number of fatalities caused by the emerging threat.

REFERENCES

Bihl, F., & Negro, F. (2010). Hepatitis E virus: A zoonosis adapting to humans. *The Journal of Antimicrobial Chemotherapy*, *65*(5), 817–821. https://doi.org/10.1093/jac/dkq085

Bryan, J. P., Tsarev, S. A., Iqbal, M., Ticehurst, J., Emerson, S., Ahmed, A., Duncan, J., Rauf Rafiqui, A., Malik, I. A., Purcell, R. H., & Legters, L. J. (1994). Epidemic Hepatitis E In Pakistan: Patterns Of Serologic Response And Evidence That Antibody To Hepatitis E Virus Protects Against Disease. *The Journal of Infectious Diseases*, *170*(3), 517–521. https://doi.org/10.1093/infdis/170.3.517

Chandra, N. S., Sharma, A., Rai, R. R., & Malhotra, B. (2012). Contribution of hepatitis E virus in acute sporadic hepatitis in north western India. *Indian Journal of Medical Research*, *136*(3), 477.

Chauhan, A., Webb, G., & Ferguson, J. (2019). Clinical presentations of Hepatitis E: A clinical review with representative case histories. *Clinics and Research in Hepatology and Gastroenterology*, *43*(6), 649–657. https://doi.org/10.1016/j.clinre.2019.01.005

Conte, D. D., Luna, L. K. de S., Passarini, A., Alves, V. R. G., Caldeira, D. B., Cruz, J. S., Gouveia, V. A., Bellei, N., & Granato, C. F. H. (2021). Hepatitis E virus infection among patients with altered levels of alanine aminotransferase. *The Brazilian Journal of Infectious Diseases*, *25*(6), 101655. https://doi.org/10.1016/j.bjid.2021.101655

Denner, J. (2019). Hepatitis E virus (HEV)-The Future. *Viruses*, *11*(3), 251. https://doi.org/10.3390/v11030251

Din, I. ud, Khan, M. A., Ishaq, M., Ranjha, M. A., Ansari, J. A., & Rathore, T. R. (2018). OUTBREAK OF HEPATITIS-E IN AMANKOT, MINGORA, SWAT, PAKISTAN IN 2013: A CASE CONTROL STUDY. *Pakistan Journal of Public Health*, *8*(2), Article 2. https://doi.org/10.32413/pjph.v8i2.95

Donnelly, M. C., Scobie, L., Crossan, C. L., Dalton, H., Hayes, P. C., & Simpson, K. J. (2017a). Review article: Hepatitis E-a concise review of virology, epidemiology, clinical presentation and therapy. *Alimentary Pharmacology & Therapeutics*, *46*(2), 126–141. https://doi.org/10.1111/apt.14109

Donnelly, M. C., Scobie, L., Crossan, C. L., Dalton, H., Hayes, P. C., & Simpson, K. J. (2017b). Review article: Hepatitis E-a concise review of virology, epidemiology, clinical presentation and therapy. *Alimentary Pharmacology & Therapeutics*, *46*(2), 126–141. https://doi.org/10.1111/apt.14109

Drexler, J. F., Seelen, A., Corman, V. M., Fumie Tateno, A., Cottontail, V., Melim Zerbinati, R., Gloza-Rausch, F., Klose, S. M., Adu-Sarkodie, Y., Oppong, S. K., Kalko, E. K. V., Osterman, A., Rasche, A., Adam, A., Müller, M. A., Ulrich, R. G., Leroy, E. M., Lukashev, A. N., & Drosten, C. (2012). Bats worldwide carry hepatitis E virus-related viruses that form a putative novel genus within the family Hepeviridae. *Journal of Virology*, *86*(17), 9134–9147. https://doi.org/10.1128/JVI.00800-12

emhj. (n.d.). *Hepatitis E in Pakistan*. World Health Organization - Regional Office for the Eastern Mediterranean. Retrieved January 8, 2023, from http://www.emro.who.int/emhj-volume-2-1996/volume-2-issue-1/article17.html

He, J. (2006). Molecular detection and sequence analysis of a new hepatitis E virus isolate from Pakistan. *Journal of Viral Hepatitis*, *13*(12), 840–844. https://doi.org/10.1111/j.1365-2893.2006.00774.x

