Sero-prevalence and Genetic characterization of Hepatitis E Virus in people living with HIV/AIDS in Nepal

Submitted to

CENTRAL DEPARTMENT OF BIOTECHNOLOGY

Tribhuvan University

Kirtipur, Kathmandu, Nepal

A thesis report submitted in partial fulfillment of the requirement of the

M Sc. degree in Biotechnology

Submitted By

Nirmal Aryal

Roll No. : **BT 117/069**

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RECOMMENDATION

This is to certify that Mr. Nirmal Aryal has successfully completed dissertation work entitled "**Sero-prevalence and Genetic characterization of Hepatitis E Virus in people living with HIV/AIDS in Nepal**" under my supervision. This thesis work was performed for the partial fulfillment of Master of Science in Biotechnology under the course code BT 601. The result presented here is her original findings. I, hereby, recommend this thesis for final evaluation.

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CERTIFICATE OF EVALUATION

This is to certify that this thesis entitled "**Sero-prevalence and Genetic characterization of Hepatitis E Virus in people living with HIV/AIDS in Nepal"** by Mr. Nirmal Aryal found satisfactory for the partial fulfillment of Master of Science in Biotechnology.

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Nirmal Aryal

June, 2016

Abstract

Hepatitis E virus (HEV) infection causes major epidemics of infectious hepatitis, with high mortality rates in pregnant women and can cause chronic infection and cirrhosis in the immunosuppressed, including patients with HIV infection. It is an emerging infection in developed countries and is thought to be zoonotic disease. Different reports indicate that HEV co-infections with human immunodeficiency virus (HIV) may have a more protracted course.

The sero-prevalence study was conducted by enzyme-linked immunosorbent assay. CD4 counts and HIV viral load were examined by Flowcytometry and Real Time PCR respectively. The conserved genomic sequences of open reading frame 1 (550 bp) and open reading frame 2(191 bp) was detected using reverse transcription-PCR, and Sequencing by Sangar method. Sequence analysis was done by BLASTN and MEGA 6.0, and phylogenetic tree construction by neighbor-Joining method.

A total of 270 patients with HIV infection were included in the study. In overall, it was found that out of 270 HIV patients; 32 %(n=87) patients were anti-HEV IgG positive and 6 %(n=17) were anti-HEV IgM positive but only 4 %(n=12) were observed HEV Ag Positive. There was highly sero-prevalence of anti-HEV IgG, IgM and HEV Ag in patients infected with HIV with Adult age, less CD4 counts and high HIV viral load. Additionally, out of 12 HEV Ag positive samples, it was found that only in two samples were detected HEV RNA. HEV genotype 1a was detected in sample after Sequence analysis.

Our findings showed that HEV infection is common in people's living with HIV/AIDS and a high prevalence of HEV in patients infected with HIV. In patients with adults, low CD4 count and high HIV viral load; it is strongly associated with HEV sero-prevalence. Commonly HEV genotype 1a is found in Nepalese blood samples infected with HIV.

Key Words

Hepatitis E virus (HEV), Human Immunodeficiency Virus (HIV)**,** Immunocompromised, Sero-prevalence**,** Genotype

Glossary Acronyms

HEV	Hepatitis E Virus
HIV	Human Immunodeficiency Virus
AIDS	Acquired Immuno Deficiency Syndrome
Ab	Antibody
Ag	Antigen
µl	Microliter
RNA	Ribonucleic Acid
DNA	Deoxy-ribonucleic Acid
cDNA	complementary Deoxy-ribonucleic Acid
CD	Cluster of Differentiation
ELISA	Enzyme-linked Immunosorbent Assay
IgG	Immunoglobulin G
IgM	Immunoglobulin M
PCR	Polymerase Chain Reaction
^{o}C	Degree Centigrade
kDa	Kilo Dalton
GT	Genotype
nt	nucleotide(s)
ORF	Open reading frame
g	gram
ng	nanogram
µg	Microgram
M	Molarity

Mg	Milligram
Ml	Milliliter
mM	milimolar
V	Volt
min	minute(s)
s	second(s)
UV	ultraviolet
pmol	picomolar
PolyA	polyadenylic acid
qRT-PCR	quantitative RT-PCR
RT-PCR	reverse transcriptase PCR
Mt	Methyl transferase
RdRp	RNA-dependent RNA-polymerase
dNTP	deoxynucleoside triphosphate
ddH2O	deionized distilled water
EDTA	ethylenediamine tetraacetic acid
IC	Internal Control
NC	Negative Control
OD	Optical Density
PC	Positive control
RPM	Revolution per Minute
RT	Room Temperature
TMB	Trimethyle Benzidine
HRP	Horse Reddish Peroxidase

aa	Amino acids
RPM	rotations per minute
ALF	Acute liver failure
ALT	Alanine aminotransferases
FHF	Fulminant hepatic failure
PBS	Phosphate Buffered Saline
BSA	Bovine Serum Albumin
HLA	Human Lymphocyte and Antigen
IFN-γ	Interferon-gamma
IN	Interleukin
TNF-α	Tumor Necrosis Factor-alpha
ET-NANBH	Enterically transmitted non-A and non-B hepatitis
HAV	Hepatitis A virus
IEM	Immune electron microscopy
mAbs	Monoclonal antibodies
mRNA	Messenger RNA
NCR	non-coding region
NK	Natural killer cells
VLP	Virus-like particles
CMV	Cytomegalovirus
HAART	Highly Active Anti-Retroviral Therapy
ART	Antiretroviral Therapy
CDC	Center for Disease Control and Prevention
WHO	World Health Oraganization
NPHL	National Public Health Laboratory

