Molecular Typing of Enteroviruses: Current Status and Future Prospects

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By

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A thesis submitted in partial fulfillment of the requirements for the degree of

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DECLARATION

I certify that this research work titled **"Molecular Typing Current Status and Future Prospects"** is my own work. The work has not been presented elsewhere for assessment. The material that has been used from other sources has been properly acknowledged/referenced.

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I dedicate this thesis to my grandfather, **Abdul Habib Khan**, who always wished to see me prosper academically and pursue all my dreams. I hope that one day I will truly be able to fulfil all dreams that he associated with me. I am extremely thankful to both my parents for always supporting my decisions, for believing in me and praying for me endlessly.

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"Preparation can only take you so far, after that, you've got to take a few leaps of faith"

- Prison Break

LIST OF ABBREVIATIONS

AFP – Acute Flaccid Paralysis

ALF – Acute Liver Failure

BLAST – Basic Local Alignment Search Tool

CAR - Coxscakievirus-adenovirus receptor

CV – Coxsackievirus

ESCRT - Endosomal sorting complexes required for transport

EV – Enterovirus

FLF – Fulminant Liver Failure

HAV – Hepatitis A virus

HBV – Hepatitis B virus

HEV – Hepatitis E virus

ICAM1 - Intercellular Adhesion Molecule 1

IPV – Inactivated Poliovirus Infection

IRES – Internal Ribosomal Entry Sites

MVBs - multivesicular bodies

NPEV – Non-polio enterovirus

OPV – Oral Poliovirus Infection

ORF – Open Reading Frame

PCR – Polymerase Chain Reaction

PV – Poliovirus

RPM – Revolutions per minute

RT-PCT – Reverse Transcription PCR

SCARB – Scavenger Receptor class B

WHO – World Health Organisation

Abstract

Molecular techniques for isolation and characterization of viruses from clinical and environmental samples are routinely used in virological laboratories. Identification and genotyping of enteroviruses through molecular typing is crucial since the enteroviruses are commonly present within our environment and are also associated with a number of diseases. Enteroviruses are spread through the faeco-oral route and the prevalence of these viruses in the environment is possibly a route of viral spread in the community. This study has explored the presence of different enteroviruses among the environmental as well as clinical samples from Islamabad and Rawalpindi (the twin cities), and Lahore. The samples included sewage, drinking water and blood, all of which were processed and analysed through RT-PCR based detection assays to determine the presence of virus in our community. A total of 80 sewage samples were collected from the twin cities and 10 from Lahore. Among the 80 samples from the twin cities, 45 were positive for NPEV (56.2%), 4 samples contained enterovirus (6.2%) and 1 was positive for HAV (1.2%). On the contrary, no NPEV was detected among the sewage samples from Lahore, 4 samples were positive for enterovirus (40%) and 1 was positive for HAV (10%). In the case of drinking water, 74 samples were obtained from the twin cities and 15 from Lahore. Out of the 74 samples, 2 contained NPEV (2.7%), 2 were positive for enterovirus (2.7%) and 1 sample was found to be positive for HAV (1.35%). The 15 drinking water samples from Lahore were tested and 2 of these samples were found positive for enterovirus (13.3%). Clinical samples were collected only from the twin cities and revealed 15 cases of HAV (23% out of the total 65 blood samples) all of which were associated with infection in children and predominantly in the male gender. Among the 65 samples, 28

were positive for HEV infection (43%), all found among the female. Out of the 28 HEV positive females 17 (60.7%) were in the third trimester of their pregnancy. The genotyping of HEV revealed the presence of HEV1 in our study population. No other genotype was found during the course of this study. The findings of the HEV genotyping results and their dominant infections among the pregnant females is in line with evidences from other studies which have reported HEV1 to be the most predominant genotype associated with complications during the later stages of pregnancy. These findings indicate the need of wider screening and analysis within the community to identify the true disease burden associated with HEV infections. Sequencing and phylogenetic analysis of the strains isolated in this study showed close molecular relation of the NPEV strain with poliovirus strains previously reported from China. The HAV strain was in close relation with that of strains reported from India and the HEV1 strains in our study had phylogenetic relatedness with the strains reported from China and Pakistan. Overall, the sewage water from the twin cities contained high percentage of NPEV, having close phylogenetic relation with poliovirus. The clinical cases of HAV have been identified among the children and HEV has been found to be infecting the female population, with greater percentage of infections among the pregnant females. Together, the results from the environmental and the clinical samples suggest that different enteroviruses are present in Pakista

1. Introduction

1.1 Background

Enteroviruses cause a significant disease burden worldwide, particularly effecting children (Lukashev et al., 2014). The enterovirus genus contains four distant species, including the poliovirus (type 1 – 3), coxsackie A viruses, coxsackie B viruses and echoviruses. There are also certain numbered enteroviruses, such as Enterovirus D-68 to Enterovirus D-71, which are classified under the coxsackie B viruses' species (Charlotte Carina Holm-Hansen, Midgley, & Fischer, 2016). The diversity in the enteroviruses, increases the diversity of diseases caused by these viruses. The diseases associated with the enteroviruses include poliomyelitis, diseases of the cardiac system, the central nervous system, acute flaccid paralysis and gastric diseases (Charlotte C Holm-Hansen, Midgley, Schjørring, Fischer, & Infection, 2017). Till date, more than 110 different enteroviruses have been reported, each having genetic diversity and associated with human as well as non-human diseases (Lukashev & Vakulenko, 2017).

The worldwide burden of enterovirus infections is quite significant. These infections occur in a seasonal pattern and are generally on peak during the summer seasons (Ivanova et al., 2019). The most widely studied and documented among the enteroviruses is the poliovirus. The widespread studies on poliovirus have been carried out due the uncontrolled disease burden across the globe (Tebbens et al., 2006); (Grassly & Orenstein, 2018). The global efforts for eradicating poliovirus have been rigorous and continuous since the early 1960s (Kew & Pallansch, 2018). Other viruses among the enterovirus genus which are regularly associated with human diseases include rhinovirus,

coxsackie A viruses, coxsackie B viruses, hepatitis A virus (HAV), Hepatitis E virus (HEV) and the non-polio enteroviruses (NPEV) (Y. Li et al., 2017).

The viruses in the enterovirus genus are mainly water-borne and are spread through the faeco-oral route (Phyu, Ong, Wong, & infections, 2017). Other sources of enterovirus transmission include contaminated food (Phyu et al., 2017), droplets and human to human transfer through aerosols that pass during coughing and sneezing (Carroll, 2018). Areas having poor sanitation, lack of potable water and improper sewage treatment plants are at the highest risk of being the root of spreading enterovirus infections (O'Brien et al., 2017). The mode of transfer makes it probable that the developing countries would have the highest burden of such infections (Alhamlan, Al-Qahtani, & Al-Ahdal, 2015). Pakistan has poor sanitary conditions and the drinking water is often contaminated with sewage, which makes the prevalence of enteroviruses quite likely across the country (Amin et al., 2019; K. Khan et al., 2018). The lack of environmental surveillance and improper treatment of water bodies in majority of the areas in Pakistan severely increase the chances of disease outbreaks in the country (Ahmad et al., 2015).

1.2 Problem Statement

Environmental surveillance and scrutiny of diseases associated with enteric viruses are rarely carried out in Pakistan. The gaps in the disease surveillance and the undocumented cases of viral diseases make it hard to estimate the true disease burden. Molecular typing of enteroviruses is crucial for epidemiological studies and for studies related to molecular evolution of enteroviruses (Farkas et al., 2018). Detection, correct diagnosis and genotyping of the enteroviruses is crucial for identifying their environmental presence and disease burden in a given region at any given time (Cowger et al., 2017). The

deplorable state of sanitation in Pakistan makes it important to carry out disease surveillance so as to be prepared for disease outbreaks. Improvements in the identification methods for successful detection of enteroviruses are constantly needed. Such studies can help in gaining a better understanding of the transmission and epidemiological burden of enteroviruses (Benschop et al., 2017).

1.3 Research Objectives

The objectives for this research study include:

- Molecular typing of enteroviruses isolated from environmental and clinical samples within the twin cities (Islamabad and Rawalpindi) of Pakistan.
- Genotyping of Hepatitis E virus for identification of the genotype dominant in our selected region.
- Sequencing and phylogenetic analysis of the strains isolated in this study for analysing evolutionary history and molecular relatedness with strains reported previously.

1.4 Contribution to Knowledge

This research study is aimed at adding to the existing knowledge about the disease burden associated with enteroviruses. The gaps in epidemiological data of enterovirus infections from Pakistan pose a threat to public health. The results of this study can contribute towards documentation of the enteroviruses present in our environment and effecting our population. Till date, very few studies have been carried out for identifying the enterovirus disease burden in a comprehensive manner. The number of such studies from Pakistan is nearly negligible since previous studies have been limited in terms of the

number of enteroviruses explored, it is possible that this study will help in providing a broader range of knowledge about the enteroviruses prevalent in Pakistan.

2. Literature Review

2.1 Enteroviruses

The enteroviruses group comprises of a number of different viruses, causing a significant disease burden worldwide (Abzug, 2014). All of these viruses are positive, single-stranded RNA viruses, which are non-segmented and non-enveloped (Shaukat et al., 2012). The classification of the enterovirus genus further divides the viruses into different species and types (serotype or genotype) (Hyypiä, Hovi, Knowles, & Stanway, 1997). Initially, the enteroviruses were divided into polioviruses, rhinoviruses, hepatitis A virus, coxsackievirus A, coxsackievirus B and echoviruses (Tapparel, Siegrist, Petty, Kaiser, & Evolution, 2013). The taxonomy has been updated over time to categorize more viruses. The revised taxonomy of enteroviruses was presented and approved in 2012 by the International Committee on Taxonomy of Viruses (ICTV) (Lukashev & Vakulenko, 2017). This classification has since then been updated and corrected on various occasions. The current classification includes 8 species of enterovirus; enterovirus A, enterovirus B, enterovirus C, enterovirus D, enterovirus E, enterovirus F, enterovirus G, enterovirus H, enterovirus I and enterovirus J. The rhinovirus species (A, B and C) are also included among the enteroviruses (M. Li et al., 2019). Each species further contains a number of different viruses, divided into serotypes.

The enterovirus genome is packaged within a capsid that protects more or less 8000 nucleotides in the form of a single stranded RNA (Vogt & Andino, 2010). A diagrammatic representation of the enterovirus genome is shown in figure 1.1. Different species among the enteroviruses may have a slightly different arrangement of proteins but the general pathway of infection common to all species is the translation of the single

stranded RNA into a single polyprotein upon entry into the host cell (Chan, Sam, AbuBakar, & Evolution, 2010). The capsid protein of the enterovirus contains different proteins, including VP1, VP2, VP3 and VP4 (Shaukat et al., 2013). The virus enters the cell through either exposure to acidic environment inside the endosome or interaction with the host receptors (Shingler et al., 2013). In any case, conformational changes are introduced into the VP proteins and pores are developed in the capsid which provide area for releasing the viral genome inside the host cell (Nikonov, Chernykh, Garber, & Nikonova, 2017; Ren et al., 2013).
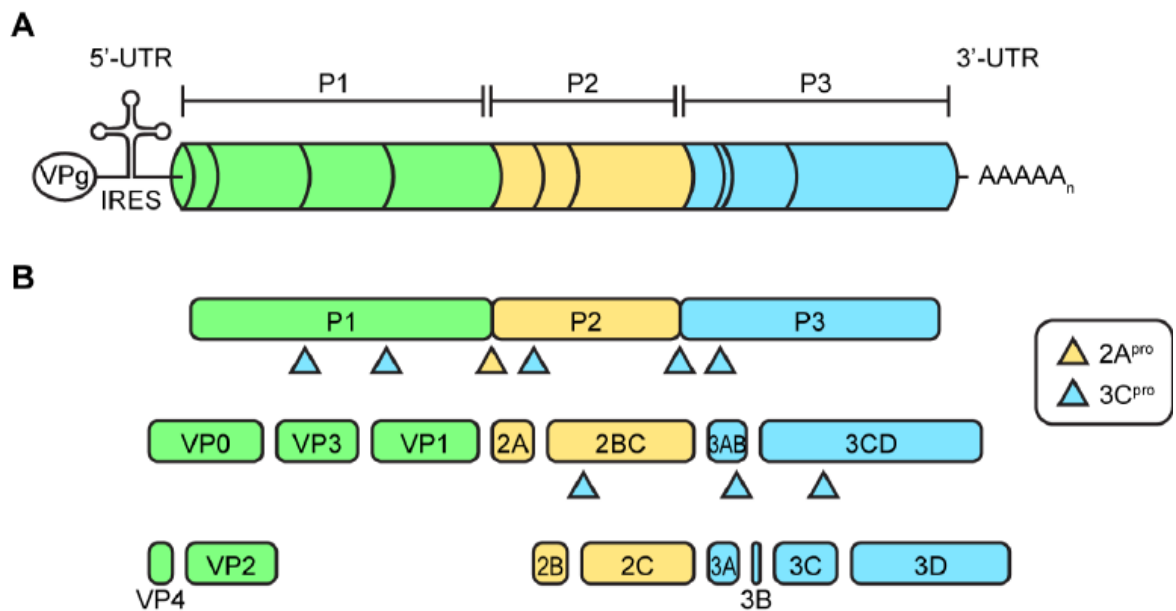
Figure 1.1: Enterovirus Genome. (A) The single stranded RNA genome having the VPg protein, the IRES, 5' UTR and the 3' UTR. (B) The protein translated from the RNA genome of the enteroviruses. Firstly, a polyprotein is produced which is later cleaved into structural and non-structural proteins constituting the virus structure and enzymes (Nikonov et al., 2017)

The enterovirus lifecycle is represented schematically in the figure 1.2. Briefly, the virus attaches to its specific receptor on the host cell and is up taken by the process of

endocytosis. In this way, the viral genome is delivered into the host cell cytoplasm. The replication of the positive stranded RNA genome takes place with the help of the viral protein VPg attached to the 5' end of the viral genome (Laitinen et al., 2016). The initial translation leads to a single polyprotein that is cleaved into structural and non-structural proteins in a proteolytic manner. The proteins include the capsid proteins such as VP0, VP1 and VP4 while the proteins which act as the viral enzymes include 2A, 2B, 2C, 3A, 3B, 3C and 3D (Lloyd, 2016). The viral replication takes place with the action of the viral RNA dependent RNA polymerase (RdRp). Initially, a negative strand RNA is synthesized from the positive sense genome and this template strand is then used for the synthesis of new genome molecules of the virus (Sofer et al., 2011). The entire replication process takes place in the cytoplasm, close to the membranous structures present in the cell which make an environment that is rich in lipids and favourable for viral replication and packaging (Baggen, Thibaut, Strating, & van Kuppeveld, 2018). The new RNA strands synthesized during the replication cycle can either get involved in subsequent replication cycles or get packaged within the capsids to become progeny virions (McIntyre, 2013). The capsid proteins are organized into pentamers and protomers along with the genomic RNA, followed by assembly into provirions which become mature when the capsid is cleaved. The mature viruses leave the host cells via lysis or through the extracellular vesicles (Lyu, Wang, et al., 2015).
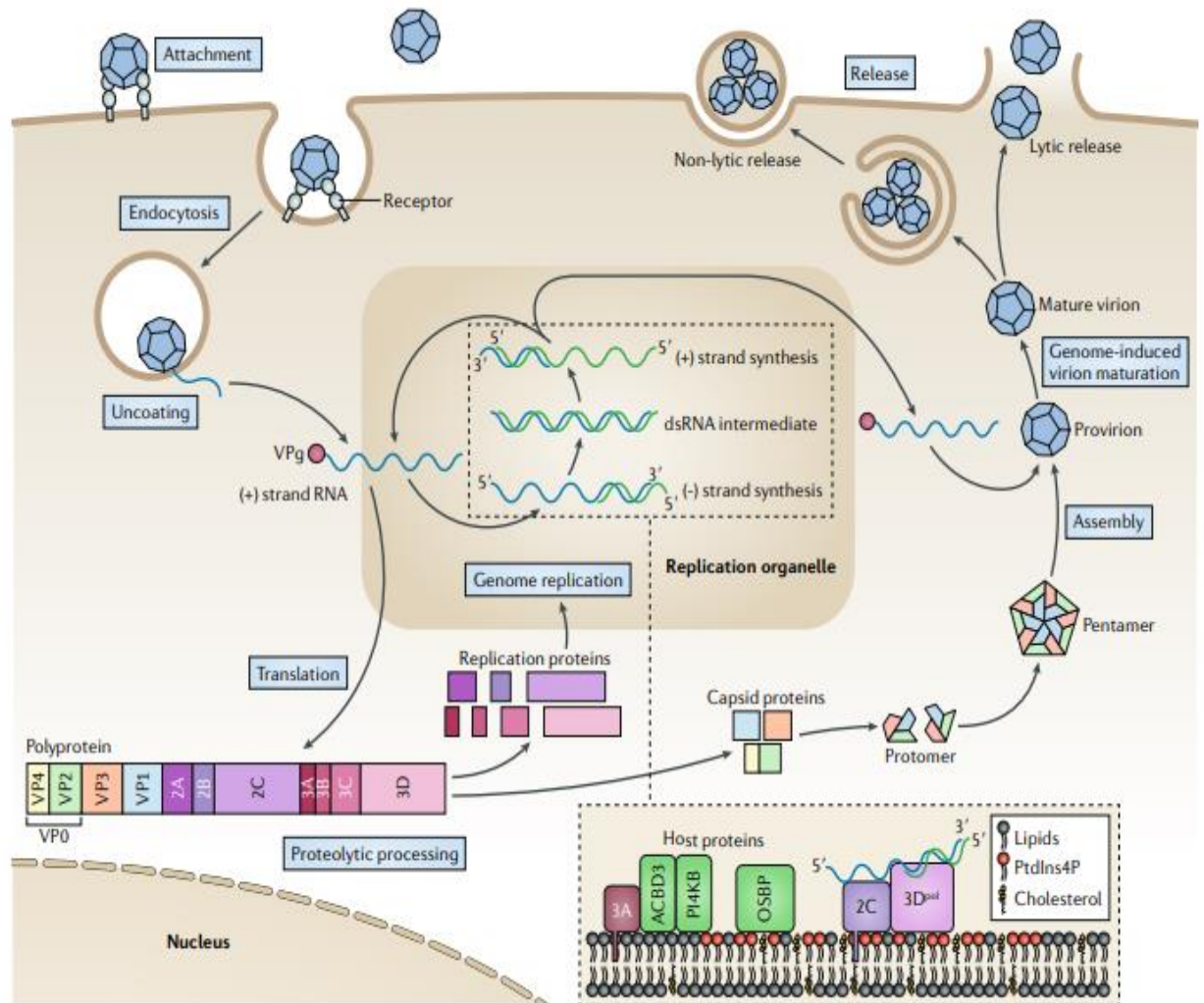
Figure 1.2: Enterovirus lifecycle. The virus enters the host cell through endocytosis by attaching to its respective receptor followed by uncoating of the virus within the endosome. The viral lifecycle proceeds within the cytoplasm by means of translation of the viral proteins and replication of the viral genome. The copies of genome are packaged within the structural proteins newly translated within the cytoplasm, followed by release of the virus from the host cell either by lytic or non-lytic pathway. (Baggen, Thibaut, Strating, et al., 2018)