Hepatitis E. (n.d.). Retrieved December 31, 2022, from https://www.who.int/news-room/fact-sheets/detail/hepatitis-e

Hepatitis E virus infection—UpToDate. (n.d.). Retrieved January 8, 2023, from https://www.uptodate.com/contents/hepatitis-e-virus-infection

Iqbal, M., Ahmed, A., Qamar, A., Dixon, K., Duncan, J. F., Islam, N. U., Rauf, A., Bryan, J. P., Malik, I. A., & Legters, L. J. (1989). An outbreak of enterically transmitted non-A, non-B hepatitis in Pakistan. *The American Journal of Tropical Medicine and Hygiene*, *40*(4), 438–443. https://doi.org/10.4269/ajtmh.1989.40.438

Iqbal, T., Idrees, M., Ali, L., Hussain, A., Ali, M., Butt, S., Yousaf, M. Z., & Sabar, M. F. (2011). Isolation and characterization of two new hepatitis E virus genotype 1 strains from two mini-outbreaks in Lahore, Pakistan. *Virology Journal*, *8*(1), 94. https://doi.org/10.1186/1743-422X-8-94

JPMA - Journal Of Pakistan Medical Association. (n.d.). Retrieved January 8, 2023, from https://jpma.org.pk/article-details/2618.

Ju, X., & Ding, Q. (2019). Hepatitis E Virus Assembly and Release. *Viruses*, *11*(6), 539. https://doi.org/10.3390/v11060539

Khan, A., Tanaka, Y., Kurbanov, F., Elkady, A., Abbas, Z., Azam, Z., Subhan, A., Raza, S., Hamid, S., Jafri, W., Shih, J., Xia, N., Takahashi, K., Mishiro, S., & Mizokami, M. (2011a). Investigating an outbreak of acute viral hepatitis caused by hepatitis E virus variants in Karachi, South Pakistan. *Journal of Medical Virology*, *83*(4), 622–629. https://doi.org/10.1002/jmv.22036

Khan, A., Tanaka, Y., Kurbanov, F., Elkady, A., Abbas, Z., Azam, Z., Subhan, A., Raza, S., Hamid, S., Jafri, W., Shih, J., Xia, N., Takahashi, K., Mishiro, S., & Mizokami, M. (2011b). Investigating an outbreak of acute viral hepatitis caused by hepatitis E virus variants in Karachi, South Pakistan. *Journal of Medical Virology*, *83*(4), 622–629. https://doi.org/10.1002/jmv.22036

Khan, R. U., Beeching, N. J., Tong, C. W., Baker, B., Malik, I. A., Siddiqi, S., Tariq, W. U. Z., & Hart, C. A. (1995). Sporadic hepatitis E in Pakistan. *Annals of Tropical Medicine & Parasitology*, *89*(1), 95–97. https://doi.org/10.1080/00034983.1995.11812937

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*(8), 952–961. https://doi.org/10.1093/aje/kwq225

Lee, G.-H., Tan, B.-H., Teo, E. C.-Y., Lim, S.-G., Dan, Y.-Y., Wee, A., Aw, P. P. K., Zhu, Y., Hibberd, M. L., Tan, C.-K., Purdy, M. A., & Teo, C.-G. (2016). Chronic Infection With Camelid Hepatitis E Virus in a Liver Transplant Recipient Who Regularly Consumes Camel Meat and Milk. *Gastroenterology*, *150*(2), 355-357.e3. https://doi.org/10.1053/j.gastro.2015.10.048

Liang, H., Chen, J., Xie, J., Sun, L., Ji, F., He, S., Zheng, Y., Liang, C., Zhang, G., Su, S., & Li, S. (2014). Hepatitis E virus serosurvey among pet dogs and cats in several developed cities in China. *PloS One*, *9*(6), e98068. https://doi.org/10.1371/journal.pone.0098068

Nadeem, M., Ahmad, T., Kakar, S. J., Adnan, F., & Anjum, S. (2021). Hepatitis E virus genotyping in Pakistan: A regional study to explore the implications for pregnant females. *Future Virology*, *16*(9), 611–617. https://doi.org/10.2217/fvl-2020-0337

Purcell, R. H., & Emerson, S. U. (2008). Hepatitis E: An emerging awareness of an old disease. *Journal of Hepatology*, *48*(3), 494–503. https://doi.org/10.1016/j.jhep.2007.12.008