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Chapter I

INTRODUCTION

[A] HUMAN IMMUNODEFICIENCY VIRUS (HIV)

1.1 Background

1.1.1 Early history

Both HIV-1 and HIV-2 are believed to have originated in non-human primates in West central Africa, and are transferred to humans in the early 20th Century (Keele *et al*., 2006)

HIV-1 appears to have originated in southern Cameroon through the evolution of SIV(cpz),a simian immunodeficiency virus (SIV) that infects wild chimpanzees endemic in the chimpanzee subspecies Pan troglodytes troglodytes (Gao *et al*.,1999).But, the closest relative of HIV-2 is SIM (smm), a virus of the sooty mangabey (*Cercocebus atys atys*), an old world monkey living in West Africa.

However, SIV is thought to be weak virus and easily suppressed by Human immune system within weeks of infection. It is believed that several transmissions of the virus from individual to individual in quick succession are necessary to allow it enough time to mutate into HIV (Marx *et al*., 2001).

AIDS was first clinically recognized in 1981 in the United States. The initial cases were observed in injecting drug users and homosexual men with no known cause of impaired immunity who showed symptoms of *Pneumocystis carinii* pneumonia (PCP),a rare opportunistic infection and rare skin cancer called Kaposi's sarcoma (KS) that was known to occur in people with very compromised immune systems (Gottlieb *et al*.,1981; Friedman-Kien AE *et al.*, 1981).

The human immunodeficiency virus (HIV) is a lentivirus (a subgroup of retrovirus) that causes HIV infection and acquired immunodeficiency syndrome (AIDS). One of the principal cellular targets of HIV infection is the CD4+T helper lymphocyte (Th); the body immune system. This disables the immune system to defend the body against diseases and tumors.

1.1.2 Systemic Position (Classification)

Group : Group VI (SS RNA –RT)

Order : Unassigned

Family : *Retroviridae*

Sub Family : *Orthoretrovirinae*

Genus : Lentivirus

Species : Human immunodeficiency Virus 1

Human immunodeficiency Virus 2

1.1.3 Structure of HIV

Infections with lentiviruses typically show a chronic course of disease, a long period of clinical latency, persistent viral replication and involvement of the central nervous system. Electron microscopy analysis of HIV-1 and HIV-2 resemble each other mostly. However, they differ with regard to the molecular weight of their proteins, as well as having differences in their accessory genes. Both HIV-1 and HIV-2 replicate in CD4 T cells and are regarded as pathogenic in infected persons, although the immune deficiency may be less severe in HIV-2-infected individuals.

1.1.3.1 Morphology and Structure of HIV-1

HIV-1 viral particles have a diameter of 100 nm and are surrounded by a lipoprotein membrane and it contains 72 glycoprotein complexes, which are integrated into this lipid membrane, and are each composed external glycoprotein gp120 and a transmembrane spanning protein gp41. The matrix protein p17 is anchored to the inner

side of the viral lipoprotein membrane and p24 core antigen contains two copies of HIV-1 RNA. The HIV-1 RNA is part of a protein-nucleic acid complex, which is composed of the nucleoprotein p7 and the reverse transcriptase p66 (RT). The viral particle contains all the enzymatic equipment that is necessary for replication: a reverse transcriptase (RT), an integrase p32 and a protease p11 (Gelderbloom *et al*., 1993).

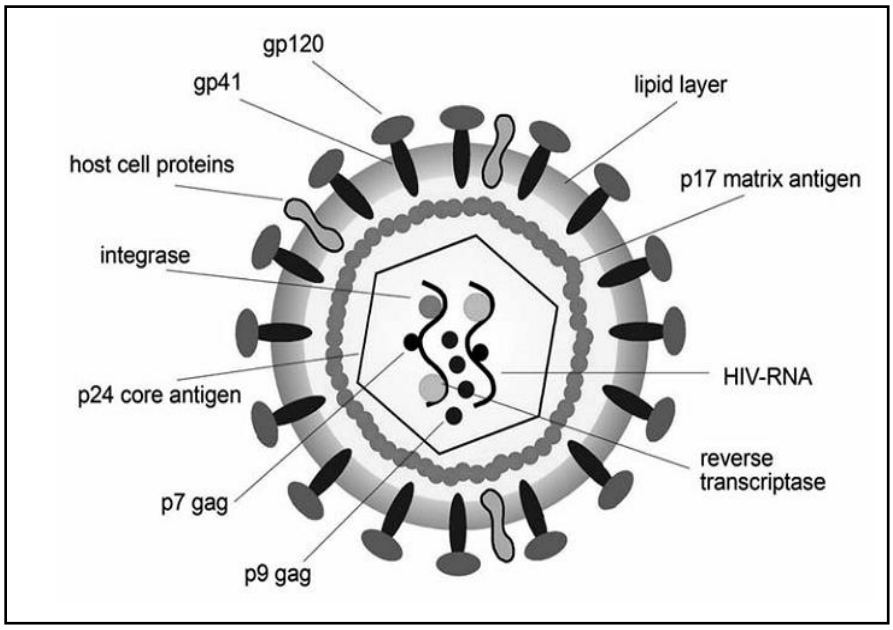

Figure 1.1: Structure of an HIV virion particle (Gelderbloom, 1993)

1.1.4 Genome Organization of HIV

The classical structural scheme of a retroviral genome is 5'LTR-gag-pol-env-LTR 3'. The LTR (long terminal repeat) regions represent the two end parts of the viral genome, that are connected to the host cellular DNA after integration and do not encode for viral proteins. The gag and env genes code for the nucleocapsid and the glycoproteins of the viral membrane; the pol gene codes for the reverse transcriptase and other enzymes. In addition, HIV-1 contains six genes (vif, vpu, vpr, tat, rev and nef) in its 9kB RNA that contribute to its genetic complexity.