The structure of enterovirus has been analysed which reveals that almost all of the viruses within the group have a similar structure. The three dimensional (3D) structure of enteroviruses has been resolved to show that all enteroviruses share similar structures (Wang et al., 2012). The basic structure contains 60 repeating units of protomers, each comprising of four capsid proteins, VP1, VP2, VP3 and VP4 (Plevka, Perera, Cardosa,

Kuhn, & Rossmann, 2012). These units together assemble to make up an icosahedral structure. The VP4 protein is present on the internal side of the capsid while the other 3 proteins are present on the external surface of the virus (Ren et al., 2013). The surface proteins are arranged in strands of 8 that represent a β barrel structure (Levy, Bostina, Filman, & Hogle, 2010). The capsid surface contains loops that are highly variable and can be easily accessible to the host immune system, thereby contributing to the antigenic diversity of the virus. A surface depression has been identified in most of the enteroviruses (Plevka et al., 2012). Most enteroviruses have a canyon structure or a deep, circular surface depression encircling each fivefold axis of symmetry that is known to serve as the receptor binding site for the viruses. The general similarities are sometimes deflected in certain viruses within the enterovirus group and unique features are also reported among these viruses (Nunes-Alves, 2015). The general structure and the unique features are highlighted through figure 1.3. The rhinovirus, for example has a protrusions covered surface in contrast to the other enteroviruses, which generally have smooth surfaces. These protrusions make the canyons of rhinovirus narrower than the other enteroviruses (Garriga et al., 2012). These differences have not been explored in depth in regards with their biological consequences. A hydrophobic pocket has been identified at the base of all enteroviruses canyons with the exception of rhinovirus (Martikainen et al., 2015). The hydrophobic pocket is generally filled with either a pocket factor or a lipid molecule which helps in maintaining the stability of the viral particle (Abdelnabi et al., 2019). Despite the high similarities in the structure of all enteroviruses, there are certain unique features which hold these viruses apart. Examples include the angular structure of HAV, the presence of protrusions on rhinovirus C, the presence of type II helices on the

capsid of Aichi virus and the absence of a canyon structure on the Ljungan virus which is instead comprised of protrusions arising from the viral surface at the five-fold axis (J. Chen et al., 2018; Egorova, Ekins, Schmidtke, & Makarov, 2019). These differences have been studied through the use of X-ray crystallography and cryo-electron microscopy (cryo-EM). Structural diversity and uniqueness among the viruses may further be studied in near future to add to the existing knowledge about the differences that exist within the enterovirus group (J. Chen et al., 2018).
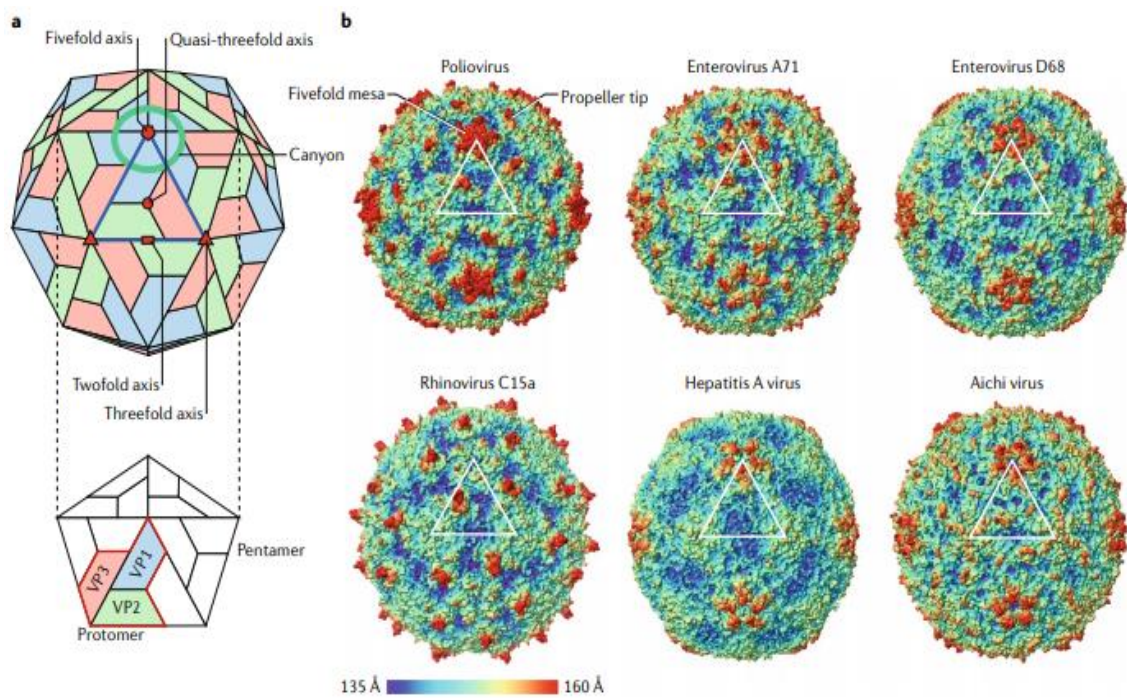
Figure 1.3: The virion structure of different enteroviruses. a) The overall structure common across all the enteroviruses. b) The structural uniqueness of different members of the group (Ren et al., 2013).

The entry of the virus into the host cell is through the surface receptors. The receptors are the major determinants of the viral tropism and the pathogenesis of specific viruses (Ren et al., 2013). The first receptor identification for enterovirus tropism came through studies on poliovirus which showed that the PV enters host cells through the use of either

the CD155 receptor or the intercellular adhesion molecule 1 (ICAM1) (Racaniello, 2016). Evidences revealed that the same receptors are used by rhinovirus for entry into the host cells (Greve et al., 1989). These studies led to many other similar explorations, which revealed that the cell surface receptors used by the NPEV belong to either the integrin receptor family or the immunoglobulin-like cell surface receptors. The identification of a wide variety of surface receptors provided some insights about the diversity of the enteroviruses (Y. Liu et al., 2015).

The viral receptors are majorly divided into two main categories, the receptors used for attachment and the ones that play a role in the uncoating of the virus (Baggen, Thibaut, Strating, et al., 2018). The attachment receptors are used for the attachment of the virus to the host cell and promote its uptake within the cell while the uncoating receptors mediate the uncoating of the viral capsid. Majority of the uncoating receptors are protein in nature with some exceptions such as the receptor that uncoats EV-D68, which is a sialylated glycan that complements the canyon structure of the virus and helps in uncoating of the virus (Wei et al., 2016). This finding provides proof that enteroviruses can bind to carbohydrates and be uncoated through those receptors. Also, the ICAM5 receptor was found to be an uncoating receptor for EV-D68 within the grey matter in the telencephalic region. This explains the ability of EV-D68 to cause AFP (Wei et al., 2016). However, why or how the virus engaged with two different uncoating receptors has not been explained yet.

The attachment receptors can also prove to be important for the pathogenesis of viruses (Y. Liu et al., 2015). These receptors can be useful in triggering downstream signaling processes within the host cell to ease the viral entry, uncoating and overall pathogenesis

(Sullivan, Seidman, & Muller, 2013). The binding of the coxsackievirus B to the decay accelerating factor (DAF) also known as the CD155 receptor, which is an attachment receptor mediates the activation of signaling molecules in the cell. The epithelial cells begin liberating the uncoating receptor, which is known as the coxscakievirus-adenovirus receptor (CAR) such that it becomes accessible for viral entry (Coyne & Bergelson, 2006). The attachment receptors are important in determining the tropism of a virus as viruses can only enter those cells that have specific receptors needed by the virus for attachment and entry (Grove & Marsh, 2011). The adaptation of a coxsakievirus A variant provided evidence about evolution in tropism through modification in its attachment mode to attach with a sialic acid receptor and lead to the development of hemorrhagic conjunctivitis in the host (Seitsonen et al., 2012). Furthermore, the presence of fatal strains of EV-71 have been known to gain access to the spinal cord and lead to damage in the brainstem but the exact receptor that facilitates the entry of the virus in the brainstem or spinal cord have not been identified yet (Yamayoshi, Fujii, Koike, & infections, 2014). Till now, there are two different receptors that have been reported to be used by EV-A71, the SCARB2 receptor (Scavenger receptor B) and the glycoprotein ligand 1. It is likely that with the evolution of the virus, more receptors have been added to the list of receptors used by EV-A71 to gain access to cells that it was previously unable to enter (Zhang et al., 2014). Evidences from studies in mice suggest that SCARB2 is found on the glial cells and neurons, suggesting that a possible pathway for entry to brain cell could be present in humans (Xie et al., 2016). As far as receptor identification is concerned, it can help in gaining an understanding about the pathogenicity of viruses but there is a lot more that needs to be discovered for

understanding how a virus overtakes cellular machinery of the host and manipulates it for

its own benefit (R. Li, Zou, Chen, Zhang, & Wang, 2011). An overview of the different

receptors used by the enteroviruses is shown through table 1.1.

Table 1.1: Receptors used by Enteroviruses

Receptor	Virus	Role of Receptor
CD155	Poliovirus	Uncoating
CAR	Coxsackievirus	Uncoating
DC-SIGN	EV-A71	Attachment
ICAM	Rhinovirus	Uncoating
Integrin $\alpha_V\beta_3$	CV-A9	Attachment
SCARB2	EV-A71	Uncoating
Sialic acid	EV-A71, EV-D70	Attachment

The Enterovirus C comprises of the poliovirus (Type 1, 2 and 3). This is one of the most

widely studied enterovirus species and has been the cause of major health concerns

worldwide (Tebruegge & Curtis, 2009). The initial classification of enteroviruses was

developed around keeping the poliovirus infection as a separate group. This was further

differentiated on the basis of paralytic or non-paralytic poliomyelitis (Niepmann, 2009).

The 19th century presented an immense challenge of dealing with poliomyelitis. Vaccine

development and a worldwide campaign to fight poliomyelitis in the 20th century proved

to be a positive approach in reducing the disease burden (Cochi, Hull, Sutter, Wilfert, &

Katz, 1997). Today, the poliovirus is present only in Afghanistan and Pakistan. Strategic

planning and vaccination programs are still underway to ensure that the global eradication of poliovirus turns out to be successful (Kew & Pallansch, 2018).

Infections associated with enteroviruses have been reported to have a wide variety of clinical manifestations. These manifestations include mild and self-limiting conditions as well as severe symptoms. The severe cases include meningitis, acute flaccid paralysis, myocarditis, respiratory illnesses, neurological diseases, gastric diseases, haemorrhagic conjunctivitis and perinatal infections (Kumar et al., 2012). The enteroviruses also cause severe chronic diseases among the immunocompromised patients and the transplant recipients (Chia, Chia, Voeller, Lee, & Chang, 2010). Many of the clinical features have been assigned to specific groups among the enteroviruses, for example myocarditis with coxsackie B virus haemorrhagic conjunctivitis with EV70 and paralysis with polioviruses. However, these conditions are likely to be added with modified clinical manifestations over time. As the viruses evolve, their clinical manifestations and the severity of diseases associated with them are also modified (M. Chen et al., 2017).

Almost 90% of the infections caused by enteroviruses are reported to be non-symptomatic (T.-C. Chen et al., 2008). This leaves only 10 % of the enterovirus infections that have defined clinical manifestations. The detection and treatment of infections caused by enteroviruses is therefore not fully elucidated. The most common and reliable approach for the detection of enteroviruses is the PCR based detection (Archimbaud et al., 2009). Since many of the enteroviruses have not been successfully grown on cell culture yet, the detection studies are mainly based on molecular analysis and typing of viruses. Moreover, the PCR based detection of viruses is much quicker and

sensitive method than the cell culture approach (Oberste, Peñaranda, Rogers, Henderson, & Nix, 2010).

The initial days of enterovirus research and detection were mainly focused on the identification of VP1 protein (Oberste et al., 1999). It has been reported that the VP1 region is the most crucial for determining the serotype of the virus. The antigenic typing has been conducted through the use of the VP1 region of different enteroviruses (Shih et al., 2000). Neutralization studies using VP1 region have been conducted for identifying enterovirus variants. However, the limitation to this approach lies in the fact that the serotype of a virus is not the most reliable for detecting all of the enteroviruses. Although the VP1 region is known to be largely conserved across all enteroviruses, detection of all these viruses through a single set of primers is not possible due to specie-specific variations (Oberste et al., 2010). The serotype and genotype specific variations within the enterovirus group make it highly unlikely for the VP1 region to be used for detection of all the viruses within the group (Brown, Kilpatrick, Oberste, & Pallansch, 2000). It is possible to employ the VP1 based detection for the development of a phylogenetic tree and studying the evolutionary history of the enteroviruses (Oberste et al., 2006).

The evolution of enteroviruses is dependent on error-prone replication and mutations. Recombination is also an important mode of evolution for the enteroviruses (Davis et al., 2008). Recombination has been reported to mend mutations in the enterovirus genome and lead to restoration of the essential genes, thereby rescuing the fit genes from a mutated virus to a hospitable and fit environment for the viral evolution (Savolainen-Kopra & Blomqvist, 2010). The development of divergent genomes and higher levels of fitness genes gathered in a pool create opportunities for more adaptable viral genomes

being sustained over time (Lowry, Woodman, Cook, & Evans, 2014). Recombination between different enteroviruses requires the co-infection of a host cell by two viruses (Lowry et al., 2014; Xiao et al., 2016). Two possible methods of recombination can occur, both of which are currently based on theories. The first theory suggests that recombination between two viruses occurs during active replication in both the viruses. On the contrary, another theory proposed the occurrence of recombination in the absence of replication. Both of these theories are valid so far and no proof has yet been seen which can rule out either of the two theories (X. Chen et al., 2010).

The exact history of recombination events and the evolution of enteroviruses is not completely understood. It has been found that recombination events are more common among members of the same species than between members of different species (Holmblat et al., 2014). The most widely documented recombination events are among the enterovirus B species (Han et al., 2012). One of the limitations in studying the recombination events across different species of enteroviruses is the absence of whole genome sequences of all the enteroviruses. This makes it extremely difficult to draw conclusions regarding the recombination events between different enterovirus species (Muslin, Joffret, Pelletier, Blondel, & Delpeyroux, 2015).