Purdy, M. A., Harrison, T. J., Jameel, S., Meng, X.-J., Okamoto, H., Van der Poel, W. H. M., Smith, D. B., & Ictv Report Consortium, null. (2017). ICTV Virus Taxonomy Profile: Hepeviridae. *The Journal of General Virology*, *98*(11), 2645–2646. https://doi.org/10.1099/jgv.0.000940

Rab, M. A., Bile, M. K., Mubarik, M. M., Asghar, H., Sami, Z., Siddiqi, S., Dil, A. S., Barzgar, M. A., Chaudhry, M. A., & Burney, M. I. (1997). Water-borne hepatitis E virus epidemic in Islamabad, Pakistan: A common source outbreak traced to the malfunction of a modern

water treatment plant. *The American Journal of Tropical Medicine and Hygiene*, *57*(2), 151–157. https://doi.org/10.4269/ajtmh.1997.57.151

Rasche, A., Saqib, M., Liljander, A. M., Bornstein, S., Zohaib, A., Renneker, S., Steinhagen, K., Wernery, R., Younan, M., Gluecks, I., Hilali, M., Musa, B. E., Jores, J., Wernery, U., Drexer, J. F., Drosten, C., & Corman, V. M. (2016). Hepatitis E Virus Infection in Dromedaries, North and East Africa, United Arab Emirates, and Pakistan, 1983–2015. *Emerging Infectious Diseases*, *22*(7), 1249–1252. https://doi.org/10.3201/eid2207.160168

Rashid, M., Khan, M. N., & Jalbani, N. (2021). Detection of Human Adenovirus, Rotavirus, and Enterovirus in Tap Water and Their Association with the Overall Quality of Water in Karachi, Pakistan. *Food and Environmental Virology*, *13*(1), 44–52. https://doi.org/10.1007/s12560-020-09448-8

Siddiqui, A. R., Jooma, R. A., & Smego, R. A., Jr. (2005). Nosocomial Outbreak of Hepatitis E Infection in Pakistan with Possible Parenteral Transmission. *Clinical Infectious Diseases*, *40*(6), 908–909. https://doi.org/10.1086/428357

Smith, D. B., Simmonds, P., Izopet, J., Oliveira-Filho, E. F., Ulrich, R. G., Johne, R., Koenig, M., Jameel, S., Harrison, T. J., Meng, X.-J., Okamoto, H., Van der Poel, W. H. M., & Purdy, M. A. (2016). Proposed reference sequences for hepatitis E virus subtypes. *The Journal of General Virology*, *97*(3), 537–542. https://doi.org/10.1099/jgv.0.000393

Stanaway, J. D., Flaxman, A. D., Naghavi, M., Fitzmaurice, C., Vos, T., Abubakar, I., Abu-Raddad, L. J., Assadi, R., Bhala, N., Cowie, B., Forouzanfour, M. H., Groeger, J., Hanafiah, K. M., Jacobsen, K. H., James, S. L., MacLachlan, J., Malekzadeh, R., Martin, N. K., Mokdad, A. A., … Cooke, G. S. (2016). The global burden of viral hepatitis from 1990 to 2013: Findings from the Global Burden of Disease Study 2013. *Lancet (London, England)*, *388*(10049), 1081–1088. https://doi.org/10.1016/S0140-6736(16)30579-7

Thakur, V., Ratho, R. K., Kumar, S., Saxena, S. K., Bora, I., & Thakur, P. (2020). Viral Hepatitis E and Chronicity: A Growing Public Health Concern. *Frontiers in Microbiology*, *11*. https://www.frontiersin.org/articles/10.3389/fmicb.2020.577339

Woo, P. C. Y., Lau, S. K. P., Teng, J. L. L., Cao, K.-Y., Wernery, U., Schountz, T., Chiu, T. H., Tsang, A. K. L., Wong, P.-C., Wong, E. Y. M., & Yuen, K.-Y. (n.d.). *New Hepatitis E Virus Genotype in Bactrian Camels, Xinjiang, China, 2013—Volume 22, Number 12—December 2016—Emerging Infectious Diseases journal—CDC*. https://doi.org/10.3201/eid2212.160979

Yin, X., & Feng, Z. (2019). Hepatitis E Virus Entry. *Viruses*, *11*(10), 883. https://doi.org/10.3390/v11100883

Zhou, J.-H., Li, X.-R., Lan, X., Han, S.-Y., Wang, Y.-N., Hu, Y., & Pan, Q. (2019). The genetic divergences of codon usage shed new lights on transmission of hepatitis E virus from swine to human. *Infection, Genetics and Evolution: Journal of Molecular Epidemiology and Evolutionary Genetics in Infectious Diseases*, *68*, 23–29. https://doi.org/10.1016/j.meegid.2018.11.024

Digital Receipt

This receipt acknowledges that Turnitin received your paper. Below you will find the receipt information regarding your submission.