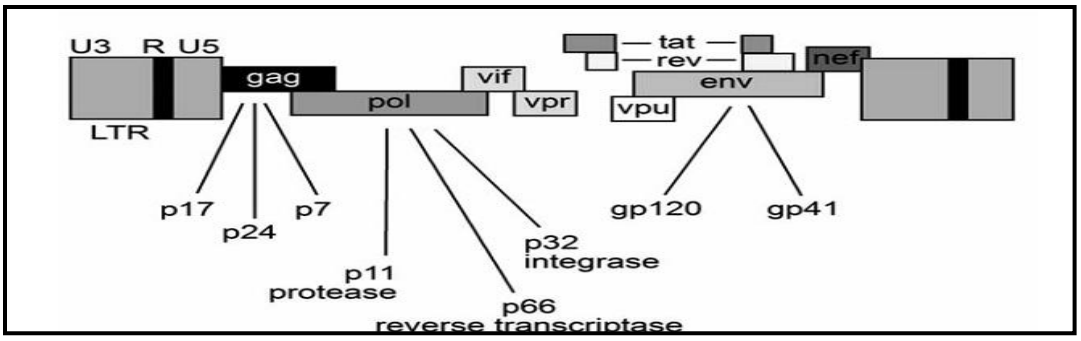

Figure 1.2: Genome Organization of HIV (Wong-Staal, 1991)

1.1.5 HIV Replication Cycle

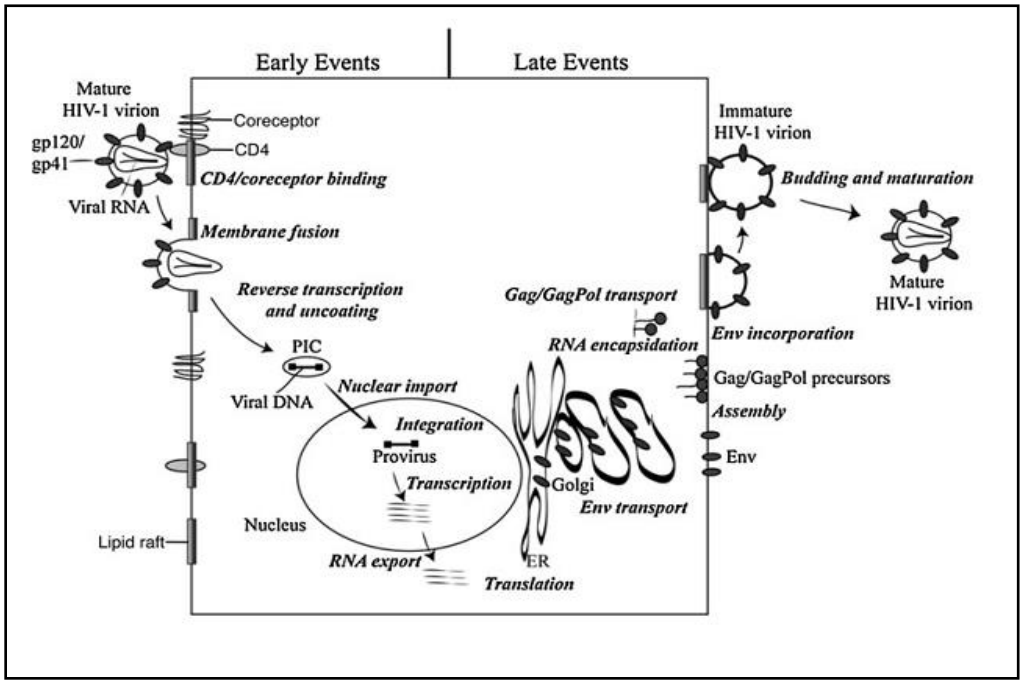

Figure: 1.3 Schematic representation of the HIV-1 replication cycle (Freed, 2004)

1.1.6 Transmissions

The main transmission routes of HIV are; unsafe sex with an HIV-infected partner, sharing injection tools with an HIV-infected partner and vertical transmission of HIV from the HIV-infected mother to the newborn (before or at birth; or later, due to breastfeeding). Among these are transmissions due to transfusion of blood or blood products in countries where blood donations are not routinely screened for HIV is most probable.

1.1.6.1 Unsafe Sex

The highly transmission route for HIV is sexual contact. The prerequisite for sexual transmission is direct exchange of infectious body secretions / fluids. The highest viral concentrations are found in blood and seminal fluid.

Although sexual transmission among homosexual males is still a significant part of epidemic spread, in the most populous regions of the world, sexual transmission among heterosexuals is the dominant mode of spread.