2.1.1 Poliovirus

Poliovirus is the causative agent of poliomyelitis, a paralytic disease in humans. Poliovirus is part of the *Picornaviridae* family which contains various plus strand RNA viruses that infect humans and other animal species (Gong & Peersen, 2010). Poliovirus was first reported in the early 20th century, when paralytic diseases in human beings were reported in the USA. This was followed by intensive research on identifying the

treatment options and vaccine developments. Successful development of the Salk's inactivated polio vaccine (IPV) in 1955 led to a significant reduction in the poliovirus epidemiology by the 1960s. The live oral polio vaccine (Sabin's OPV) was marketed and used from 1962 onwards. The second vaccine was developed on the basis of the idea that an oral vaccine could better mimic the natural lifecycle of poliovirus and thus had a chance of higher efficacy (Hird & Grassly, 2012). Since then, efforts for global eradication of poliovirus have been in place and a number of different countries around the world, particularly the developed countries have been declared polio free. However, unfortunately, the incidence of poliovirus is reported sporadically from the underdeveloped countries and the environmental prevalence and spread has not been totally controlled. (Hovi et al., 2012)

There are three known poliovirus serotypes, all of which are associated with paralytic disease in humans. The route of transmission of the virus is faeco-oral and ingestion of the virus is followed by its multiplication in the intestinal mucosa (Minor, 2016). The entry of poliovirus in the central nervous system leads to the development of paralytic symptoms. The virus reaches the central nervous system through the blood stream (M.-M. Georgescu et al., 1994). Type 1 poliovirus is the most potent for causing paralysis while type 2 poliovirus is the least paralytogenic (Kew et al., 2002). In most cases, the poliovirus is excreted out of the patient's body through faeces after causing only mild symptoms and there is no carrier state associated with the immunocompetent individuals (M. M. Georgescu et al., 1997). However, individuals having an immunocompromised state are likely to carry the virus in their bodies for a longer period of time and can secrete poliovirus for up to 24 months (Khetsuriani et al., 2003). Depending on the

immune status of an individual, the poliovirus infection may range from mild illness to paralysis or aseptic meningitis (Kamboj, Sepkowitz, & Epidemiology, 2007). The genome of poliovirus is translated into a single polyprotein, as shown through figure 1.4.
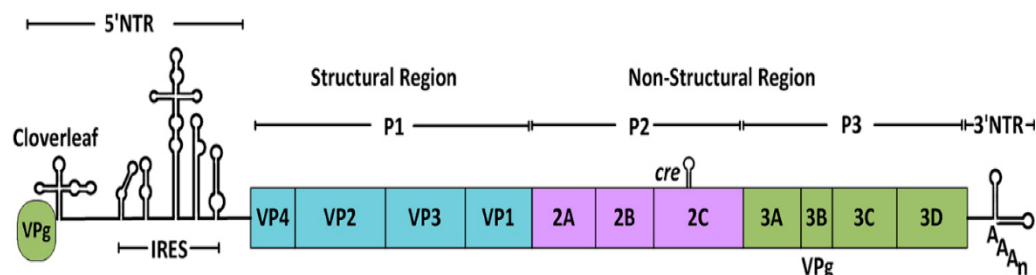
Figure 1.4: Genome of Poliovirus showing the untranslated regions, the internal ribosomal entry sites (IRES) and the division of the structural and non-structural regions within the open reading frame (Vogt & Andino, 2010)

Poliovirus vaccines have been linked with risk of activating disease in the immunized individuals. The first dose of OPV and IPV are known to have a predictable line of action. However, the attenuated strains of poliovirus in the Sabin OPV has been positively linked with vaccine-derived poliomyelitis (Avellón et al., 2008). Vaccine-derived poliovirus infection is a major health concern and holds a negative impact in the post eradication era. The removal of OPV from the markets can lead to complications in containing the epidemiology of poliovirus infections. The disease causing ability of the Sabin poliovirus vaccine lies in its instable nature (Korotkova et al., 2016). The cases of vaccine-derive poliomyelitis are sporadic. These cases have led to an increasing interest among the public health and policy making bodies across the world. Identifying ways in which the vaccine derived strains of poliovirus can be controlled is crucial in the post-poliovirus eradication era (Razafindratsimandresy et al., 2018).

The polio eradication program has been running since decades and the updates in 2010 revealed that the polio cases were reduced up to 99% in endemic countries like Nigeria, India, Afghanistan and Pakistan. within a few years, polio cases further dropped from 125 to 4 cases in ("Progress toward eradication of polio - worldwide, january 2011-march 2013," 2013). In 2010, 1292 cases of infection with wild type poliovirus were reported, out of which 1060 (82%) cases were found in the non-endemic countries. The distribution of case was; 458 cases from Tajikistan and 384 from Congo. Newer vaccination strategies were pondered upon by the World Health Organization in 2013 ("Global polio eradication: not there yet," 2013) (Chatterjee, Vidyant, & Dhole, 2013). According to the latest reports by WHO, the total number of polio cases recorded in 2018 were 33, out of which, 12 cases were reported from Pakistan. Also, the surveillance of sewage bodies of Pakistan has revealed continuous presence of wild type poliovirus in the major cities such as Karachi, Lahore, Rawalpindi, Peshawar and Quetta (F. Khan et al., 2018). Vaccine efficacy of Oral Poliovirus Vaccine (OPV) can depend on the geographical distribution. Regular OPV program can be sufficient to interrupt wild type poliovirus (WPV) transmission, but it has been seen that where the transmission of WPV is high; the efficacy of OPV is low. The areas where the wild type poliovirus transmission is low, was found to have regular OPV program. Pakistan, Nigeria, and Afghanistan are among the regions which failed to prevent WPV even after the extensive vaccination program (Owais, Khowaja, Ali, & Zaidi, 2013).

2.1.2 Non-Polio Enterovirus

The non-polio enterovirus (NPEV) includes some crucial pathogens within the enterovirus group. These viruses can include rhinoviruses, coxsackieviruses, numbered

enteroviruses and echoviruses (Faleye & Johnson, 2015). Like the other enteroviruses, infections with NPEV are generally asymptomatic and self-limiting. However, there are chances of severe infections within children and immune-compromised patients. The diseases associated with NPEV include sepsis, myocarditis, pericarditis, acute flaccid paralysis, febrile illness, encephalitis and pleurodynia. The rhinovirus is associated with common cold, and also severe respiratory diseases such as chronic obstructive pulmonary disease (COPD) and asthma (Pons-Salort, Parker, & Grassly, 2015). The diversity of these viruses and the range of diseases caused by them has made it difficult for the development of vaccines or preventive measures to treat patients suffering from NPEV infections (Baggen, Thibaut, & Strating, 2018).

NPEV infections are known to follow a seasonal pattern and are commonly reported in the tropical regions of the world (Pons-Salort et al., 2018). Despite many of the enterovirus infections being asymptomatic and self-limiting, it is possible that some of the NPEVs can lead to debilitating diseases and sporadic cases or outbreaks of NPEV infections are not uncommon (Pons-Salort et al., 2015). Moreover, NPEV are now largely being considered to be associated with neurological diseases, but the exact cause and pathogenesis of the infections being unfathomed as yet (Huang & Shih, 2015). The incidence of coxsackievirus A16 and EV-A71 have reported to have increased within the Asia-Pacific region in the recent years (Lugo & Krogstad, 2016). Moreover, the presence of NPEV poses a continued disease burden of AFP, despite the eradication of wild type poliovirus from many regions across the world. The positive association of NPEV with AFP is alarming and poses threats to human health worldwide (Maan, Dhole, & Chowdhary, 2019).

2.1.3 Hepatitis E virus

The Hepatitis E virus (HEV) is a single stranded, positive sense RNA virus, having a genome size of approximately 7.2 Kb and is non-enveloped. HEV belongs to the *Hepeviridae* family (Kamar, Dalton, Abravanel, & Izopet, 2014). The genome of HEV contains three open reading frames (ORF), a cap of 7-methylguanosnine cap at the 5' end and a poly-adenylated 3' end (Meng, 2010), as shown through figure 1.5. The ORF 1 of HEV encodes 1700 amino acids which contain the several non-structural protein domains including the methyltransferase, the viral RNA dependent RNA polymerase (RdRp), cysteine protease, guanyltransferase and a macrodomain (domain X) (Engle, Yu, Emerson, Meng, & Purcell, 2002). In the ORF1 region of the HEV-1, there is an additional ORF present, known as the ORF4 which helps in the stimulation of the polymerase activity in HEV1 in the case of stress generated through the endoplasmic reticulum (Raj et al., 2012). The ORF2 forms a 660 amino acid portion comprising of the structural protein; the capsid. The capsid has three different kind of domains, the shell domain, the middle domain and the protruding domain which is the major target of the neutralizing bodies against HEV. The protruding region has also been reported to contain a putative receptor binding site and has thus been used in various studies for identification of possible vaccine epitopes (Xing et al., 2010). The ORF3 comprises of 114 residues (except in the case of HEV 4, which contains an ORF3 of 113 amino acid residues) and is responsible for translating into a small sized protein which helps in the exit of HEV from the host cells (Kamar et al., 2017). There is a conserved motif on ORF3

(Pro-Ser-Ala-Pro) which helps it in interacting with the sorting complexes of the endosomes for viral transport.
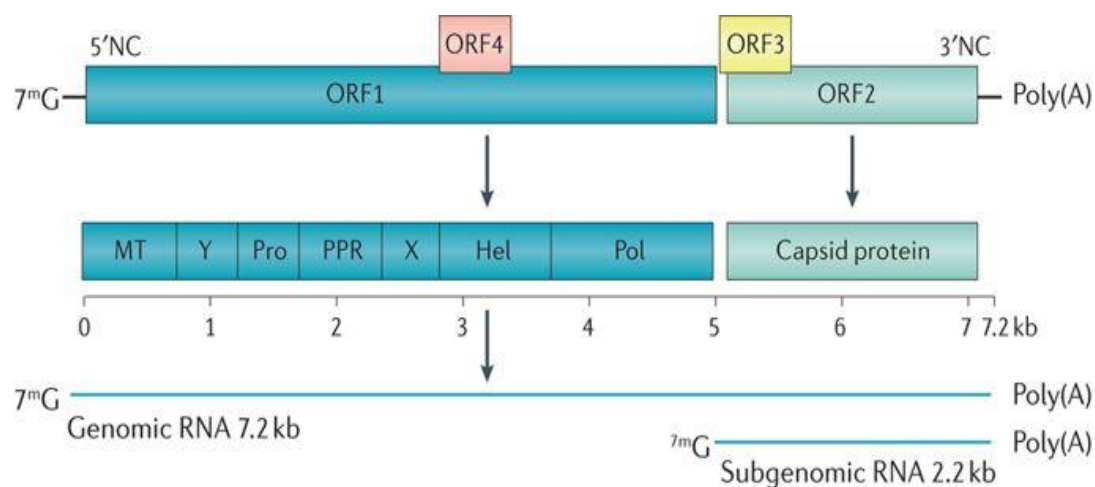
Figure 1.5: Organisation of the 7.2 Kb HEV genome. A 5' methyl guanosine cap and a 3' poly adenosine tail is present in the HEV genome. The genome consists of ORF1 which codes for the non-structural proteins of the virus, the ORF2 and an overlapping ORF3 which together code for the capsid protein. An additional ORF (ORF4) is present only in the HEV-1 which takes part in cleavage of the viral proteins after the polyprotein translation. During replication, sub-genomic RNA of 2.2 kb are produced which code for the capsid protein of HEV (Kamar et al., 2017)

The phylogenetic studies on HEV reveal that the different genotypes of the virus contain a slightly diverged path of evolution, which also sheds light on the differences in the epidemiological patterns of the different genotypes (P. Liu et al., 2012). The divergence studies have showed that the HEV ancestors forked into two kinds of genotypes; the anthropotropic genotype which contains HEV-1 and HEV-2 and the enzootic genotype, which comprises of HEV-3 and HEV-4 (Purdy & Khudyakov, 2010). HEV is among the self-limiting viruses that have been implicated to be transmitted via contaminated water and food sources (Purdy & Khudyakov, 2011). Recent evidences suggest that the HEV is also transmitted through the zoonotic routes (Brayne, Dearlove, Lester, Pond, & Frost,

2017). The animals generally infected with HEV include rabbits, swine, wild boars, deer and rats (Doceul, Bagdassarian, Demange, & Pavio, 2016). The genotypes 1 and 2 are generally found in humans while zoonotic transmission to humans involve the HEV serotype 3 and 4 (Kamar et al., 2014). These genotypes cause infections in humans which are generally self-limiting. However, recent evidences suggest that HEV has been found in the serum of the infected people and the long term prevalence of the virus can lead to fulminant liver infections. The chronic infection with HEV is widely reported in the liver transplant patients (Lee et al., 2016). It has been seen that the complications and chronic infections are highly associated with the pregnant females. These complications include hyper immune response, liver failure, fetal death and even death of the mother. Moreover, the prevalence of the virus in the blood is a cause of concern (Rasche et al., 2016). Therefore, it is important to ensure that blood is screened before transfusions and the pregnant females should be tested for the presence of the virus so as to avoid any complications or transmission to the neonates (Sehgal et al., 2015).

The disease burden of HEV is particularly alarming in the case of pregnant females. It has been found that HEV primarily causes debilitating infection among the pregnant females. Fulminant liver disease has been reported to be associated with HEV among the pregnant females (Bose et al., 2011). Such cases are mainly associated with the genotype 1 and 2 of HEV. However, a few cases of fulminant liver disease have also been reported to be associated with genotype 3 and 4, but these cases are very limited and have been reported from the developed countries (Anty et al., 2012). The major reports of fulminant hepatitis among the pregnant females is associated with HEV genotype 1. These females are reported to be majorly from the developing countries or the regions endemic for HEV

infections (Nelson, Labrique, & Kmush, 2019). The occurrence of different genotypes of HEV has been highlighted through figure 1.6. It can be seen that genotype 1 and 2 are commonly found in the developing countries, particularly in the Asian continent while the genotypes 3 and 4 are commonly found in the developed countries (Song, 2010).
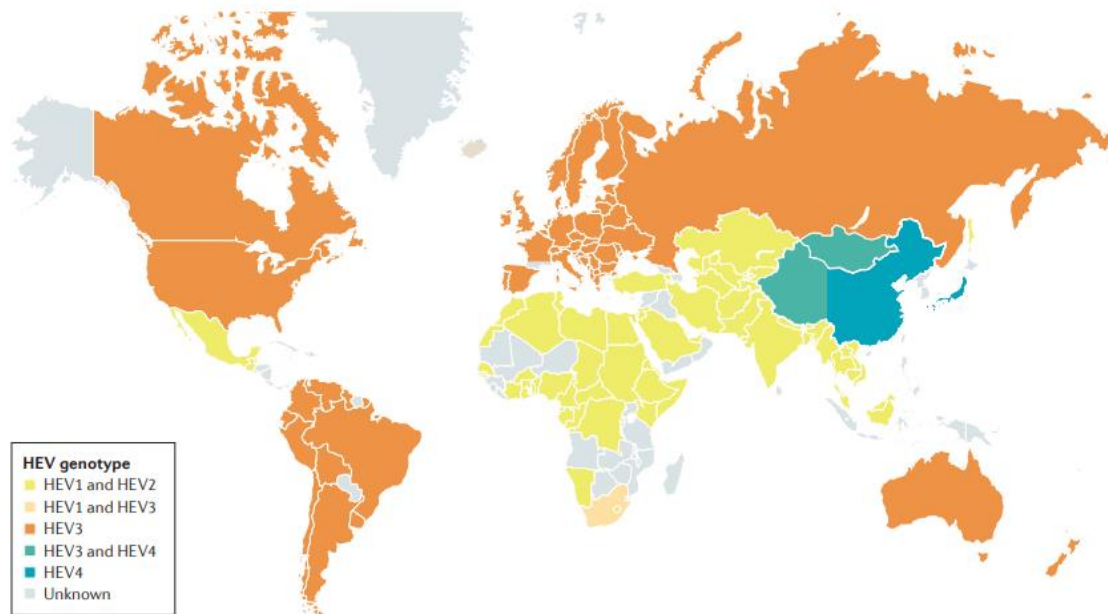
Figure 1.6: HEV genotypes found worldwide. The HEV-1 and HEV-2 are common in South Asia and Africa, HEV-3 is predominantly found in North America, South America, Europe and Australia while HEV-4 is reported from some parts of East Asia and Europe (Kamar et al., 2017).

HEV, like all other enteroviruses, is a waterborne virus that infects humans through the faeco-oral route. The genotypes 1 and 2 of HEV are solely associated with disease in humans (Nelson et al., 2019). On the contrary, the HEV-3 and HEV-4 have been found to be associated with zoonotic transmission from pigs (Tessé et al., 2012). The contamination of HEV-3 and 4 is found in sewage water as well, where it is shed by the infected animals in the form of faeces (Doceul et al., 2016). Ingestion of water contaminated with any genotype of HEV can lead to the development of HEV infection

(Ahmad et al., 2010). The consumption of food products obtained from HEV infected animals is also a possible source of developing infection of HEV-3 and 4. Evidences of HEV-3 and HEV-4 have been found in domestic animals including sheep, cows, cats, dogs, rabbits, buffaloes and horses, suggesting that these animals can be reservoirs of the zoonotic strains of HEV (Cook, Van der Poel, & virology, 2015) .

The zoonotic potential of HEV-3 and HEV-4 are a cause of concern for those regions where pig farming and swine based foods are common (Corman et al., 2019). Many areas across Europe and China have been reported to have 100% rate of contamination and transmission of HEV (Khuroo, Khuroo, & Khuroo, 2016). These areas pose threat to human health as the virus is shed in excessive quantity from the pigs. Infected pork is the major source of transmission of HEV to humans in these regions and raw or undercooked pork can lead to immediate transmission. Evidences of HEV being transmitted from rabbits have been reported from France (Bouthry et al., 2018). These evidences have been linked with the presence of HEV-3 among infected persons, who contracted the virus from rabbits, suggesting that the strains infecting rabbits hold zoonotic potential (Dalton & Izopet, 2018). Similarly, HEV-7 has been reported in humans who consume milk or meat sourced from camels (Bassal et al., 2019). These cases were reported among the immunocompromised patients, who had to undergo liver transplantation following HEV infection. There are yet no evidences of HEV-5 and HEV-6 being present in humans, but there are reports from Japan where these viruses have been reported in wild boars (Mirazo et al., 2018).

The transmission of HEV is also possible through the transfusion of contaminated blood (Al-Sadeq, Majdalawieh, & Nasrallah, 2017). The United Kingdom has made it

necessary for blood donors to be screened for HEV infections to ensure that the population is not exposed to HEV risk arising from the transfusion of contaminated blood (Domanović et al., 2017). Many of the European countries have reported the contraction of HEV through blood products. Evidences of HEV-1 and HEV-4 infections have commonly been found in Europe while that of HEV-3 and HEV-4 have been reported from Japan (Minagi et al., 2016). Zoonotic transmission of HEV is common in the developed countries and the infections are generally asymptomatic (Sasaki, Haruna, Uema, Noda, & Yamada, 2018).