The first page of your submissions is displayed below.

Submission author:	Mis Momina
Assignment title:	papers
Submission title:	Hepatitis E virus
File name:	Momina.pdf
File size:	1.94M
Page count:	73
Word count:	15,307
Character count:	78,491
Submission date:	09-Feb-2023 01:36AM (UTC-0800)
Submission ID:	2010008502

Copyright 2023 Turnitin. All rights reserved.

DR. ANEELA JAVED

Digital Receipt

This receipt acknowledges that Turnitin received your paper. Below you will find the receipt information regarding your submission.

The first page of your submissions is displayed below.

Submission author:	Mis Momina
Assignment title:	papers
Submission title:	Hepatitis E virus
File name:	Momina.pdf
File size:	1.94M
Page count:	73
Word count:	15,307
Character count:	78,491
Submission date:	09-Feb-2023 01:36AM (UTC-0800)
Submission ID:	2010008502

Copyright 2023 Turnitin. All rights reserved.

CamScanner

ORIGINALITY REPORT

15%	13%	8%	6%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

#	Source	%
1	Submitted to Higher Education Commission Pakistan Student Paper	2%
2	it.scribd.com Internet Source	1%
3	Submitted to Nelson Mandela Metropolitan University Student Paper	1%
4	www.domusmedica.be Internet Source	1%
5	www.ncbi.nlm.nih.gov Internet Source	1%
6	www.mdpi.com Internet Source	1%
7	hdl.handle.net Internet Source	<1%
8	J. He. "Molecular detection and sequence analysis of a new hepatitis E virus isolate from Pakistan", Journal of Viral Hepatitis, 12/2006 Publication	<1%

CamScanner

9	www.sid.ir Internet Source	<1 %
10	Submitted to Manipal University Student Paper	<1 %
11	apps.who.int Internet Source	<1 %
12	academic.oup.com Internet Source	<1 %
13	www.magbiogenomics.com Internet Source	<1 %
14	link.springer.com Internet Source	<1 %
15	Geng, Yansheng, Chenyan Zhao, Jinping Fan, Tim J. Harrison, Hongxin Zhang, Haichen Lian, Kunjing Geng, and Youchun Wang. "Genotype analysis of hepatitis E virus from sporadic hepatitis E cases in northern China", Infection Genetics and Evolution, 2013. Publication	<1 %
16	Submitted to Swinburne University of Technology Student Paper	<1 %
17	www.pubfacts.com Internet Source	<1 %

18	Submitted to Kwame Nkrumah University of Science and Technology Student Paper	<1 %
19	www.food.gov.uk Internet Source	<1 %
20	doaj.org Internet Source	<1 %
21	journals.plos.org Internet Source	<1 %
22	doi.org Internet Source	<1 %
23	Molecular Pathology Library, 2011. Publication	<1 %
24	porto.polito.it Internet Source	<1 %
25	eprints.hud.ac.uk Internet Source	<1 %
26	mdpi.com Internet Source	<1 %
27	Scott P. Kenney, Xiang-Jin Meng. "Hepatitis E Virus: Animal Models and Zoonosis", Annual Review of Animal Biosciences, 2019 Publication	<1 %
28	tudr.thapar.edu:8080 Internet Source	<1 %