1.1.6.2 Injection Paraphernalia

Sharing injection paraphernalia is the most important HIV transmission route for persons who use drugs intravenously through HIV infected blood contamination. Due to the usually quite large amount of blood that is exchanged when sharing of needles, syringes, and other injection equipment.

1.1.6.3 Vertical Transmission

Perinatal transmission or Vertical transmission of human immunodeficiency virus accounts for virtually all new HIV infections in children. Without intervention up to 40% of newborns born to HIV-1-positive mothers are infected with HIV-1. The most important risk factor is viral load at the time of delivery.

The relative contributions of in utero and intrapartum HIV transmission are unknown. One proposed scheme for differentiating these 2 modes of transmission suggests that the virus was transmitted early or in utero if HIV is detected in the infant within the first 48 hours of live. Late or intrapartum transmission is said to have occurred if virologic evaluations are negative during the first week of life but there is subsequent HIV detection between 7 and 90 days of age and suggest that 50% to 70% of HIV vertical transmission occurs intrapartum. Breast feeding substantially increases the risk of HIV vertical transmission, therefore bottle feeding is currently recommended for all infants born to HIV infected mothers.

1.1.6.4 Blood products, tissue transplantation and artificial insemination

Transmission of HIV-1 can occur following transfusion of a blood product derived from an infected person's blood and processed into a blood component (i.e., whole blood, packed red cells, fresh frozen plasma, cryoprecipitate, and platelets).

HIV has been transmitted through transplantation of kidney, liver, heart, pancreas, bone, and skin: all blood containing organs or highly vascular tissues. There are no reports of HIV tissue transmission from HIV-seropositive donors of cornea. Both intrauterine insemination and cervical insemination result in HIV transmission.

1.1.6.5 Insects

All studies that have investigated the possible transmission of HIV via insects have come to the same conclusion, that it is not possible. This holds true as well for studies performed in Africa with a high AIDS prevalence and large insect populations (Castro *et al*., 1988).

1.1.7 Epidemiology

The Human Immunodeficiency Virus had probably emerged in the 1920s or '30s when the Simian Immunodeficiency Virus (SIV) jumped host from the chimpanzee to the human in Western Africa (Worobey *et al*., 2008).The first serological evidence for HIV infection in human serum sample was found in Kinshasa (Zaire, now the Democratic Republic of Congo) in 1959, suggested that HIV was circulating in Africa at those times (Zhu *et al*., 1998). After the first description of AIDS in 1981 by now almost all countries in the world have been affected by HIV.

1.1.7.1 HIV Scenario in World (WHO July 21, 2014)

There were approximately 35 million people worldwide living with HIV/AIDS. Estimated value of 2.1 million individuals worldwide became newly infected with HIV .This includes over 240,000 children (<15 years). Most of these children live in sub-Saharan Africa and

were infected by their HIV-positive mothers during pregnancy, childbirth or breastfeeding. Report shows that 19 million of the 35 million people living with HIV today do not know that they have the virus.

The majority of people living with HIV are in low- and middle-income countries. Sub-Saharan Africa is the most affected region with 24.7 million people living with HIV.71 percent of all people who are living with HIV in the world live in this region.HIV is the world's most infectious killer disease. According to WHO, estimated 39 million people have died since the first cases were reported in 1981 and 1.5 million people died of AIDS-related causes in 2013.

At the end of 2013, 12.9 million people living with HIV were receiving antiretroviral therapy (ART) globally, of which 11.7 million were receiving ART in and middle-income countries. About 740,000 of those were children. This is a 5.6 million increase in the number of people receiving ART since 2010. However, almost 22 million other people living with HIV are still not accessing ART.

Preventing mother to child transmission of HIV and keeping mothers alive has been progressing. According to WHO, in 2013, 67% of pregnant women living with HIV in low and middle income countries (970,000 women) received ART to avoid transmission of HIV to their children. This is up from 47% in 2010.

Table: 1.1 Global Summary of the AIDS epidemic (2013) [Source: WHO- HIV department July 21, 2014]

Number of People living with HIV in 2013	**Total**	**35.0 million**
	Adults	31.8 million
	Women	16.0 million
	Children (< 15 years)	3.2 million
People newly infected with HIV in 2013	**Total**	**2.1 million**
	Adults	1.9 million
	Children (< 15 years)	2,40,000
AIDS deaths in 2013	**Total**	**1.5 million**
	Adults	1.3 million
	Children (< 15 years)	1,90,000