HEV infections are asymptomatic and non-cytopathic. There are three different stages of HEV infection, including; the incubation period, the acute infection period and the end period which is also known as the convalescent phase which is associated with steady recovery (Tedder et al., 2016). HEV infection is generally self-limiting and asymptomatic but in certain cases, there may be the development of chronic infection. These chronic infections are mostly found among the immune-compromised patients (Walker, 2018). HEV is basically a hepatic virus that multiples rapidly in the liver and can at times cause extra-hepatic manifestations by reaching the digestive system through bile (Kamar et al., 2015). The active infection can be detected within 7 days of contracting the virus but there is no way of identifying the percentage of infected hepatocytes. Immunohistochemistry has revealed that despite being a hepatic virus, HEV is found to be actively replicating in the kidney, digestive tract, placenta and the central nervous system. The infected individuals shed the virus through stool and urine (Kamar, Marion, Abravanel, Izopet, & Dalton, 2016). Once the virus is cleared out of a person's body, they develop immunity against it. However, the chances of reinfection cannot be ruled

out as there are different genotypes of the virus and even the same genotype has been reported to reinfect individuals. In the case of reinfection, the chances of symptoms being developed and severity being increased are quite high (Blasco-Perrin & Peron, 2018).

The presence of HEV in human placenta is a major cause of maternal health concerns. Maternal and fetal morbidity and mortality have been reported due to HEV infection and are associated with acute liver failure (ALF) (Gouilly et al., 2018). Immunity, viral load, hormones and signaling molecules are important factors in determining the outcomes of HEV infections during pregnancy. The third trimester of pregnancy is known to be the most critical in terms of viral infection since the sex steroid hormones are very high during this stage which facilitates viral replication. The spread of virus from mother to fetus is common during the early stages of pregnancy and fetal death can occur due to infection. Studies have revealed the presence of actively replicating virus particles in human placenta, which suggests that HEV replicates readily in the placental environment. In the case of pregnant females suffering from HEV infection and having ALF, there have been evidences of preterm delivery (Chaudhry, Verma, & Koren, 2015). While immune mediated damage of placenta has also reported to occur in pregnancy. The complications of HEV infection arise during pregnancy due to the release of high levels of pregnancy related hormones and the general lowering of immunity (Baptista-González, Trueba-Gómez, Rosenfeld-Mann, Roque-Álvarez, & Méndez-Sánchez, 2017). High titers of virus have been reported in the placenta of the pregnant females and the placental tissues have been implicated as favourable replicating sites for HEV (Cartwright & Whitley, 2017). Interestingly, during the active replication of HEV in the

placenta, the viral load in the serum is comparatively quite low, indicating higher concentrations of the virus within the placenta (Trivedi & Shah, 2019).

Fulminant hepatic failure (FHF) has been reported among pregnant females from developing countries who present with HEV infection. In such regions, around 30% of the patients suffer from FHF (Bouthry et al., 2018). The condition is associated with complications such as haemorrhage, eclampsia, membrane rupture, still births or spontaneous abortions. The presence of the pathogen leads to dysfunction in the maternal-fetal interface or the decidua (the endometrium lining in the placenta) (Sahai & Kiran, 2015). These conditions are mainly associated with HEV-1 infections. The infections of HEV-3 during pregnancy are reported to be rather harmless. It has been found that infection with either HEV-1 or HEV-2 during the second and third trimester of pregnancy account for around 25% of maternal deaths (Mishra, Jha, Thakur, Tiwari, & Gynecology, 2016). One of the possible reasons behind the differences in infection severity between the different genotypes is the presence of an additional ORF in HEV-1. The ORF4 has been implicated as a possible source of recruiting host factors in the placenta during HEV1 infection which lead to adverse outcomes for the pregnancy (Kamar et al., 2017). HEV-1 has been reported to cause higher rates of apoptotic death and necrosis within the placental tissues (Gouilly et al., 2018). The presence of the virus in the placenta develops a pro-inflammatory environment, which recruits many of the pro-inflammatory targets towards the placenta, thereby creating a positive feedback loop which leads to increased tissue damage within the placenta (Pereira, 2018).

The diagnosis of HEV infections can be carried out either by detecting the RNA in the serum or by detecting anti-HEV antibodies. The RNA based detection is possible up to 6

weeks as the HEV RNA remains persistent in blood and stool till this time period (Marrone et al., 2019). The antibody response against HEV initiates after the incubation period of the virus and persists for around 9 months post infection. The levels of immunoglobulin M (IgM) can be detected at the same time that alanine aminotransferase (ALT) increases in the blood and persists for about 6 to 9 months in the patient's serum (Abravanel et al., 2018). The immunoglobulin G (IgG) response against HEV can be delayed in terms of activation but it can persist for several years after the infection has cleared (Kamar et al., 2017). Given the response time and durations, immunoassays detecting anti-HEV-IgM are used to detect recent infections while the presence of IgG points towards the past infections. The specificity of the immunoassays against HEV has been reported to be around 80 to 85% (Ankcorn et al., 2018). For detecting the active HEV infections, WHO has regarded the viral RNA detection and quantification methods as the gold standards in HEV detection and diagnosis. HEV genotyping is crucial in terms of maintaining records of HEV epidemiology as different genotypes are reported among different parts of the world and the mode of transmission also differs depending on the genotype (Kamar et al., 2015). The appearance of HEV RNA in blood and the antibody response phases are illustrated through figure 1.7.
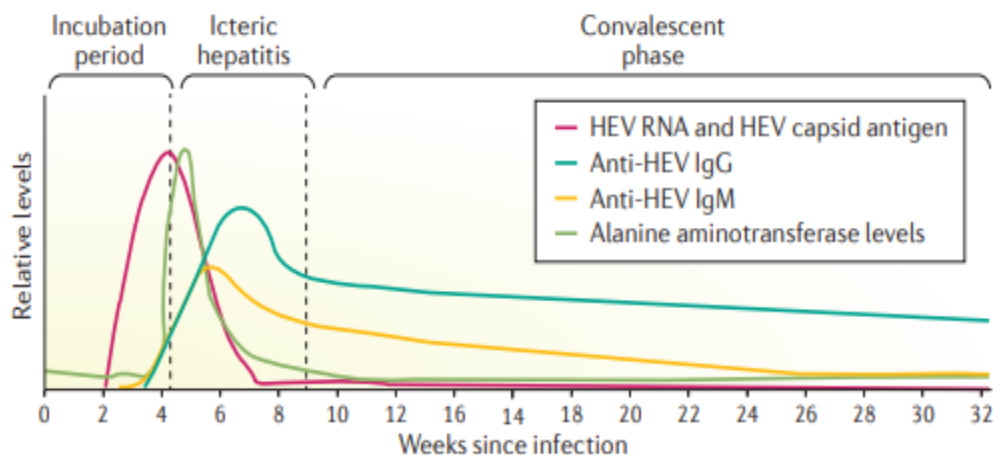
Figure 1.7: HEV RNA and Immunoglobulin appearance in blood. The HEV RNA and capsid antigen are present in the blood in high concentration during the incubation period and icteric hepatitis but the concentration drops significantly during the convalescent phase (Kamar et al., 2017).

2.1.4 Hepatitis A virus

Hepatitis A virus (HAV) is a member of the enterovirus genus, having a single stranded positive sense RNA. The transmission of HAV, like other enteroviruses follows the faeco-oral route. The virus has mainly been associated with infection in children, particularly those who have no history of previous infections with HAV or have not been vaccinated against the virus (Ikobah, Okpara, Ekanem, & Udo, 2015). The clinical manifestations of HAV are known to be age-dependent and the symptoms tend to be more prominent with progressing age than in young children (Joon, Rao, Shenoy, & Baliga, 2015). A great majority of the HAV infection cases are reported to be self-limiting, with only a few cases relapsing after a few weeks of infection resolution (Lemon, Ott, Van Damme, & Shouval, 2018). The hepatitis A virus infection is regarded as an ancient infection, dating back to the times that human beings first began to live in the form of communities. The initial evidences of HAV infections date back to 1912 and during this era the infection was known as the 'catarral jaundice' (Teo & Infection,

2018). The evidences from varying studies were gathered and studied much later with the use of electron microscopes, which helped in analysing the viral particles. The differentiation of HAV from Hepatitis B virus (HBV) was also made possible through advancements in experimental methods and it was concluded that the HAV genomic characteristics were similar to that of poliovirus (Block, Alter, London, & Bray, 2016).

The genome of HAV is 7.5 kb in length and contains a long untranslated region (UTR) at the 5' end with a protein (VPg) covalently linked with the genome at the same terminus (McKnight & Lemon, 2018). The viral RNA is translated as a single large polyprotein upon infecting the host cell. The translation is aided by the internal ribosomal entry sites (IRES) present in the long 5' UTR of the viral genome. The 3' end of the virus ends in a lengthy poly A tail (Lemon et al., 2018). It has been reported that the infectious HAV occur in two forms, including the naked HAV particles and the semi-enveloped HAV or eHAV particles. The naked HAV particles are similar to the other enteroviruses and have been known to cause infections in humans for long. The naked infectious virus exits the infected cell through lysis while the eHAV are known to exit from the infected cells without lysing the cells, possibly taking the secretory routes (Kirkegaard, 2017). The eHAV have only been reported recently when blood from infected individuals revealed the presence of infectious virus in membrane vesicles, without any complex proteins on the viral surface (Lemon et al., 2018). This feature has been implicated to be an important part of the HAV pathogenesis.

The HAV replication cycle resembles that of the other picornaviruses and occurs through the direct translation of the RNA genome into a polyprotein. The replication of the viral RNA occurs within the cytoplasm like the majority of other RNA viruses. The replication

occurs close to the host cell membrane and overexpression of the non-structural proteins of HAV lead to modifications within the host cells' intracellular membranes, indicating a possible method by which the viral particles could be packaged within the membrane vesicles for a non-lytic exit from the host cells (Seggewiß, Paulmann, & Dotzauer, 2016). The exit of eHAV from the host cells resembles the method through which multivesicular bodies (MVBs) are released from the cells. It is suggested that the viral capsids are packaged through endosomes within the MVBs to be secreted out of the cell by the fusion of MVB and the plasma membrane (Petrik & Science, 2016). The budding of the late endosome and the MVB is mediated by the endosomal sorting complexes required for transport (ESCRT). The overall exit pathway of eHAV from the host cell is summarised through figure 1.8 (McKnight et al., 2017).
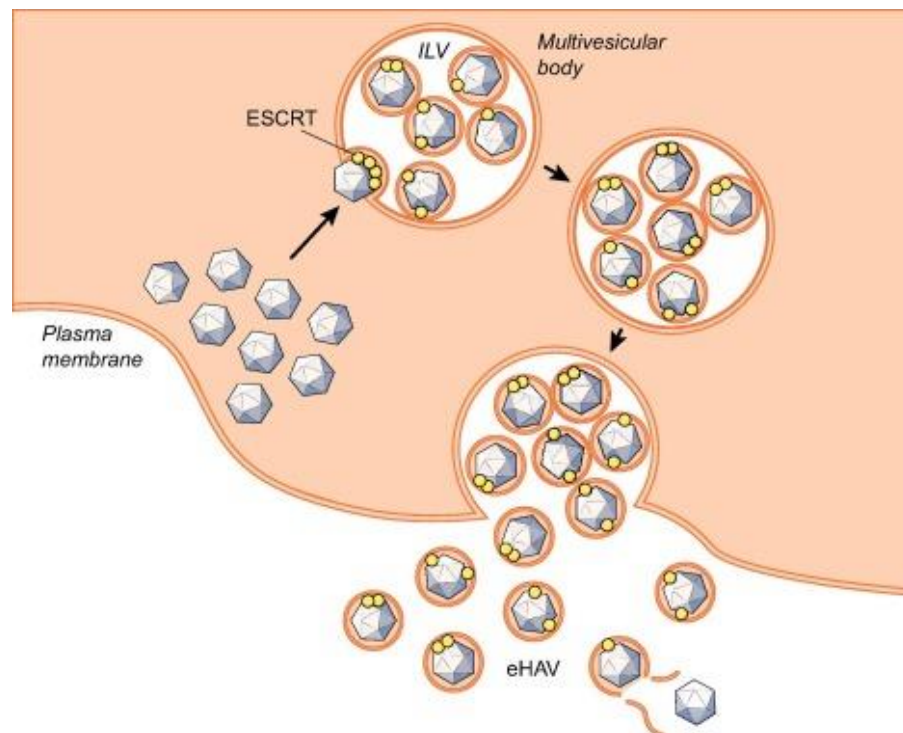
Figure 1.8: HAV exit from the host cell through the multicellular vesicles. This method of exit is followed by the eHAV (McKnight et al., 2017).

The HAV pathogenesis depends on the ingestion of the virus through the faeco-oral route. Despite the route, the initial infection with HAV is known to affect the hepatocytes (Hirai-Yuki et al., 2016). The exact pathway used by the virus to reach the liver from the intestine is not established yet and so far there have only been theories for understanding the exact path of infection followed by HAV. The virus shed through the faeces has been found to be naked, despite the evidences of quasi enveloped HAV being present within the blood stream (Pandya & Chaudhary, 2015). The shedding of the virus is maximum during the last stages of the incubation period within the host that is the third or fourth week of incubation (Satsangi & Dhiman, 2016). As the symptoms of infection or the indications of acute liver injury become prominent, the viral shedding drops significantly. However, the modern and sensitive molecular detection methods such as the RT-PCR for viral detection can help in detecting the virus from stool or liver tissue samples for several weeks post infection. Detection of the virus within the blood is most likely during the early incubation period and has not been reported post infection (Randazzo, Piqueras, Rodríguez-Díaz, Aznar, & Sánchez, 2018). Hence, viraemia is significantly present only during the incubation period of the virus and drops down as the antibodies against HAV begin their action within the host body (Lemon et al., 2018).

2.2 Disease Burden in Pakistan

The disease burden of enteroviruses is not completely documented in Pakistan. Epidemiological data of the poliovirus is well documented but the eradication of the virus has not been achieved yet. Moreover, sporadic cases of infections with different enteroviruses arise in Pakistan regularly. The rise in the enteric infections during the

summers and monsoon seasons are quite common in the country (Shaukat et al., 2017). Reports of infections with individual viruses from the enterovirus group are also reported. However, there exists a gap in complete epidemiological data regarding the true disease burden owing to enteroviruses in Pakistan.

Various enteroviruses have been reported from Pakistan at different occasions. Enteric diseases in children and infants have revealed the prevalence of variants of enteroviruses in the country. The monsoon seasons and summers are particularly laden with cases of enterovirus infections across Pakistan. Different serotypes of NPEV have been reported from Pakistan. Children found to be infected with different enteroviruses have been reported to be suffering from AFP. In 2007, 1775 cases of AFP were identified in a study, all of these patients were below the age of 15 and 474 of these cases were found to be suffering from NPEV mediated AFP. Serotyping of the virus revealed that 13% of the NPEV isolated belonged to the coxsakievirus B species. Almost 180 cases were untypable through the anti-serum assays and were subjected to RT-PCR assays to reveal that all of the untypable viruses also belonged to NPEV species (Saeed et al., 2007). In the post-polio eradication era, the NPEV associated AFP needs to be analysed and strategies need to be developed to prevent the spread of such infections. Sporadic cases of AFP among children continue to burden the healthcare system of Pakistan (Angez et al., 2017).

Environmental surveillance in Pakistan is although quite weak, studies carried out in different regions of Pakistan point towards the presence of different enteroviruses within the water sources in the country. In 2016, the water sources of rural Khyber Pakhtunkhwa (KPK, Pakistan) were tested to reveal high prevalence of enterovirus and HAV, pointing

towards the levels of viral contamination in the water sources (Ahmad et al., 2016). The presence of enterovirus and HAV in the sewage and drinking water samples was confirmed from Lahore, Islamabad and Rawalpindi. Additionally, clinical cases of HAV were also reported from Islamabad at the same time (Ahmad et al., 2018).

2.3 Treatment Strategies

Treatment strategies for dealing with enterovirus infections can be diverse due to the diversity of the viruses present within the group. Apart from antiviral drugs, the use of vaccines is also an important strategy to cope with infections caused by enteroviruses (Lugo & Krogstad, 2016). In the case of poliovirus, two vaccines have been used worldwide, the oral poliovirus vaccine (OPV) and the inactivated poliovirus vaccine (IPV). Both these vaccines have proved to be crucial in combating and overcoming the spread of poliomyelitis. The use of OPV has been regarded as a better option as compared to IPV due to the requirement of using less virus to gain higher immunity. OPV limits the spread of the virus within the body due to mucosal immunity being activated. The drawback of using OPV is the chance of the virus reverting to its infectious state and recombining with other related viruses to develop vaccine derived poliomyelitis in the vaccinated individual (Sepúlveda, 2017). Developing safer OPV is currently a major concern and a few alterations such as mutations in the capsid region and targeting the recombination capacity of the virus are being explored (Toole, 2016).