No.	Source	Type	Match
29	Submitted to Coventry University	Student Paper	<1%
30	s3-ap-southeast-1.amazonaws.com	Internet Source	<1%
31	digital.maag.ysu.edu:8080	Internet Source	<1%
32	dr.ntu.edu.sg	Internet Source	<1%
33	"Hepatitis E Virus", Springer Nature, 2016	Publication	<1%
34	Chalupa, P., P. Vasickova, I. Pavlik, and M. Holub. "Endemic Hepatitis E in the Czech Republic", Clinical Infectious Diseases, 2014.	Publication	<1%
35	Laura Serracca, Roberta Battistini, Irene Rossini, Walter Mignone et al. "Molecular Investigation on the Presence of Hepatitis E Virus (HEV) in Wild Game in North-Western Italy", Food and Environmental Virology, 2015	Publication	<1%
36	research.birmingham.ac.uk	Internet Source	<1%
37	www.entomoljournal.com	Internet Source	<1%
38	Hepatitis E Virus, 2013.	Publication	

<1 %

	Source	
39	macsphere.mcmaster.ca Internet Source	<1 %
40	Submitted to Regis College Student Paper	<1 %
41	virologyj.biomedcentral.com Internet Source	<1 %
42	Submitted to Glasgow Caledonian University Student Paper	<1 %
43	Submitted to London School of Hygiene and Tropical Medicine Student Paper	<1 %
44	core.ac.uk Internet Source	<1 %
45	ecommons.aku.edu Internet Source	<1 %
46	dspace.lib.cranfield.ac.uk Internet Source	<1 %
47	financedocbox.com Internet Source	<1 %
48	Mara Lúcia Gravinatti. ">b/b<", Universidade de Sao Paulo, Agencia USP de Gestao da Informacao Academica (AGUIA), 2022 Publication	<1 %

No.	Source	Type	Match
49	Submitted to University of Glamorgan	Student Paper	<1%
50	ourarchive.otago.ac.nz	Internet Source	<1%
51	tel.archives-ouvertes.fr	Internet Source	<1%
52	"Hepatitis E Virus", Transfusion Medicine and Hemotherapy, 2015	Publication	<1%
53	Aftab Ahmed, Ijlal Akbar Ali, Hira Ghazal, Javid Fazili, Salman Nusrat. "Mystery of Hepatitis E Virus: Recent Advances in Its Diagnosis and Management", 'Hindawi Limited', 2015	Internet Source	<1%
54	Sergei A. Tsarev. "Infectivity titration of a prototype strain of hepatitis E virus in cynomolgus monkeys", Journal of Medical Virology, 06/1994	Publication	<1%
55	Soumi Sukla, Tarun K. Bhattacharya, R. T. Venkatachalapathy, Pushpendra Kumar, Arjava Sharma. " Cloning and characterization of α -casein gene of Riverine buffalo ", DNA Sequence, 2009	Publication	<1%
56	creativecommons.org	Internet Source	<1%

57	etheses.bham.ac.uk Internet Source	<1%
58	mce.nust.edu.pk Internet Source	<1%
59	parasitesandvectors.biomedcentral.com Internet Source	<1%
60	www.science.gov Internet Source	<1%
61	Bo Wang, Dominik Harms, C. Patrick Papp, Sandra Niendorf et al. "Comprehensive Molecular Approach for Characterization of Hepatitis E Virus Genotype 3 Variants", Journal of Clinical Microbiology, 2018 Publication	<1%
62	Labrique, A. B., K. Zaman, Z. Hossain, P. Saha, M. Yunus, A. Hossain, J. R. Ticehurst, and K. E. Nelson. "Epidemiology and Risk Factors of Incident Hepatitis E Virus Infections in Rural Bangladesh", American Journal of Epidemiology, 2010. Publication	<1%
63	Piyawat Komolmit, Vinita Oranrap, Sirinporn Suksawatamnuay, Kessarin Thanapirom et al. "Clinical significance of post-liver transplant hepatitis E seropositivity in high prevalence	<1%

area of hepatitis E genotype 3: a prospective study", Scientific Reports, 2020
Publication

64	Rajib Kishore Hazam, Rajiv Singla, Jugal Kishore, Saudan Singh, Ram Kumar Gupta, Premashis Kar. "Surveillance of hepatitis E virus in sewage and drinking water in a resettlement colony of Delhi: what has been the experience?", Archives of Virology, 2010 Publication	<1%
65	Submitted to University of Sheffield Student Paper	<1%
66	academicjournals.org Internet Source	<1%
67	dokumen.pub Internet Source	<1%
68	krishikosh.egranth.ac.in Internet Source	<1%
69	www.qiagen.com Internet Source	<1%
70	Bo Wang, Chun-Lin Cai, Bei Li, Wei Zhang, Yan Zhu, Wei-Hong Chen, Fei Zhuo, Zheng-Li Shi, Xing-Lou Yang. "Detection and characterization of three zoonotic viruses in wild rodents and shrews from Shenzhen city, China", Virologica Sinica, 2017 Publication	<1%