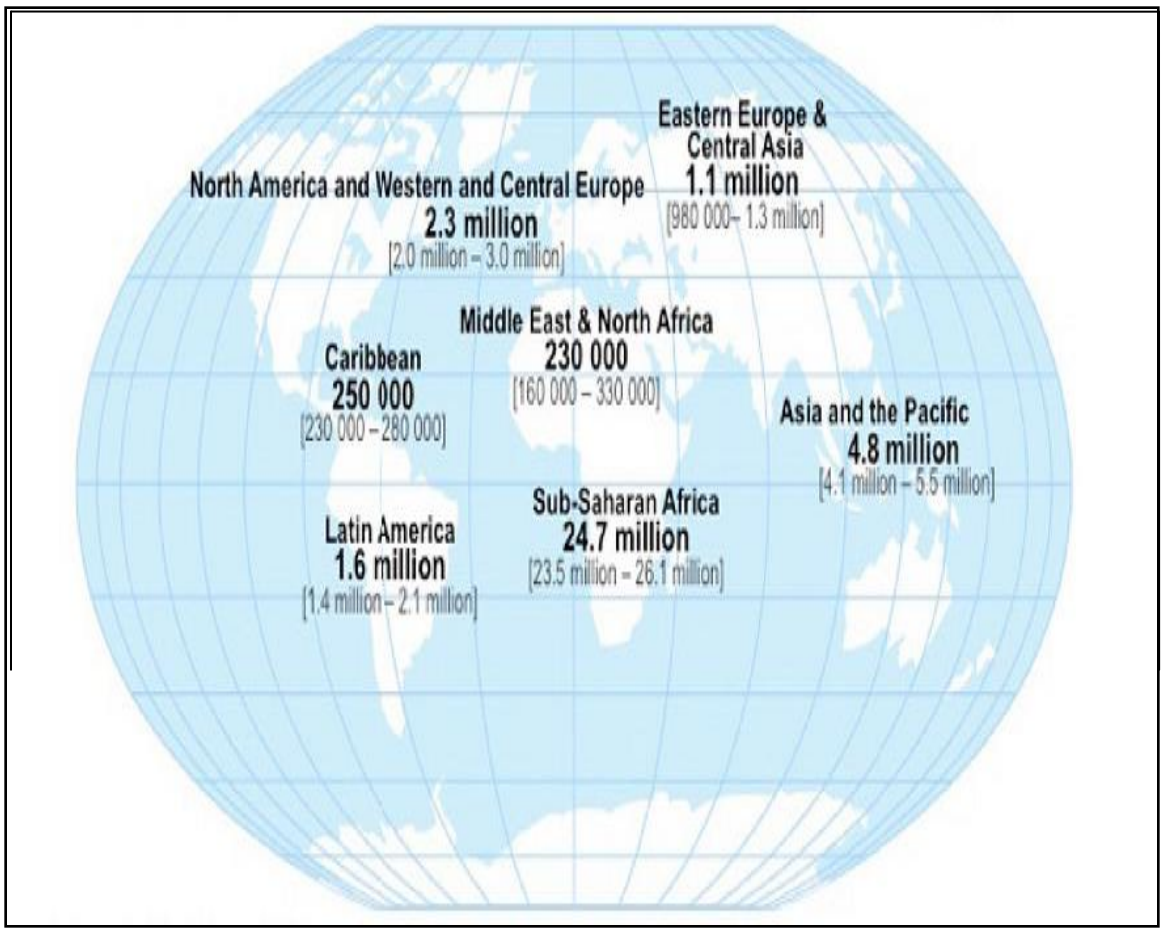

Figure: 1.4 People worldwide living with HIV/AIDS 2013 (Source: UNAIDS)

1.1.7.2 HIV Scenario in Nepal

Since the detection of the first HIV case in 1988, the HIV epidemic in Nepal has evolved from a low to concentrated epidemic. In Nepal approximately 23000 people are living with HIV/AIDS (NCASC, 2013). This is the highly prevalence of HIV/AIDS, as compared to the total populations in Nepal. HIV in Nepal is characterized as concentrated epidemic, where majority of infections are transmitted through sexual transmission. People who inject drugs, men who have sex with other men and female sex workers are the key populations at higher risk of HIV in Nepal.

Table 1.2 Cumulative HIV Infections by Sub Group, Age and Sex, Reported (July 2013) [Source: Routine reporting, NCASC, July 2013]

Sex Group	**Male**	**Female**	**Third Gender**	**Total**
Sex Workers(SW)	68	1083	0	1,151
Injecting Drug Users	2,866	71	6	2,943
Men having Sex with Men(MSM)	285	-	17	302
Blood or Organ Recipients	61	25	0	86
Clients of Sex Worker	8,985	141	0	9,126
Housewives	-	5,662	1	5,663
Male Partners of FSW/Female Migrant	125	-	0	125
Migrant Workers	944	43	0	987
Spouse of migrants	35	611	0	646
Prison Inmates	-	-	-	-
Children	980	647	0	1,627
Sub-group NOT identified	211	125	2	388
Total	**14,560**	**8,408**	**26**	**22,994**
Age Group				
0-4	347	222	0	596
5-9	412	292	0	704
10-14	188	130	0	318
15-19	325	366	3	694
20-24	1,573	1,229	4	2,806
25-29	2,917	1,862	4	4,783
30-39	5,840	2,983	10	8,833
40-49	2,244	1026	4	3,274
50- above	687	298	1	986
Total	**14,560**	**8,408**	**26**	**22,994**

1.1.8 HIV and the immune system

1.1.8.1 Humoral immune response

The association between an HIV-1-specific humoral immune response and the course of disease is less well characterized. A slow progression of immunodeficiency was observed in patients with high amount of anti-p24 antibodies and persistence of neutralizing antibodies against primary and autologous viruses (Hogervorst *et al*., 1995; Montefiori *et al*., 1996). It has been observed that the individuals may demonstrate a local (mucosal) IgA response against HIV-1 proteins that are not detected by the usual

antibody testing methods. Thus, detection of IgA, rather than systemic IgG, may be associated with protection against HIV-1 infection (Saha *et al*., 2001).