The treatment of other enteroviruses and the NPEV is being explored globally to identify ways in which these viruses could be limited. It is possible to develop vaccines against NPEV by using inactivated or attenuated viruses. This is being done in China since long and vaccines have been developed against EV-A71 and HEV in China (Lyu, He, Li, &

Chen, 2015). Development of a broad spectrum, polyvalent vaccine against many serotypes of enteroviruses is being explored since long. Studies on rhinoviruses have revealed that the development of a polyvalent vaccine is quite possible. It was found that using 50 different rhinovirus strains for developing a single vaccine helped in gaining neutralization against 49 different types, providing evidence that a broad-spectrum vaccine can indeed be created (Gao et al., 2016). Another interesting approach is the development of vaccines using virus-like particles (VLPs). These particles have the capability to assemble when the capsid proteins are co-expressed. This approach can be extremely useful for developing vaccines against the viruses that cannot be cultured such as the rhinoviruses or those viruses which require the use of high-containment facilities such as poliovirus. It is important to note that the VLPs can be slightly different from the infectious viruses in terms of structure, stability and antigenicity. This is due to the absence of the viral genome, which provides stability to the virion (Lin et al., 2018). However, it is possible to overcome this particular limitation by inducing mutations that help in stabilizing the capsid. Therefore, there are various strategies which could be employed for successful development of vaccines against different enteroviruses.

2.4 Importance of Environmental Surveillance

Environmental surveillance for identifying the presence of enteric viruses is a much needed strategy that has been largely overlooked. Since the enteroviruses spread mainly through the water bodies, it is crucial to ensure hygienic conditions in the water reservoirs and keep the viral loads in check (Charlotte C Holm-Hansen et al., 2017). The presence of enteric viruses in the sewage water is the highest as these viruses are shed in the faeces of the infected individuals. The discharge of these viruses in the sewage often

leads to contamination in the surface water sources, ultimately contaminating drinking water (Aw & Gin, 2010). The persistent presence of the enteroviruses in the water sources and the widespread infections among the population make it a major concern for public health. Moreover, critical questions are raised regarding the methods used for monitoring potable water sources being used by the people (Benschop et al., 2017). Viral pollution in the water sources is a research area that needs to be explored in greater depths. A number of different studies carried out around the world have reported the presence of adenovirus in the highest numbers in the water samples (Iaconelli et al., 2017). These studies indicate about the possibility of using adenovirus as a marker for estimating the extent of viral loads in the water sources. However, before moving on to decide about pollution markers and possible indicators of viral contamination, it is crucial to carry out extensive studies to determine the types of enteroviruses most common in a given country or region. It is quite likely that the viral loads will be varying in different regions.

The traditional approach to public health monitoring revolves around analysing the health status of individual patients. The increasing urbanization has increased the chances of viral diseases being spread across different regions. Moreover, frequent traveling across different countries is also a factor which can aid in the spread of viruses. This makes it crucial to broaden the approaches used for public health monitoring and include environmental surveillance as a measure towards identifying the presence of different viruses in a given area. Waste water monitoring has been implicated as a logical approach for determining the types of viruses and their prevalence in a given community.

However, such an approach has not been implemented on wider levels within communities which leaves gaps in the documentation of enterovirus prevalence.

3. Methodology

3.1 Water Samples

3.1.1 Sample Collection

Water sampling comprised of collecting both, drinking and sewage water samples. These samples were collected from Lahore and the twin cities (Rawalpindi and Islamabad). In total, 25 samples, each of 100 ml were collected from Lahore, out of which 15 were drinking water samples while 10 were sewage samples. While 154 samples (100 ml each) were collected from the twin cities, such that 74 were drinking water samples and 80 samples were that of sewage. These samples were then processed and analysed for the presence of enteroviruses, as explained in the following sections.

3.1.2 Sample Processing and RNA extraction

The water samples were processed by initial centrifugation (cold centrifugation) at 4000 rpm for 20 minutes to get rid of any sediments. Once the sediments were settled as pellet, they were removed and the supernatant was centrifuged once again at 4000 rpm for 20 minutes. This was followed by collecting the supernatant in a glass bottle and keeping the pellet (labelled as pellet I) stored overnight at 4°C. The supernatant was added with 8 gms of Poly Ethylene Glycol (PEG 6000) per 100 ml of sample and then kept overnight at 4°C. The next day, the PEG treated samples were centrifuged at 4000 rpm, at 4°C, after which the supernatant was discarded and the pellet was saved. The pellet was then mixed with the pellet I stored previously. The mixture of pellets was then treated with 4 ml of chloroform. The mixture was shaken vigorously and allowed to settle. After the solution

separated into two distant phases, the clear solution was collected and the debris was discarded.

Once concentrated, the solution was passed through membrane filters of 0.45 µm for concentration, using a vacuum pump for setting up the filter assembly and aiding the filtration process. The water RNA/DNA purification kit (NORGEN, USA) was used for this purpose. The water samples were passed through the filter columns provided in the kit, and vacuum pump was used for aiding the filtration process. After filtration, the filter paper was removed from the column and added to the beaded tubes. This was followed by addition of ethanol to the tubes and then vortexing the tube for one minute to ensure that the filtrate was properly adhered to the beads. The tube was then washed with wash solution A (provided in kit) twice and the RNA was eluted by adding the elution solution and then spinning the column to pass the solution and elute the RNA. The overall process is explained through figure 3.1.

Pass water sample through filter column by vacuum to collect
microorganisms. Remove filter and transfer to a Bead Tube.

Vortex and incubate.
Centrifuge and transfer lysate

Add Ethanol

Bind to column

SPIN

Wash twice with
Wash Solution A

SPIN

Elute DNA and RNA
with Elution Buffer H

SPIN

Purified Total RNA and Genomic DNA
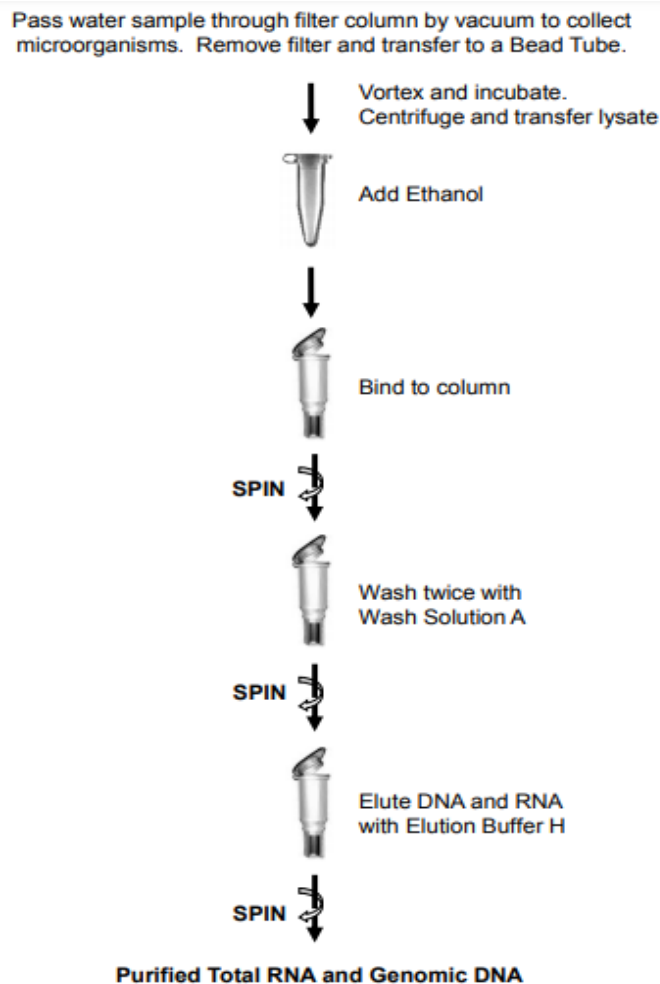
Figure 3.1 : Total RNA extraction from water through NORGEN (USA) kit method

An alternate method used for processing water samples was the concentration of viruses through negatively charged membrane filters. The water samples were centrifuged at 3000 rpm (cold centrifugation) for 45 minutes for removing the debris present within the water. Negatively charged filters having a pore sizes of 0.45 μm and 0.22 μm were aligned in a vacuum pump. The samples were centrifuged at 4000 rpm to remove solid particles. The sample was then treated with 25 mM $MgCl_2$ briefly and then passed through the filter assembly. This was followed by adding 100 ml of 0.5 mM H_2SO_4

through the filter membrane to remove cations, and then 5 ml of 1 mM NaOH (pH 10.5) was filtered through the same membrane and the filtrate was then collected in a falcon tube. The neutralization of the filtrate was carried out using 0.1 ml of 100X TE buffer along with 50 mM of H_2SO_4. The liquid eluted here was then concentrated in the DNA concentrator (Eppendorf, Germany). The concentrated samples were stored at -20 °C until the step of RNA extraction.

Nucleic acid extraction was carried out by adding 500 µl of Trizol reagent to the samples stored after filtration. After addition of Trizol, the samples were incubated for 5 minutes at room temperature, followed by the addition of 100 µl chloroform and further incubation for 5 minutes. These samples were then centrifuged in a cold centrifuge at 1200 g for 20 minutes and then 300 µl of the supernatant was taken from the centrifuged tubes and transferred to eppendorf tubes. The volume of the supernatant was matched with 1/10 of 3 M sodium acetate having pH 5.2 and then absolute ethanol was added (chilled) equals to twice the volume of that of the supernatant. This mixture was stored at -20°C overnight. The next day, this sample was centrifuged at 1200 g for 10 minutes, followed by collection of the pellet and its washing with 70% ethanol added as 250 µl in each tube and then centrifuged for 10 minutes in a cold centrifuge at 1200 g for obtaining a completely dried pellet. The pellet thus obtained was resuspended in 40 µl of nuclease free water.

3.2 Blood Samples

3.2.1 Sample Collection and Processing

Blood samples of infected patients (with HEV and HAV) were collected from a local diagnostic centre in Islamabad, the demographic sites of sampling are highlighted in

figure 3.3. The samples were centrifuged to separate serum, which was stored at – 20 °C until further processing. The samples were processed using the FAVORGEN Viral nucleic acid extraction kit. The method used for extracting the RNA from serum sample was followed exactly as instructed in the kit. The flow chart of extraction method is shown through figure 3.2.
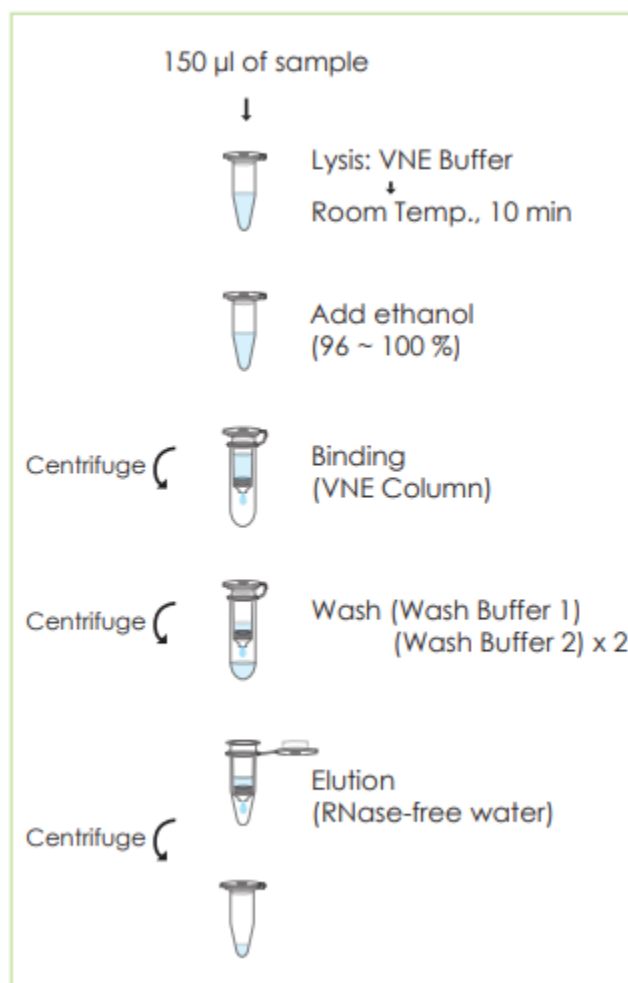
Figure 3.2 : Viral nucleic acid extraction from blood using Favorgen nucleic acid extraction kit.
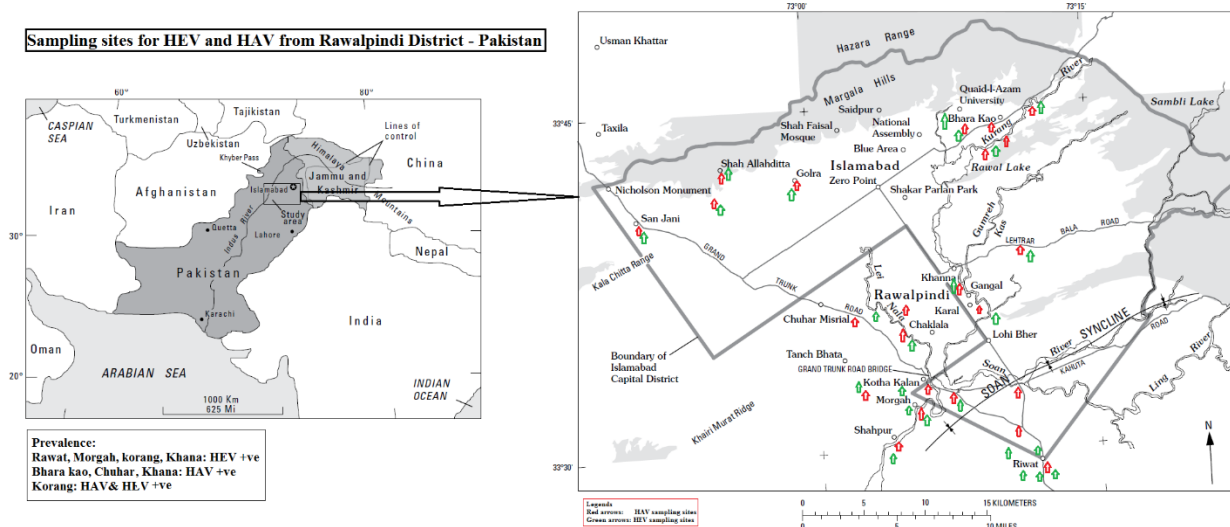
Figure 3.3 : Sites within Islamabad and Rawalpindi that were used for HEV sampling

3.3 Polymerase Chain Reaction

The molecular detection of viruses was carried out by polymerase chain reaction (PCR). The genomes of different enteroviruses were identified through using specific primer sets for each of the viruses (given in table 2). Primers were designed using the conserved regions of specific viruses. The reverse primers of each of the viruses were used for making an attempt at cDNA synthesis for the viruses present within the samples. The recipe for cDNA synthesis (for all viruses) included 4 μl M. Mulv Buffer, 2 μl dNTPS (10 mM), 10 μl RNA Template, 0.2 μl RNase inhibitor, 1.5 μl reverse primer and 0.6 μl Reverse Transcriptase enzyme. The mixture was kept in thermo-cycler at 42 °C for 90 minutes. The cDNA synthesis was followed by RT-PCR for detection. The samples of HEV were further subjected to RT-PCR reactions specific for genotyping of the virus. The specific PCR conditions for each of the viruses are discussed in the following sections.

3.3.1 PCR for NPEV

The recipe for carrying out NPEV amplification included 2 µl dNTPS (2 mM), 4 µl PCR buffer, 2 µl MgCl$_2$, 1.5 µl forward primer 1.5 µl reverse primer (2 picomole of each), 8 µl cDNA template, 0.5 µl DNA polymerase enzyme and nuclease free water to make the reaction volume equal to 20 µl. The PCR reaction was run for 35 cycles while maintaining these temperatures; 95 °C for 5 minutes, followed by 94 °C for 30 seconds, 62 °C for 30 seconds and 72 °C for 30 seconds, followed by a hold of 10 minutes at 72 °C.

3.3.2 PCR for HEV

The recipe for carrying out HEV amplification included 2 µl dNTPS (2 mM), 4 µl PCR buffer, 2 µl MgCl$_2$, 1.5 µl forward primer 1.5 µl reverse primer (2 picomole of each), 8 µl cDNA template, 0.5 µl DNA polymerase enzyme and nuclease free water to make the reaction volume equal to 20 µl. The PCR reaction was run for 35 cycles while maintaining temperatures, 95°C for 5 minutes, followed by 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 30 seconds, followed by a hold of 10 minutes at 72°C.

The genotyping PCR for HEV were also carried out using the conditions and recipe used for the detection PCR. The primer sets were different and genotype specific primers (as given in table 1) were added for testing each genotype.

3.3.3 PCR for HAV

The amplification of HAV was carried out using 5 µl cDNA template, 2.5 µl Dream taq buffer, 1.5 µl of each, forward and reverse primers, 1.5 µl dNTPs, 0.5 µl Dream Taq Enzyme and 7.5 µl Nuclease Free Water. The PCR reaction was run for 35 cycles with

the reaction profile being; 95°C for 5 minutes, 94°C for 1 minute, 55°C for 1 minute, 72°C for 45 second and a final hold of 7 minutes at 72°C.

3.3.4 PCR for Enterovirus

The recipe used for carrying out RT-PCR of enterovirus 2 µl dNTPS (2 mM), 2 µl PCR buffer, 2 µl MgCl$_2$, 2 µl of each, forward primer and reverse primer (2 pico-mole concentration of both), 8 µl cDNA template, 0.5 µl Taq DNA polymerase and nuclease free water for making up the reaction volume up to 20 µl. The PCR reaction was run for 35 cycles, with conditions set as follows; 95°C for 5 minutes, followed by 95°C for 1 minute, 58°C for 1 minute and 72°C for 1 minute, followed by a hold of 10 minutes at 72°C.