71 Oana Belei, Oana Ancusa, Adelina Mara, Laura Olariu et al. "Current Paradigm of Hepatitis E Virus Among Pediatric and Adult Patients", Frontiers in Pediatrics, 2021 <1%
Publication

72 Sarkar, Souvik, Elenita M. Rivera, Ronald E. Engle, Hanh T. Nguyen, Cathy A. Schechterly, Harvey J. Alter, T. Jake Liang, Robert H. Purcell, Jay H. Hoofnagle, and Marc G. Ghany. "An Epidemiologic Investigation of a Case of Acute Hepatitis E", Journal of Clinical Microbiology, 2015. <1%
Publication

73 Siddiqa, Abida, Maidah Zainab, Ishtiaq Qadri, Muhammad Bhatti, and Joanna Parish. "Prevalence and Genotyping of High Risk Human Papillomavirus in Cervical Cancer Samples from Punjab, Pakistan", Viruses, 2014. <1%
Publication

74 V. Tandon. "Surface fine topography and PCR-based determination of metacercaria of Paragonimus sp. from edible crabs in Arunachal Pradesh, Northeast India", Parasitology Research, 10/18/2007 <1%
Publication

75 bmcgastroenterol.biomedcentral.com <1%
Internet Source

#	Source	Type	Match
76	efsa.onlinelibrary.wiley.com	Internet Source	<1 %
77	hepatologyforum.org	Internet Source	<1 %
78	library.unisel.edu.my	Internet Source	<1 %
79	repositorio-aberto.up.pt	Internet Source	<1 %
80	static.igem.org	Internet Source	<1 %
81	upcommons.upc.edu	Internet Source	<1 %
82	v3r.esp.org	Internet Source	<1 %
83	www.ahcmedia.com	Internet Source	<1 %
84	www.scielo.org.co	Internet Source	<1 %
85	Aybüke Koyuncu, Daniel Mapemba, Iza Ciglenecki, Emily S. Gurley, Andrew S. Azman. "Setting a course for preventing hepatitis E in low and lower-middle-income countries: A systematic review of burden and risk factors", Cold Spring Harbor Laboratory, 2020	Publication	<1 %

86 Debing, Yannick, Darius Moradpour, Johan Neyts, and Jérôme Gouttenoire. "Update on hepatitis E virology: Implications for clinical practice", Journal of Hepatology, 2016. <1%
Publication

87 Mohammad Khuroo, Mehnaaz Khuroo, Naira Khuroo. "Transmission of Hepatitis E Virus in Developing Countries", Viruses, 2016 <1%
Publication

88 dspace.itg.be <1%
Internet Source

89 "Viral Hepatitis", Wiley, 2013 <1%
Publication

90 Anjum, Sadia. "Mutations in the STAT1-interacting domain of the hepatitis C virus core protein modulate the response to antiviral therapy", Molecular Medicine Reports, 2013. <1%
Publication

91 F. Bihl, F. Negro. "Hepatitis E virus: a zoonosis adapting to humans", Journal of Antimicrobial Chemotherapy, 2010 <1%
Publication

92 Nassim Kamar, Jacques Izopet, Nicole Pavio, Rakesh Aggarwal, Alain Labrique, Heiner Wedemeyer, Harry R. Dalton. "Hepatitis E <1%

virus infection", Nature Reviews Disease Primers, 2017
Publication

93	Thomas Horvatits, Julian Schulze zur Wiesch, Marc Lütgehetmann, Ansgar W. Lohse, Sven Pischke. "The Clinical Perspective on Hepatitis E", Viruses, 2019 Publication	<1 %
94	V. A. Arankalle, M. S. Chadha, S. A. Tsarev, S. U. Emerson, A. R. Risbud, K. Banerjee, R. H. Purcell. "Seroepidemiology of water-borne hepatitis in India and evidence for a third enterically-transmitted hepatitis agent.", Proceedings of the National Academy of Sciences, 1994 Publication	<1 %

Exclude quotes	On	Exclude matches	< 6 words
Exclude bibliography	On		