A number of different studies have shown that neutralizing antibodies do exist in HIV-1-infected individuals, but there is a time lag in their appearance. That is, infected individuals will develop neutralizing antibodies to their own viruses with time, however, by the time these antibodies develop, the new viruses circulating in the individual's plasma will become resistant to neutralization, even though the older ones are now sensitive to the current antibodies in the patient's serum. Selected patients with HIV infection were treated with plasma from HIV-infected patients at an earlier stage of the disease. No significant effect on the course of disease was notable (Jacobson *et al*., 1998)

1.1.8.2 Cellular immune response

Cytotoxic T-cells (CTL) are able to recognize and eliminate virus-infected cells. However, there is little evidence to assume that CTL play a major role in primary protection of HIV. HIV-specific CTL responses have been observed in individuals exposed to, but not infected by HIV-1. Nef-specific CTL have been detected in HIV-1-negative heterosexual partners of HIV-infected patients and env-specific CTL have been identified in seronegative healthcare workers after exposure to HIV-1-containing material by needle stick injuries (Pinto *et al*., 1995). Unfortunately patients with a broad and strong CTL response do not seem to be protected from superinfection by a different, but closely related HIV isolate (Pinto *et al*., 1995; Altfeld *et al*., 2002). However, it is still unclear in most patients who exhibit a potent temporary CTL response, why this CTL response diminishes later on.

Depending on the secretion pattern of cytokines, CD4+ T-cells may be divided into TH1 and TH2 cells. TH1 CD4+ T-cells primarily produce interleukin-2 (IL-2) and IFNγ, cytokines that support the effector functions of the immune system (CTL, NK-cells, macrophages) and TH2 cells predominantly produce IL-4, IL-10, IL-5 and IL-6, cytokines that favor the

development of a humoral immune response. Since TH1 cytokines are critical for the generation of CTLs, this HIV-1-specific TH1 response is regarded as being a protective immune response. Some studies on HIV-exposed but non-infected individuals have shown that stimulation with HIV-1 env antigens (gp120/gp160) and peptides, T-cells from these individuals secrete IL-2 in contrast to non-exposed control persons (Clerici *et al*., 1991).

1.1.9 Opportunistic Infections

In the early years of the AIDS epidemic, the life expectancy of individuals diagnosed with their first AIDS defining illness was at most two to three years. Today, however, many patients now live with AIDS for 15 years or longer. Up to 90% of patients who develop AIDS with severe opportunistic infections is unaware of their HIV status. Typically, these patients seek medical attention late at that time their overall health condition is serious. Since AIDS remains life-threatening, every HIV clinician should be familiar with the diagnosis of OIs and their respective therapy.

The incidence of many OIs has been reduced to less than one-tenth of their frequency in the pre-HAART era (Buchacz *et al*., 2010). ART has not only decreased the incidence of OIs, but it has also changed the course of OIs considerably.

People with healthy immune systems can be exposed to certain viruses, bacteria, or parasites and have no reaction to them but people living with HIV/AIDS can face serious health threats, that are known as *"*opportunistic" infections (OIs). These infections are called "opportunistic" because they take advantage of weakened immune system, and cause devastating illnesses. OIs are signs of a declining immune system. Most life-threatening OIs occur when CD4 count is below 200 cells/mm^3 and these are the most common cause of death for people with HIV/AIDS.

The CDC developed a list of more than 20 OIs that are highly considered AIDS-defining conditions like Cryptococcosis, cytomegalovirus diseases, Tuberculosis, Toxoplasmosis gondi, etc but Hepatitis is not included in this list. Different research showed that

Hepatitis is also Opportunistic infections in HIV patients but there is any exact data of opportunistic infections of Hepatitis has not given by CDC and WHO. Among Hepatitis, hepatitis B and C are high degree of co-infection in HIV positive patients as compared to other hepatitis but co-infection with HIV and HEV can also observed as shown by different research in different countries. In addition, persons who are co-infected with HIV and Hepatitis E can have serious medical complications, including an increased risk for liver-related morbidity and mortality.

[B] HEPATITIS E VIRUS (HEV)

1.2 Background

1.2.1 Early history

The first epidemiological study about hepatitis E came from India in the early Fifties. In the peak of this infectious acute hepatitis outbreak in Delhi, the incidence was almost 190 cases per day. During more than 6 weeks about 29,300 cases were reported; it has been estimated that approximately 68 % of the population of Delhi was infected (Viswanathan, 1957).

Without knowing the infectious agent caused this outbreak a very detailed study was performed; some epidemiological data differed from hepatitis caused by HAV. The fatality rate showed that the pathogen was of low Virulence. However, when "infectious hepatitis" occurred during pregnancy there were some complications like still-birth, neonatal death and a high case-fatality ratio. The study pointed to water borne infection, nevertheless the unusual pathogen was not identified (Naidu and Viswanathan, 1957).

Nearly 15 years after the outbreak a group of researchers analyzed patient samples from the Delhi outbreak 1955-56 and other two more infectious hepatitis outbreaks in India (Ahmadabad 1975-76 and Pune 1978-79). There was no evidence for infection with either HAV or HBV was found and it was confirmed that an unrecognized agent had been responsible for the outbreaks (Wong et al., 1980). This unknown agent was named "enterically transmitted non-A and non-B hepatitis" (ET-NANBH) (Sreenivasan *et al*., 1984a).

In 1983 a scientist infected himself ingesting fecal suspension from an ET NANBH patient. Spherical 27 to 30 nanometers virus-like particles (VLP) were observed in his feces and characterized using immune electron microscopy (IEM). Volunteer had

previously been exposed this virus like particles and not produced antibodies against HAV and HBV, but developed antibodies against the VLPs recovered in his feces. After this cynomologus monkeys were inoculated with the virus-containing stool and hepatitis was confirmed by liver enzymatic profile, specific antibody response and excretion of VLPs (Balayan *et al*., 1983).