Table 3.1: Primer sets used for molecular detection of enteroviruses

Virus	Primer	Product size
Polio (Det) Forward	5'-CACAACCCGACCAAGGTCACCT-3'	291 bp
Polio (Det) Reverse	5'-ACACAGCTTTGTTTTGGTGTCC-3'	
HEV ORF2/3 Forward	5'-GGTGGTTTCTGGGGTGAC-3'	66 bp
HEV ORF2/3 Reverse	5'-GGGGTTGGTTGGATGAA-3'	
HEV1 Forward	5'-AGCCAACCTAGCACTGGAGA-3'	100 bp
HEV1 Reverse	5'-TGGGAACAACCACTACGTCA-3'	
HEV2 Forward	5'-GGTTCCGCGCTATTGAGAACG-3'	202 bp
HEV2 Reverse	5'-TCGCCGGATTGGTTCTTCC-3'	
HEV3 Forward	5'-GTTGAGAATGCACAGCAGGA-3'	231 bp
HEV3 Reverse	5'-CATAGGGTTGGTGGATGACC-3'	
HEV4 Forward	5'TGCGAAAGCTAACGAATGTG-3'	216 bp

HEV4 Reverse	5'-CCATGCACAGAGCAAAAGAA-3'	
HAV Forward	5' -GTTTTGCTCCTCTTTATCATGCTATG-3'	246 bp
HAV Reverse	5- GGAAATGTCTCAGGT-3'	
Ent virus Forward	5'-CGGTACCTTTGTACGCCTGT-3'	362 bp
Ent Virus Reverse	5' -ATTGTCACCATAAGCAGCCA-3'	

NOTE: The universal primer (-37) was used for sequencing of HAV and HEV.

The results obtained through PCR were analysed by subjecting the PCR product to gel electrophoresis, using 1% Agarose gel in TAE buffer. The gel was visualized using the Gel documentation system (Whealtec Dolphin, Germany).

3.4 Sequencing and Phylogeny

The PCR products detected through the initial reactions were purified through gel purification kit (Invitrogen, UK). This was followed by sequencing using the Beckman Coulter Genetic Analyzer CEQ 8000 (USA). The sequencing procedure is described in detail as follows:

1) Preparing DNA samples for sequencing:

The DNA sample obtained after PCR were prepared for subsequent sequencing PCR. This included preparing a reaction mixture as follows: 10 µl DNA template, 8 µl deionized water, 2 µl of -47 sequencing primer, and 8 µl DTCS Quick Start Master Mix. The reaction mixture was prepared on ice.

2) PCR:

The sequencing PCR was carried out using the above prepared mixture and setting conditions as 96°C for 20 seconds, 50°C for 20 seconds and 60°C for 4 minutes. The reaction was run for 30 cycles followed by a hold at 4°C.

3) Ethanol Precipitation:

Each sample was precipitated with ethanol to obtain pure DNA ready for sequencing. Firstly, a stopping solution was prepared for adding to the products obtained through the sequencing PCR (defined above). The stopping solution contained 2 μl Sodium Acetate (3M, pH 5.2), 1 μl glycogen (20 mg/ml), 2 μl Na_2-EDTA (100 mM, pH 8). This mixture was then added to the PCR products, such that 5 μl of the stopping solution was added per sample. The samples were then transferred to autoclaved micro-centrifuge tubes labelled clearly for each sample. Absolute ethanol was added in a quantity of 60 μl per sample and mixed thoroughly with the sample. This solution was then subjected to cold centrifugation at 14,000 rpm for 15 minutes. The pellet was rinsed with 70% ethanol. The rinsing step was repeated twice and carried out by adding 200 μl of 70% ethanol per sample and then immediately subjecting the mixture to centrifugation at 14,000 (4 °C) for 2 minutes. The supernatant was discarded and the pellet was subjected to air drying in a sterile environment. The dried pellet was resuspended in 40 μl of sample loading solution (provided with the sequencing kit).

4) Sample loading for Sequencing:

The resuspended samples were transferred to the 96 well plate of the sequencing equipment, carefully adding individual samples to their specific destination. Each

individual sample was overlaid with one drop of mineral oil. The sample plate was then loaded into the instrument to carry out sequencing.

The sequences thus obtained were subjected to BLAST (Basic Local Alignment Search Tool) on NCBI and highly similar sequences were downloaded in FASTA format. This was followed by alignment of genes using clustal X2 software. The aligned sequences were then used for generating the phylogenetic tree using MEGAX software through the UPGMA (Unweighted Paired Group method with arithmetic mean) method.

3.5 Statistical Analysis

Statistical analysis was carried out using SPSS version 20.0. Mann Whitney test was applied to analyse the difference in the prevalence of the different types of viruses within the 2 different cities of Pakistan that have been explored in this study. The chi-square test was applied to test the relationship between viral prevalence in the different types of samples collected during this study.

4. Results

4.1 Water Samples

The sample collection points and the areas where water sources were found to be contaminated with enteroviruses were mapped to analyse whether there was any cross contamination between sewage and drinking water samples. It was found that NPEV was present in bulk from sewage collection sites within the twin cities. The map for sampling sites within the twin cities has been illustrated through figure 4.1. The contaminated samples were found across the entire region of Islamabad and Rawalpindi, with no sewage treatment plants being free from the presence of NPEV within the sewage. The drinking water samples from Pir Wadhai and Nala Lai areas were found to be contaminated with NPEV. Other viruses detected within the sewage samples included enterovirus and HAV. However, the most intriguing contamination was that with NPEV and the other viruses were detected only in rare cases. The total number of sewage samples from the twin cities were 80, out of which 45 samples (56.2%) contained NPEV, 5 (6.25%) were positive for EV and 1 (1.25%) was positive for HAV. On the contrary, 74 drinking water samples were collected from the twin cities, out of which 2 (2.7%) were positive for PV, 2 (2.7%) for EV and 1 (1.35%) for HAV. The total number of sewage water samples from Lahore were 10 out of which 4 (40%) were positive for EV and 1 for HAV (10%). No NPEV or HEV was detected from these samples. The drinking water sample from Lahore were 15 in total and 2 (13.3%) of them contained EV, no other virus was detected from these samples. No HEV was detected from sewage samples, either in Lahore or from the twin cities. The results are for testing presence of different viruses within drinking water and sewage samples are summarized through table 4.1 and the RT-PCR results are shown through figure 4.2 and 4.3.
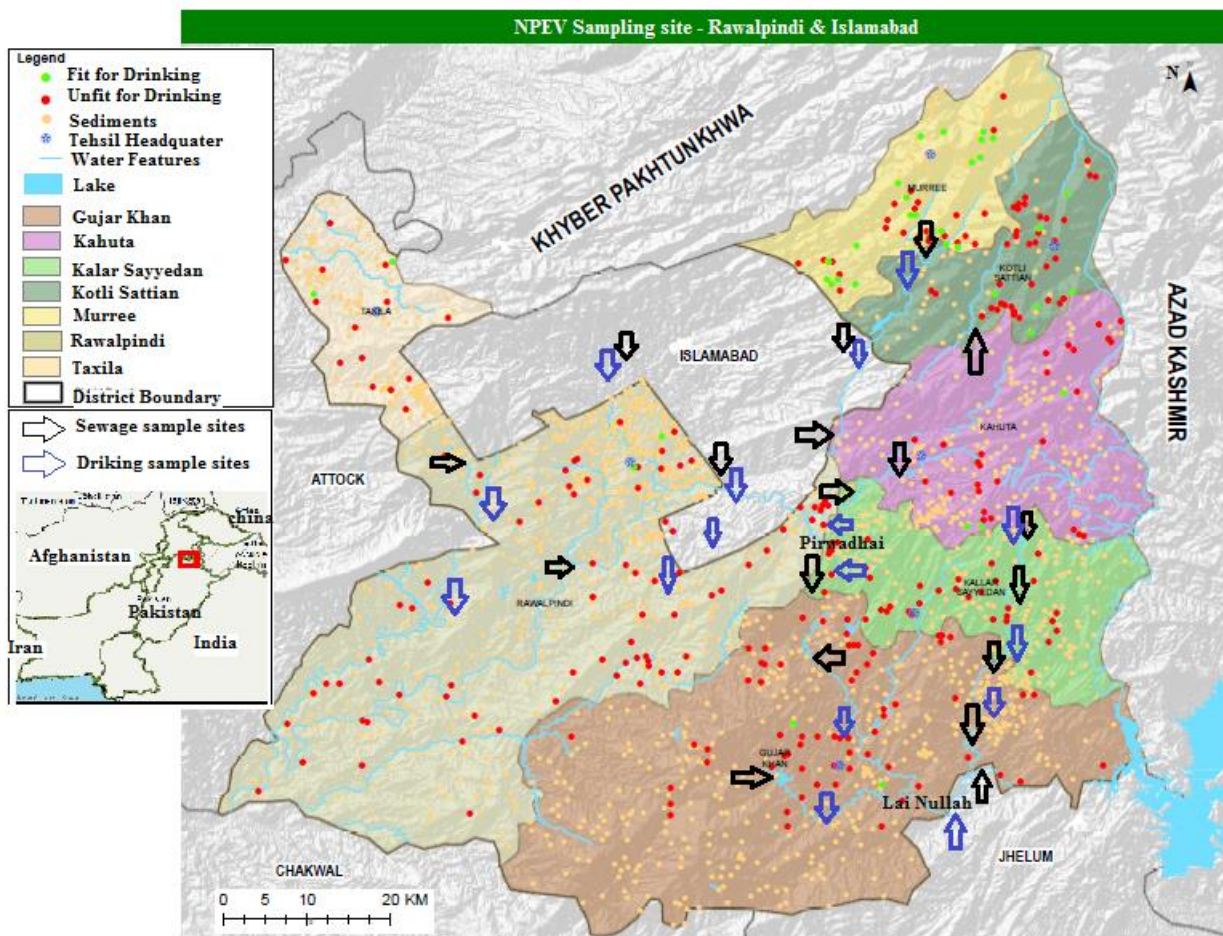
Figure 4.12: Water collection sites from Rawalpindi and Islamabad. The black arrows indicate the areas from which sewage samples were collected while the blue arrows indicate drinking water samples.

Table 4.1 : The results for detection of different enteroviruses found in sewage and drinking water samples collected from Lahore, Rawalpindi and Islamabad

City	Type of Sample							
	Drinking Water				**Sewage**			
	PV	EV	HAV	HEV	PV	EV	HAV	HEV
Lahore	0/15	2/15 (13.3%)	0/15	0/15	0/10	4/10 (40%)	1/10 (10%)	0/10
Islamabad & Rawalpindi	2/74 (2.7%)	2/74 (2.7%)	1/74 (1.3%)	0/74	45/80 (56.2%)	5/80 (6.25%)	1/80 (1.25%)	0/8
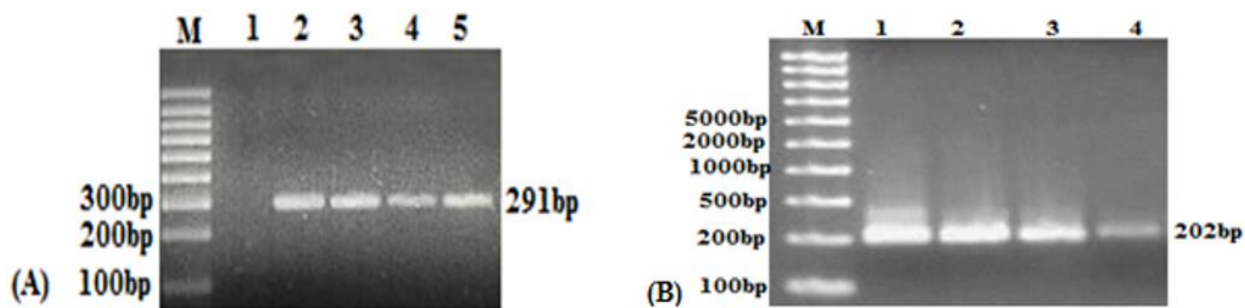
Figure 4.2 *:* Nested PCR results visualized on 2% Agarose gel. (a) The amplified product of NPEV measured 291 basepairs. The M lane contains 100bp ladder, the Lane 1 is the negative control (Nuclease Free water), while lanes 2 to 5 contain the amplified products of NPEV. (b) The nested PCR round amplified product of 202 bp is shown in lane 1 to 4 while the lane M contains 1 Kb DNA ladder.

Figure 14.3: 2% gel agarose showing amplified EV product of 360bp. The lane marked as 100bp contained the 100bp ladder, lane 1 to 13 contain the amplified products and lane 14 is the negative control (Nuclease free water).

The chi-square analysis carried out to analyse the relationship between the presence of different viruses within the drinking water samples and sewage showed no significant association, possibly suggesting that there was no significant evidence of cross-contamination of enteroviruses between sewage and drinking water during the course of this study. Upon statistically tested the association of individual viruses found in the drinking water and sewage, it was found that there was a significant relationship between the prevalence of NPEV in drinking water and sewage water, with a p value of less than 0.05. The Mann Whitney test revealed that the presence of NPEV was significantly

higher in the twin cities as compared to that in the sewage samples from Lahore, with a p value less than 0.05.

4.2 Blood Samples

Blood samples obtained from Islamabad and Rawalpindi revealed the presence of Hepatitis E virus, Hepatitis A virus and Enterovirus infection among the communities analysed during this study. HEV infection was found to be associated with female patients as we did not find any HEV positive samples that were reported among the male population. In total, 28 HEV samples were obtained during the course of this study and all of these samples were from females. Among these patients, 17 (were pregnant, all in their third trimester of pregnancy, having an age range between 23 and 35. The remaining 11 samples were from non-pregnant females, having an age range between 28 and 60 years. The PCR products are shown through the gel electrophoresis results for HEV detection PCR shown in figure 4.4. These samples were then subjected to genotyping PCR to identify the genotype present among our population. It was found that all samples were positive for the HEV genotype 1. No other genotype of HEV was found during our study. The results of the genotyping PCR are shown through figure 4.5.
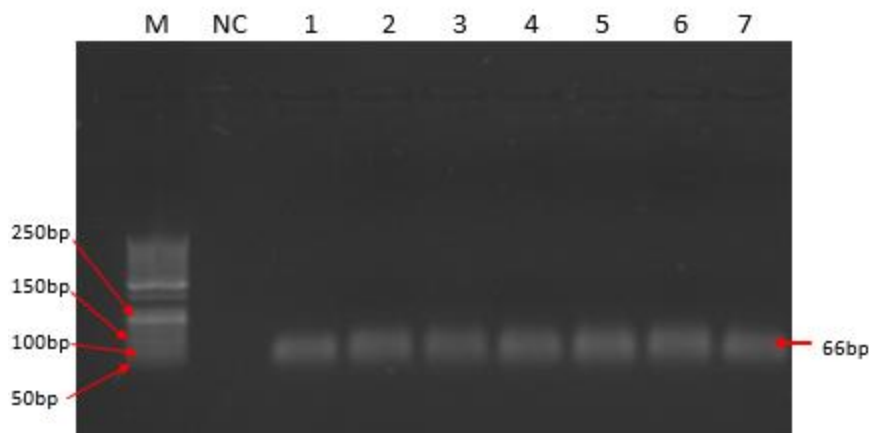
Figure 4.4 : HEV detection PCR results on the 1.5% gel agarose showing amplified HEV product of 66bp. The lane marked as M contained the 50bp ladder, the NC lane contained the negative control and the lanes 1 to 7 contain the HEV amplified product.
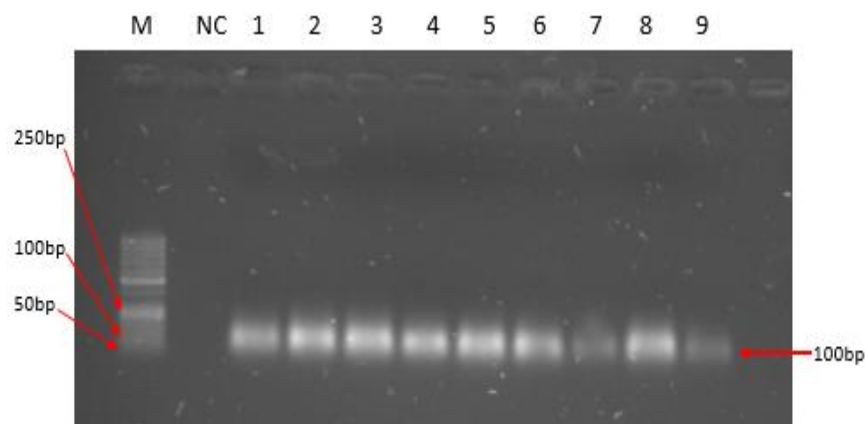
Figure 4.5 : HEV genotyping results on the 1.5% gel agarose showing amplified HEV1 product of 100bp. The lane marked as M contained the 50bp ladder, the NC lane contained the negative control and the lanes 1 to 9 contain the HEV1 amplified product.

Screening for HAV infection during this study helped us in identifying 15 positive cases. The infection was found to be predominant among children as the samples that we obtained during this study were all from children having an age range between 8 and 16 years. While only 2 samples were that of adults having ages 22 and 24. Out of the 15 samples obtained during this study, 11 samples were of males and 4 were from females. Among the two adult patients identified during this study, one was male and the other

was female. The results of the detection PCR for HAV patients are shown through figure

4.6.