Later the ET-NANBH virus from a Burmese (Myanmar) patient was inoculated in to the cynomologus monkeys and HEV cDNA was isolated for the first time. In the same study it was also demonstrated that the viral genome had a plus strand RNA genome and was polyadenylated; the name hepatitis E virus (HEV) was proposed (Reyes *et al*., 1990; Zuckerman, 1990). Afterwards the first full-length HEV genome was successfully cloned and sequenced (Tam *et al*., 1991).

1.2.2 Systemic Position (Classification)

Group : Group IV ((+)ssRNA)

Order : Unassigned

Family : *Hepeviridae*

Genus : Orthohepevirus

Type Species : Orthohepevirus A

1.2.3 Structure of HEV

HEV is small and structurally simple animal RNA virus. The virion is nonenveloped with a diameter of 27-34 nm, is composed entirely of viral protein and RNA. Electron microscopy (EM) analyses show spherical particles of possible icosahedral symmetry, with indefinite surface substructure, resembling the caliciviruses.

Both immune and negative stain electron microscopy of human stool specimens have showed that the diameter of HEV is about 32nm (Balayan *et al*., 1983; Bradley *et al*., 1988).

The surface of the virion has obvious spikes that are slightly less pronounced than those of Norovirus, but is clearly distinct from the smooth, featureless surface of the hepatitis A virus (Guu *et al*., 2009; Xing *et al*., 2010).

However, based on morphology structure alone, HEV could not be reliably distinguished from other small spherical human enteroviruses usually found in feces (Bradley *et al*., 1988).

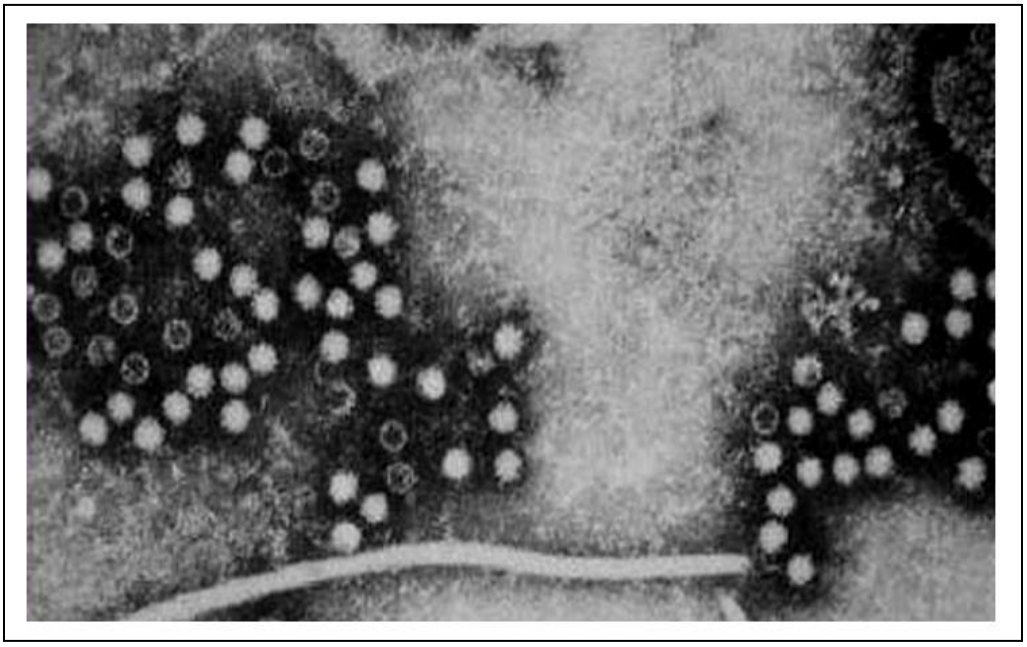

Fig: 1.5 Electron Microscopy Picture of HEV (Source: CDC)

1.2.3.1 HEV Viral Particle Structure

The HEV capsid subunits are formed by two identical molecules (homodimers), which represent the main structure responsible for the virion shell. The capsid protein comprises about 660 amino acids with a molecular size of approximately 70 kda and can be divided into three different domains: S (shell), M (middle) and P (protruding). These domains are located in position 118-317, 318-451 and 452-606,respectively (Xing *et al*., 2010).

The S domain forms the internal skeleton of the particle, forming a continuous capsid shell. The M domain is tightly associated to the S domain and linked to the P domain. The association of these two domains makes it possible for the capsid protein dimer to change its conformation, allowing a very unique topology. The P domain, it forms dimeric spikes stabilizing protein interactions across the two-folds (Guu *et al*., 2009; Mori *et al*., 2011; Yamashita *et al*., 2009).