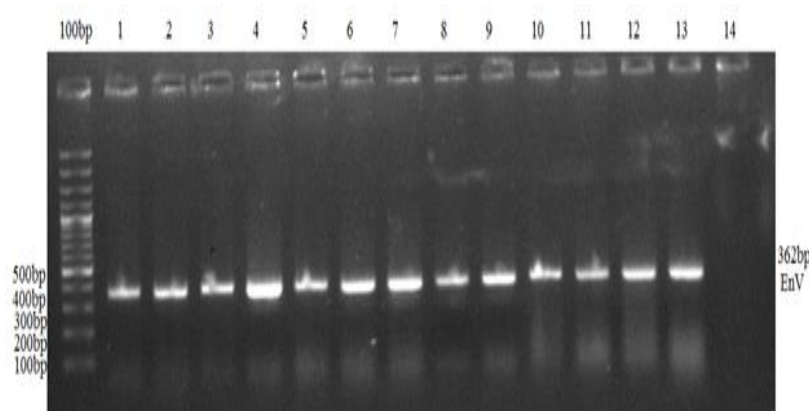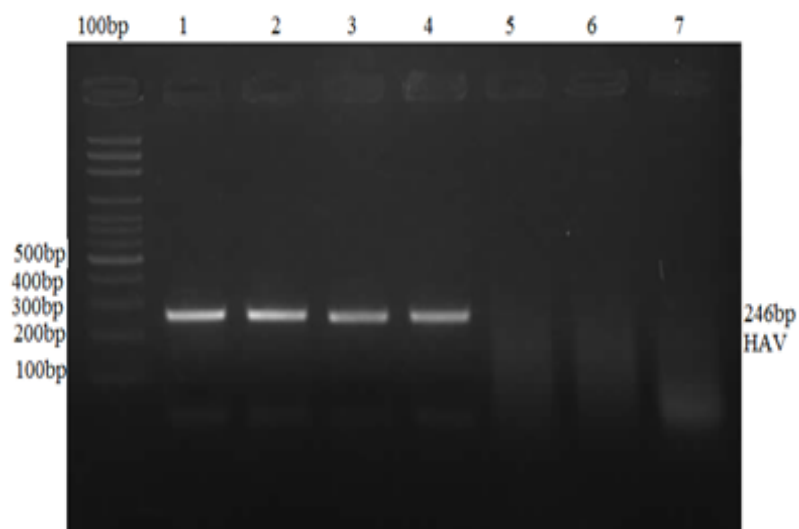**Figure 4.6** : HAV from patient samples. The 2% gel agarose showing amplified HAV product of 246bp. The lane marked as 100bp contained the 100bp ladder, lane 1 to 4 contain the amplified HAV product.

The positive samples of NPEV, HEV and HAV were subjected to sequencing using the

Sanger sequencing method. The sequences thus obtained are shown in table 4.2.

Table 4.2: Sequencing results

Virus	Sequence	Length	Accession Number
NPEV	CAACCCGACCAACGTCACCTCCAAAATCAGTGT GTATCTAAAACCCTTACACATCAGAGTCTGGTGC CCGCGTCCACCGACGGCAGAGGCGTACTACGGC CCTGGAGTGGATTACAAGGATGGTACGCTTACA CCCCTCTCCACCAAGGATCTGACCACATAT	163	MK719554
NPEV	CCGACCAAGGTCACCTCCAAAATCACAGTGTAT CTAAAACCCAATTACATCAGAGTCTGGTGCCCG CGTCCAAGGAGGGCAGAGGCGTACTACGGCCCT GGAGTGGATTACAAGGATGGTACGCTTACACCC CTCTCCACC	141	MK719555
HEV	GCCCTTGCGAATGCTGTGGTAGTTAGGCCTTTCT CTCTCACCAGCAGATTGAGATCCTTATAACCTAA TGCAACCTCGCCAGCTTGTTTTCCGCCCCGAGGT TTTCTGGAACCATCCCATCCAGCGTGTTATCCAT AATGAGCTGGAGCTTTACTGTCGCGCCCGCTCCG GCCGCTGCCTCGAAATTGGTGCCCACCCCCGCTC AATAAATGACAATCCTAATGTCGTCCACCGTTGC TTCCTCCGTCCTGCCGGGCGTGATGTTGGGCGTT	272	Applied
HEV	TAGTTAGGCCTTTTCTCTCTCACCAGCAGATTGA GATCCTTATTAACCTAATGCAACCTCGCCAGCTT GTTTTCCGCCCCGAGGTTTTCTGGAACCATCCCA TCCAGGGTGTTATCCATAATGAGCTGGAGCTTTA CTGTCGCGCCCGCTCCGGCCGCTGCCTCGAAATT GGTGCCCACCCCCGCTCAATAAATGACAATCCT AATGTGGTCCACCGTTGCTTCCTCCGTCCTGCCG GGCGTGATGTTCAGCGTTGGTATACTGCCCCTAC CCGCGGGCCGGCTGCTAATTGCCGGCGTTCC	302	Applied
HEV	CCTTTTCTCTCTCACCAGCAGATTGAGATCCTTA TTAACCTAATGGAACCTCGCCAGCTTGTTTTCCG CCCCGAGGTTTTCTGGAACCATCCCGTCCAGCGT GTTATCCATAATGAGCTGGAGCTTTACTGTCGCG CCCGCTCCGGCCGCTGCCTCGAAATTGGTGCCCA CCCCCGCTCAATAAATGACAATCCTAATGTGGTC CACCGTTGCTTCCTCCGTCCT	225	Applied
HAV	CCACTCTATCATGCTATGGATGTCACAACTCAGG TTGGAGATGATTCTGGAGGCTTCTCTACCACTGT TTCAACAAAACAAAATGTTCCAGACCCTCAAGT TGGGATCACAACACTGAAGGATCTTAAAGGTAG AGCAAACCAAGGGAAAATGGATATCTCAGGTGT CCAAGCTCCCGTGGGAGCCATTACTACTATTGA AGATCCAGTTTTGGCAA	217	Applied

HAV	GCTAAGGATGTCACAACTCAGGTTGGAGTTGAT TCTGGAGGCTTCTCTACCACTGTTTCAACAAAAC AAAATGTTCCAGACCCTCAAGTTGGGATCACAA CACTGAAGGATCTTAAAGGTAGAGCAAACCAAG GGAAAATGGATATCTCAGGTGTCCAAGCTCCTG TGGGAGCCATAACTACTATTGAA	189	Applied
HAV	ATGGATGTCACAACTCAGGTTGGAGATGATTCT GGAGGCTTCTCTACCACTGTTTCAACAAAACAA AATGTTCCAGACCCTCAAGTTGGGATCACAACA CTGAAGGATCTTAAAGGTAGAGCAAACCAAGGG AAAATGGATATCTCAGGTGTCCAAGCTCCTGTG AA	166	Applied

The sequence alignment was carried out for individual virus species that were sequenced. Firstly, the sequenced data was subjected to nucleotide BLAST for analysing the similar sequences. The highly similar sequences were downloaded in FASTA format and subjected to alignment using Clustal 2. The same was repeated for all three viruses that were sequenced. The aligned sequences for NPEV, HEV and HAV are shown through figure 4.7, 4.8 and 4.9 respectively. Next, these aligned sequences were used for developing phylogenetic trees of each virus using the MEGAX software, through the Maximum Likelihood method and keeping a bootstrap value of 100. The phylogenetic trees of NPEV, HEV and HAV are shown through figure 4.10, 4.11 and 23 respectively.
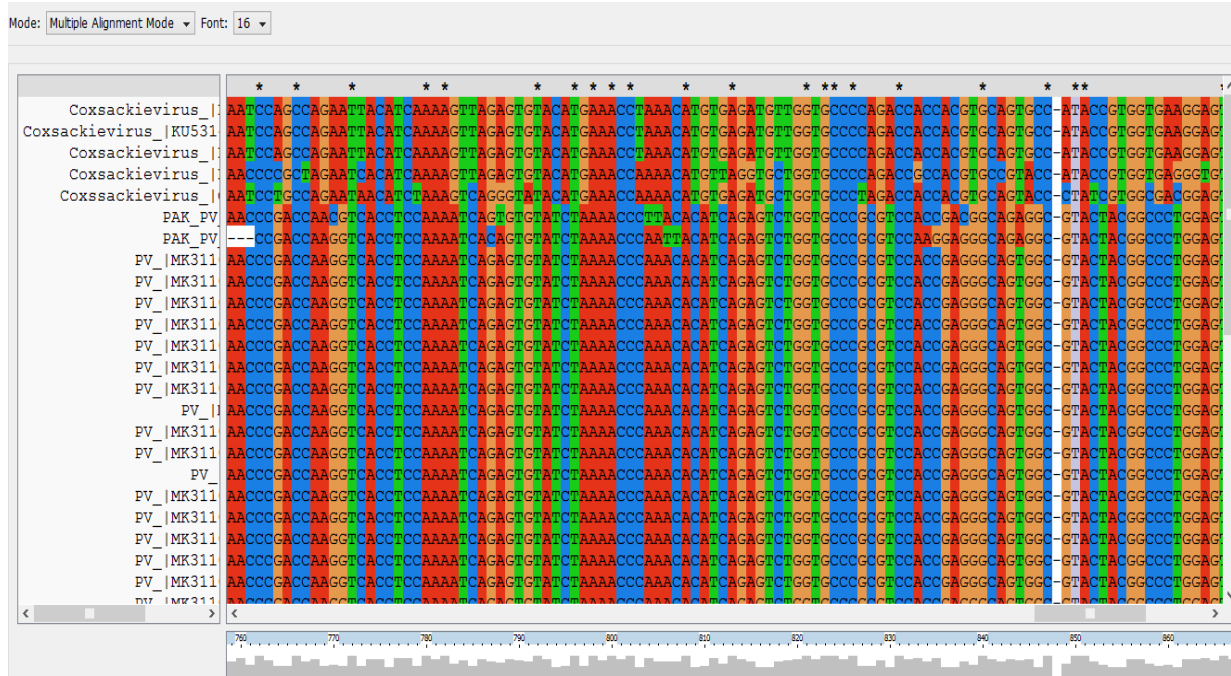
Figure 4.7 : NPEV sequence alignment through Clustal X2. The sequences marked as PAK_PV1 and Pak_PV2 were the products sequenced in this study.
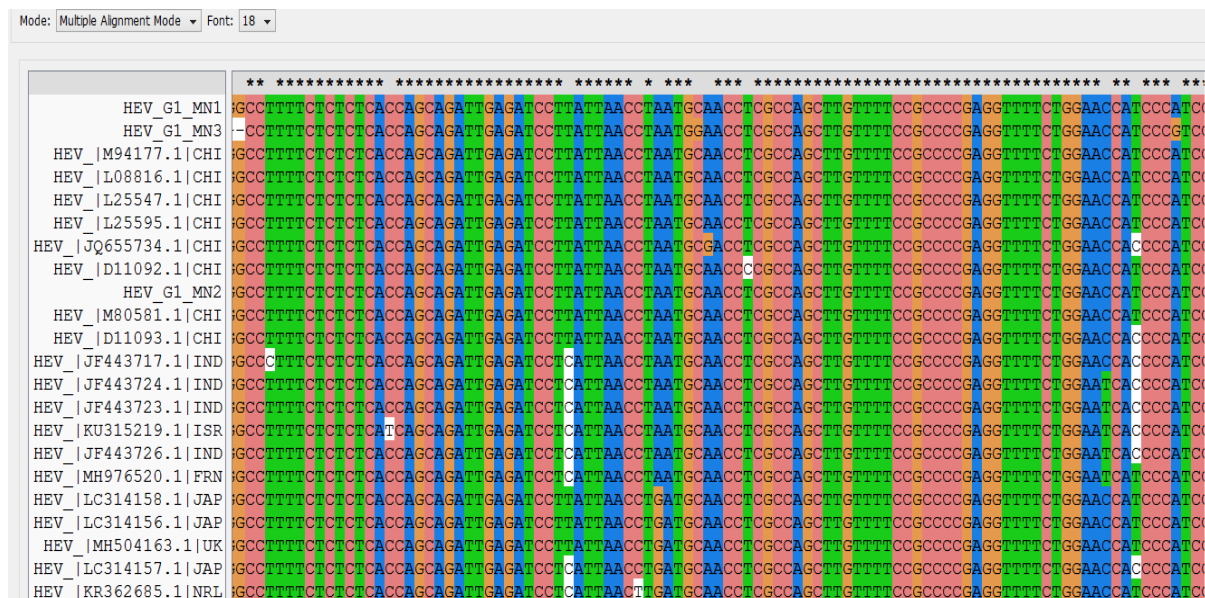
Figure 4.8 : HEV sequence alignment through Clustal X2. The sequences marked as HEV_G1_MN1, HEV_G1_MN2 and HEV_G1_MN3 were sequenced during this study.
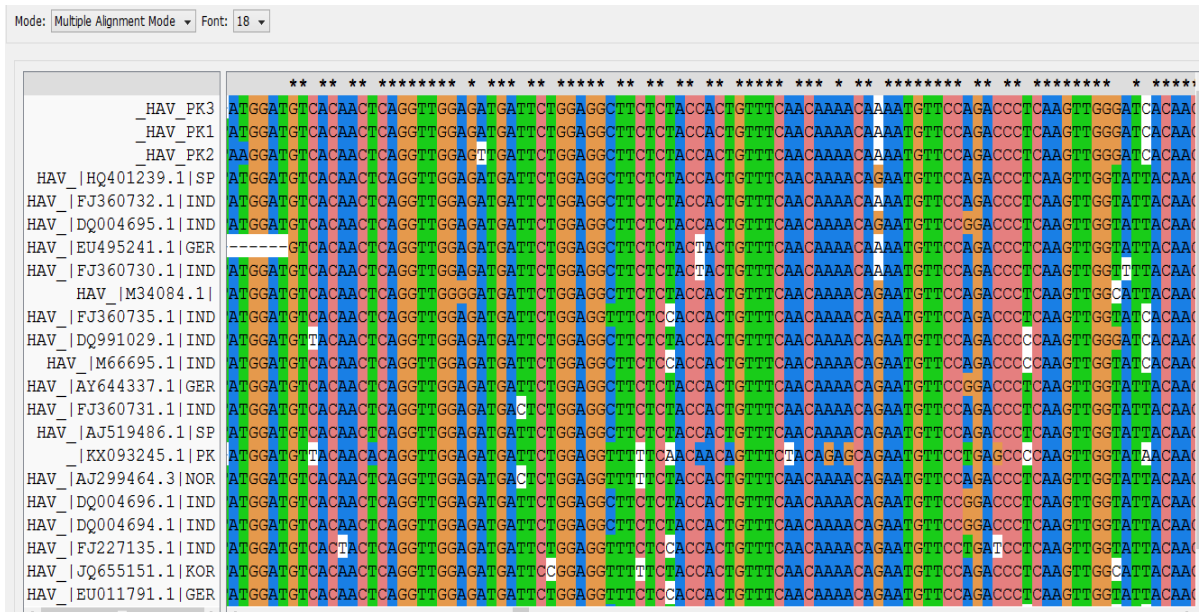
Figure 4.9 : HAV sequence alignment through Clustal X2. The sequences marked as HAV_PK1 and HAV_PK2 were sequenced during this study
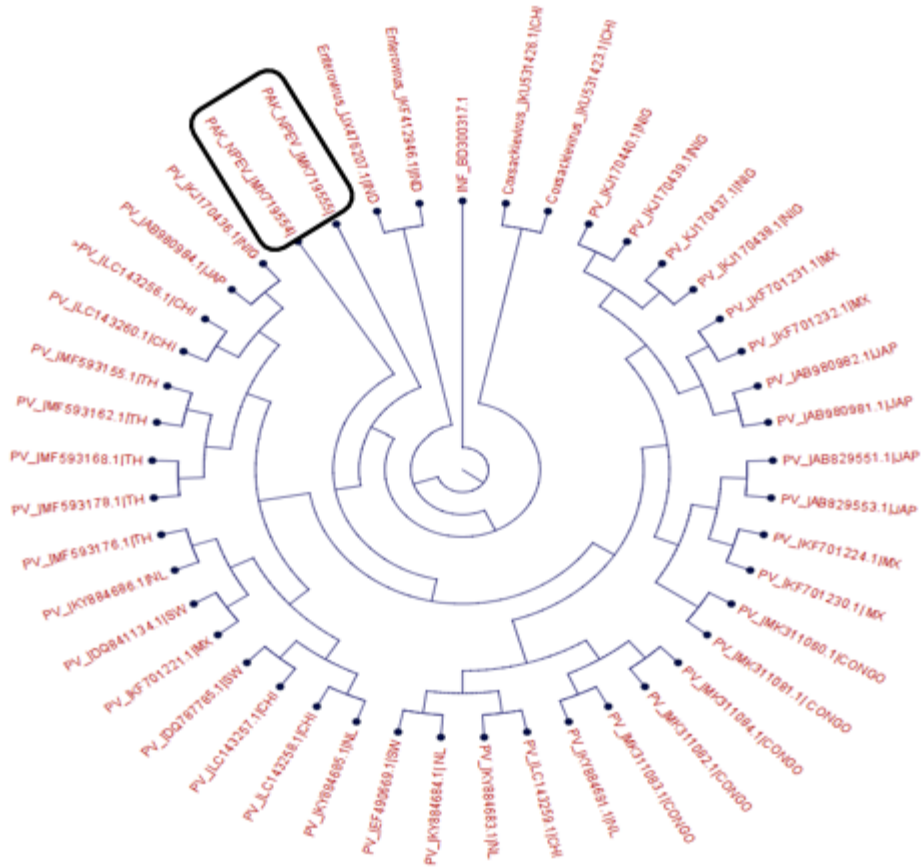
Figure 210: Phylogenetic analysis of NPEV isolated from sewage and sequenced (MK719554 and MK719555). The tree was constructed through MEGAX software, using the UPGMA method and a bootstrap value of 100. The boxed sequences represent the strains sequenced in this study. These strains have evolutionary relatedness with the strains previously reported from China.
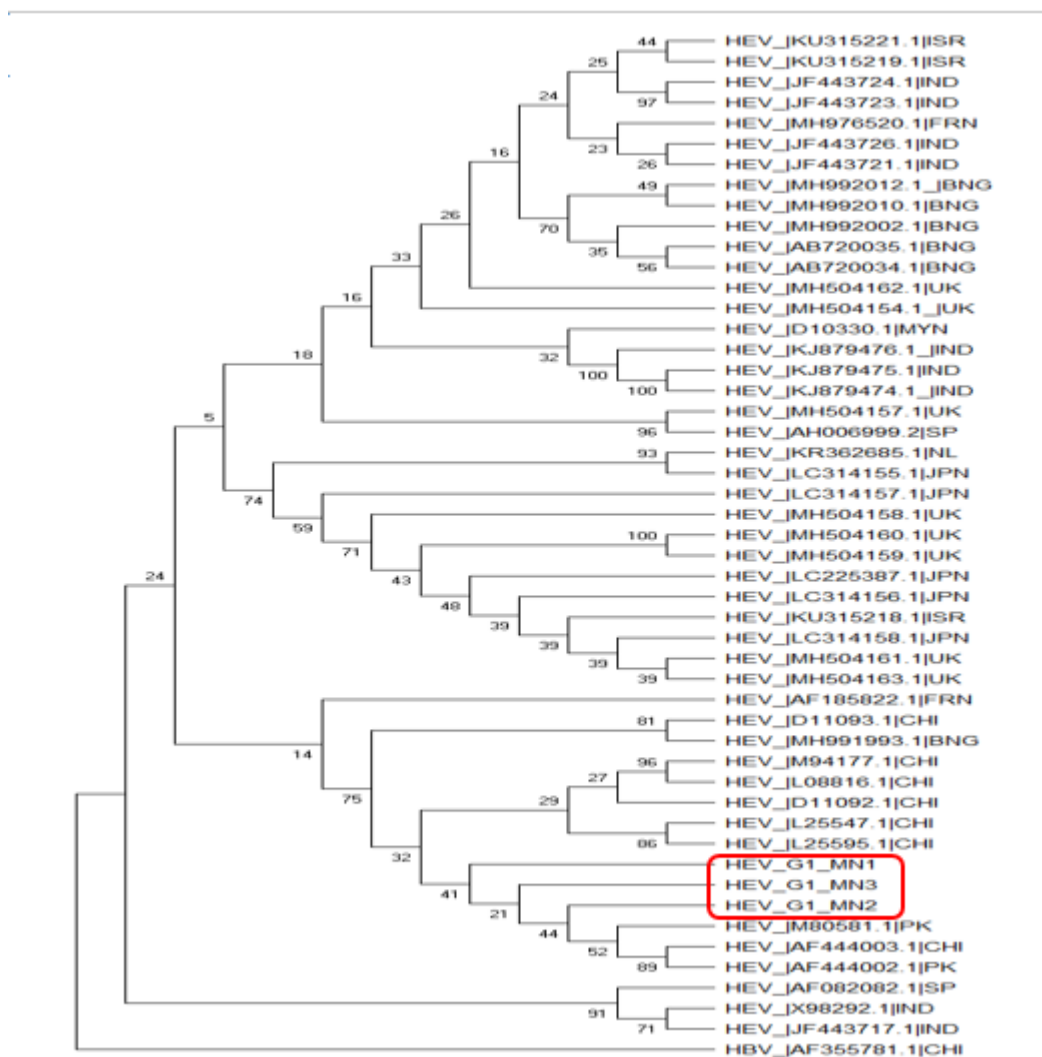
Figure 4.11 : HEV phylogenetic tree constructed through MEGAX software, using the UPGMA Method and a bootstrap value of 100. The boxed sequences represent the strains sequenced in this study. These strains have evolutionary relatedness with the strains previously reported from Pakistan and China.
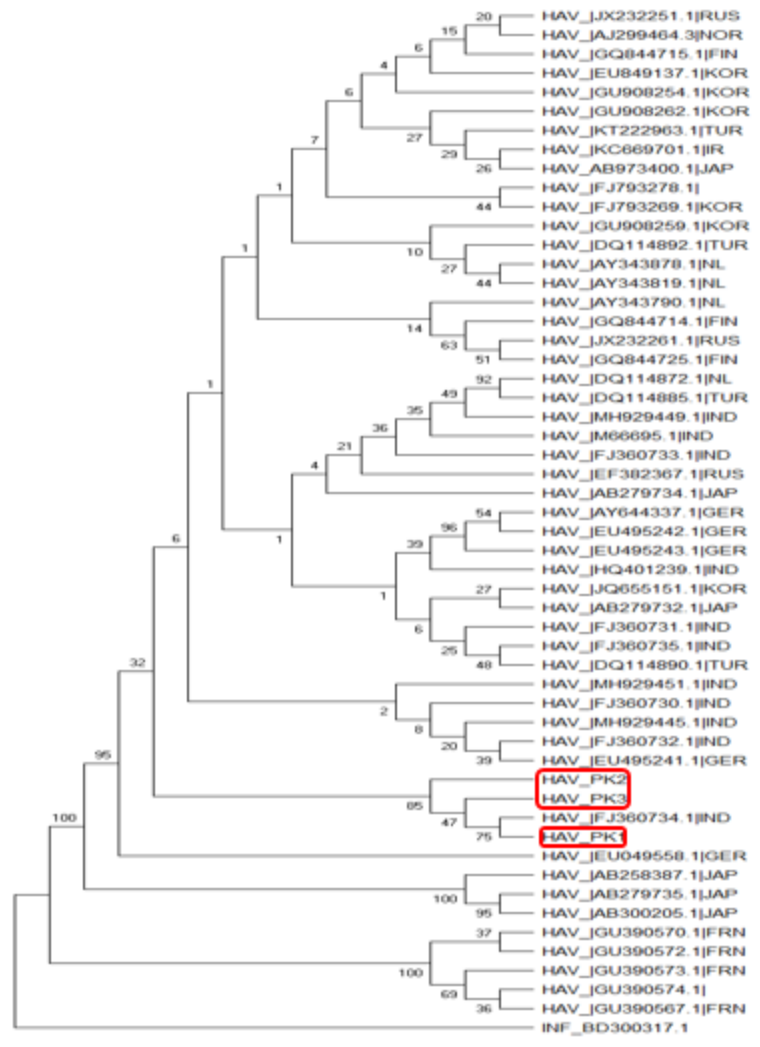
Figure 4.123: HAV phylogenetic tree was constructed through MEGAX software, using the UPGMA Method and a bootstrap value of 100. The boxed sequences represent the strains sequenced in this study. These strains have evolutionary relatedness with the strains previously reported from India.

5. Discussion

Enteroviruses are commonly found in the environment and are associated with a wide variety of diseases. The environmental surveillance of enteroviruses is a crucial method of identifying the presence of different enteroviruses in a given region. In this study, we analysed environmental as well as clinical samples to gain insights about the prevalence of enteroviruses within Pakistan. The areas selected for this study included Lahore, Islamabad and Rawalpindi. Sewage and drinking water samples were collected from Lahore and the twin cities while blood samples were only obtained from the twin cities. The results of this study reveal the presence of NPEV in bulk within the sewage samples collected from the twin cities as 45 out of the 80 samples (56.2%) from this region were positive for NPEV, 4 samples contained enterovirus (6.2%) and 1 was positive for HAV (1.2%). On the contrary, no NPEV was detected among the sewage samples from Lahore, 4 samples were positive for enterovirus (40%) and 1 was positive for HAV (10%). In the case of drinking water, 74 samples were obtained from the twin cities and 15 from Lahore. Out of the 74 samples, 2 contained NPEV (2.7%), 2 were positive for enterovirus (2.7%) and 1 sample was found to be positive for HAV (1.35%). The 15 drinking water samples from Lahore were tested and 2 of these samples were found positive for enterovirus (13.3%). Cross-contamination of NPEV in the drinking water could not be proved statistically as only 2 of the drinking water samples out of a total of 74 samples were found to have NPEV. However, it is important to note that cross-contamination of drinking water with sewage is not uncommon in Pakistan and the lack of water surveillance studies within the country make it impossible to account for the true nature and extent of viral contamination within the water bodies in the country. A few studies have been carried out in the past, focusing on the identification of enteroviruses within

the sewage and drinking water samples. Our results point towards the presence of enteroviruses among the sewage water samples in Pakistan, which is in line with a few previous studies that have explored presence of enteric viruses in the water sources. Previously, high percentage of enterovirus was reported to be present in the sewage water samples from Lahore and the twin cities (Ahmad et al., 2015). The current study has explored the same areas and found high percentages of NPEV and evidences of the presence of enterovirus. Thus, it can be said that different enteroviruses are consistently found within our environment.

The presence of NPEV strains isolated in this study were found to share close lineage with the wild type poliovirus. The phylogenetic analysis revealed close association of our NPEV strain with the ones reported from China previously.This can have immense implications on the polio eradication campaign as the presence of NPEV in high numbers in the sewage samples point towards the widespread dissemination of these viruses among the population. The statistical analysis of the results achieved in this study showed significance in the presence of NPEV within the sewage and drinking water samples, with a p value less than 0.05. The chances of cross-contamination within the water bodies and the spread of infections within the population cannot be ruled out. These results add to the existing data regarding the concerns that exist for the post-polio eradication era. The reports of wild type polio virus cases from Pakistan have not completely ceased and in such times, the presence of closely related enteroviruses can be extremely alarming. According to the latest reports by WHO, the total number of polio cases recorded in 2018 were 33, out of which, 12 cases were reported from Pakistan. Also, the surveillance of sewage bodies of Pakistan has revealed continuous presence of wild type poliovirus in

the major cities such as Karachi, Lahore, Rawalpindi, Peshawar and Quetta (F. Khan et al., 2018). Such reports are highly alarming and point towards the need of better surveillance and treatment of waste and sewage water in order to prevent excessive viral contaminations and creating sources of viral reservoirs in our country. Although this study does not report the presence of wild type poliovirus, the presence of phylogenetically similar strains is nonetheless a cause of concern. It is therefore important to consider strategies for estimating the true risk of poliovirus infections within Pakistan. Currently, the identification of AFP is considered as the major indicator for the presence of wild type poliovirus. However, the evidence of wild type strains being found in the sewage samples, along with other, genetically similar viruses point towards the need of combining different approaches and including environmental surveillance in the strategies for identifying and estimating the true burden of poliovirus infections that threatens the world today.

The clinical samples tested in this study were collected only from the twin cities and revealed 15 cases of HAV (23% out of the total 65 blood samples) all of which were associated with infection in children and predominantly in the male gender. Among the 65 samples, 28 were positive for HEV infection (43%), all found among the female. Out of the 28 HEV positive females 17 (60.7%) were in the third trimester of their pregnancy. The HEV1 strains in our study had phylogenetic relatedness with the strains reported from China and Pakistan. The evidences of HEV infection among the pregnant females within our community is alarming and calls for screening tests. The suggestion for HEV screening is backed with the data regarding the complex outcomes of HEV infection in pregnancy. Moreover, the presence of the HEV-1 is more alarming because it has been

linked with maternal and fetal death. The patients identified in this study were all females, with 60.7% of them being pregnant and hence possibly at a higher risk of facing severe consequences such as still birth, hemmorhage, spontaneous abortion or even death. The healthcare sector of Pakistan currently does not carry out population wide screening or even donor screening for HEV infections. This is a cause of concern as silent prevalence of HEV infections is a major contributor towards maternal and foetal deaths. The widespread number of maternal death cases from Pakistan which have not been linked with any defined cause necessitate the need to carry out screening of viral infections during pregnancy to identify the viral diseases in time and possibly prevent major complications owing to infectious diseases. The high rate of maternal deaths in Pakistan have been linked with cases of haemorrhage and eclampsia (F. Khan et al., 2018), which are the conditions also known to develop as a result of HEV infection during pregnancy. Previous reports of HEV infection during pregnancy among our population has revealed severe outcomes including premature labour, perinatal deaths, spontaneous abortions and death of the mother. All these cases have been reported to be associated with the third trimester of pregnancy (Javed et al., 2017). In the current study, although the patient outcomes could not be explored, but the presence of infection in the third trimester is consistent with the previous findings and indicates an alarming situation for maternal health management in Pakistan. There are currently no vaccines against HEV, the only hope of preventing severe outcomes lies in timely detection and management of the infection. Also, testing for HEV infections needs to be made routine for the pregnant females so as to identify the disease and prevent chances of acute liver failure or other complications during pregnancy due to unidentified infections.

The detection of HAV infection among children has been reported in this study. Even though the evidences of cross contamination between sewage, drinking water and blood have not been determined to be statistically significant through the chi-square analysis. Since the infections are associated mainly with children, and the route of infection is the faeco-oral route, it is highly likely that the infection is spread through contaminated water or food sources. The demographic data of clinical samples need to be analysed against the sites of sewage and drinking water sampling. Our findings are consistent with previous studies which have reported the endemic presence of HAV in Pakistan. Previously, presence of HAV in water sources and it clinical samples have been reported from different regions of Pakistan, including the twin cities, Lahore, Faisalabad and Peshawar (Ahmad et al., 2016). The HAV strains isolated in this study was in close relation with that of strains reported from India. Clinically, it has been reported that HAV accounts for around 60% of the viral hepatitis cases among children across Pakistan. The same has been found in our study as the samples positive for HAV infection were all among children.

The current study cannot do to determine the true disease burden of enteroviruses in Pakistan. Moreover, the cross-contamination of drinking water and sewage could not truy be determined through this study. Concluding whether the water bodies have cross contamination or that the clinical infections could directly be linked with the presence of enteroviruses in the water bodies cannot be done with proof in this particular study. However, it is established that the enteroviruses enter their human hosts through the faeco-oral route. This points towards the need of further studies and wider analysis with

an approach that combines clinical and environmental surveillance for determining the prevalence of different enteroviruses across Pakistan.

6. Conclusion and Future Prospects

In conclusion, enterovirus surveillance is a research area that requires focus. The current study has been done to explore the presence of different enteroviruses in the twin cities (Rawalpindi and Islamabad) of Pakistan and Lahore. The presence of NPEV has been found predominant in the twin cities, which calls for attention of the public health sector and also points towards the need of wider analysis within the country to document the data related to enterovirus prevalence and epidemiology. Since poliovirus eradication efforts are underway and many of the countries across the world have been declared polio-free, in such instances, the presence of NPEV within our environment, which is strongly associated with AFP is a cause of major concern. In this study, we did not find any significant association or indicator of cross contamination between sewage and drinking water. However, the datasets for this study were restricted to a small time frame and the sample collection duration was also limited. It is quite possible that extensive sampling and wider analysis from different regions at varying time frames and seasons can help in identifying evidences of environmental spread of different enteroviruses in Pakistan. The Mann Whitney test revealed that the presence of NPEV was significantly higher in the twin cities as compared to that in the sewage samples from Lahore, with a p

value less than 0.05. However, it is important to note that the total number of samples obtained from Lahore was limited and gaining conclusive evidences was not possible in this study. Sampling from more cities and wider cities might help in gaining better insights about the prevalence of enteroviruses within our community. Moreover, understanding the risk of clinical infections contracted due to contaminated water sources can be aided if studies across different regions of Pakistan are carried out using environmental and clinical samples in larger numbers that can represent the population size of the regions and thus help in gaining statistical data regarding the prevalence of different viruses.

The finding of HEV infection among the females in the Pakistani population is a cause of health concerns. Since HEV infection is associated with negative outcomes in pregnancy and this study found quite a few cases of pregnant females suffering from HEV infections, it may be an indicator of the need to carry out screening of the virus in our population to ensure that maternal health could be protected. Moreover, since the genotyping results are supported with data from across the globe that HEV-1 is associated with negative outcomes in pregnancy, it is crucial to consider avenues that could be explored in regards with screening of the virus during pregnancy.

This study was limited in terms of time period and sampling sites. The findings of this study have indicated that different enteroviruses are present in our environment and among our population. These findings can be used as evidences to build upon in the future and carry out surveillance of enteroviruses in the environmental as well as clinical samples. The differences in the number of samples from the twin cities and Lahore and the absence of clinical samples from Lahore are among the limitations of this study which

have made it impossible to drive conclusions about the city wise prevalence of the different enteroviruses found in this study. Moreover, statistical analysis at this point of time may not be very accurate due to gaps in datasets. However, collection of more samples and wider range of samples from clinical settings in different studies can help in providing evidences about the public health status in different cities across Pakistan. In future, it might be possible to gather data from different areas and carry out a meta-analysis regarding the environmental prevalence and epidemiological status of different enteroviruses in Pakistan.

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