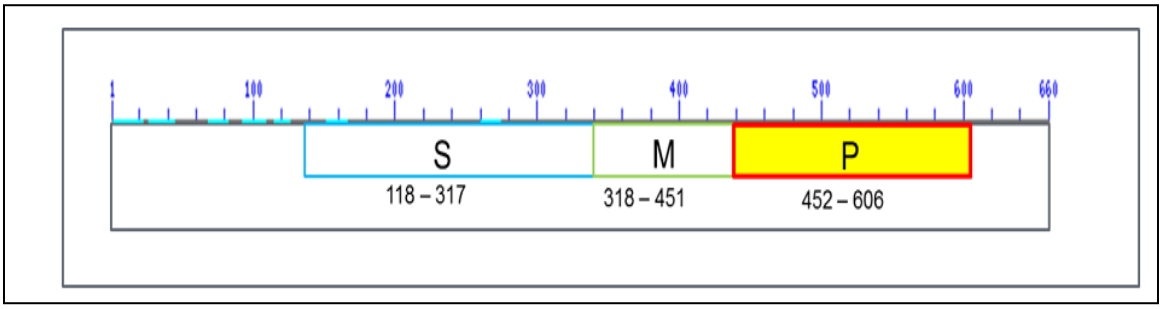

Figure 1.6 Structural domains of the HEV capsid protein. Shell (S) from aa 118 -317, middle (aa 318-451) and protruding (aa 452-606) domains (Xing *et al*., 2010)

1.2.4 Genome Organization of HEV

HEV virions are non-enveloped spherical particles with a size of 27 to 32 nm in diameter. They possess a positive strand RNA genome with a size of approximately 7.2 kb with three partly overlapping open reading frames (ORFs), a capped 5' end and polyadenylated 3' end (Mushahwar, 2008).The genome organization is the same for genotypes 1, 2 and 3 and only differs regarding the position of ORF3 in genotype 4 (Panda *et al*., 2007).

The 5' end of the genome contains a short non-coding region (NCR) with 26 to 28 nucleotides in length. The size of ORF1 is approximately 5.1 kb. This region encodes a polyprotein, which is cleaved into the viral nonstructural proteins as methyltransferase, papain-like cysteine protease, helicase and RNA dependent RNA polymerase (RdRp); these enzymes are involved in viral replication, transcription and polyprotein cleavage (Kaur *et al*., 1992).

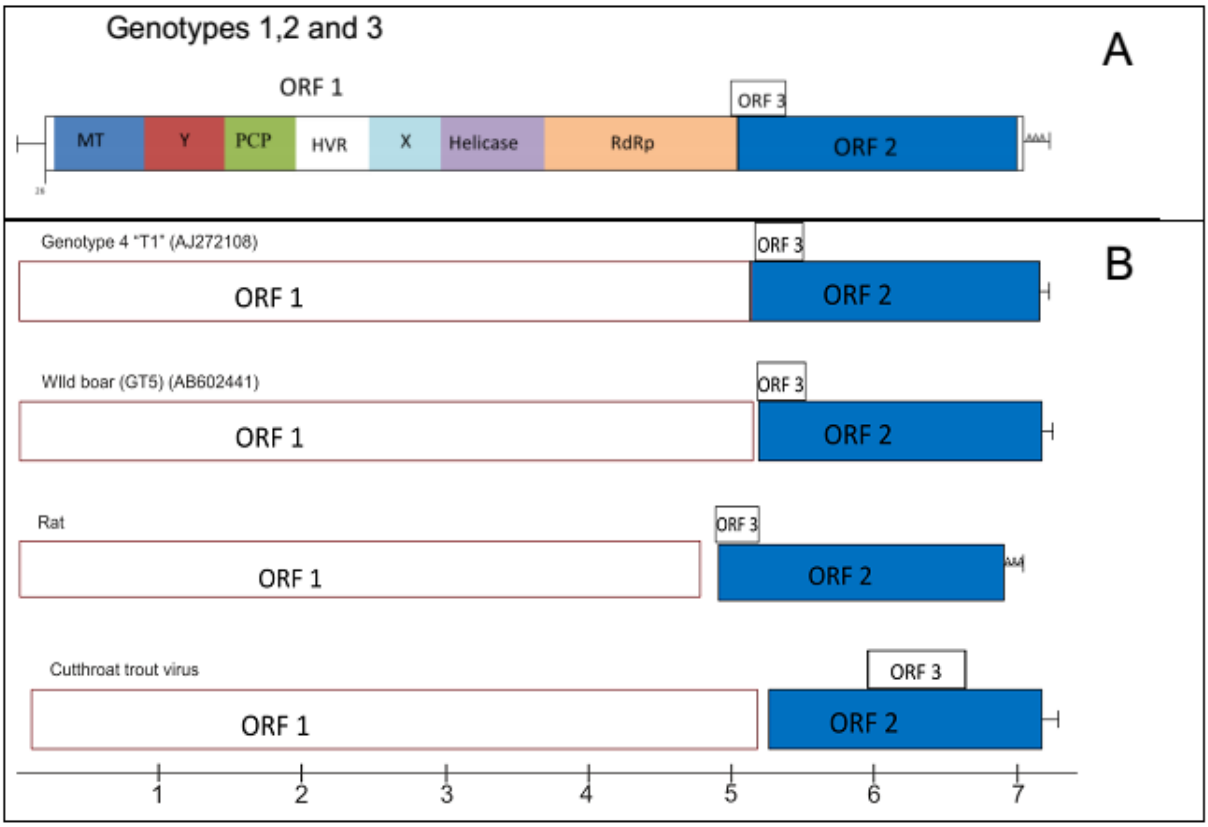

Fig 1.7 Genome organization of HEV Oliveira-Filho *et al*., 2013

The ORF 2 encodes the structural capsid protein and has a size of approximately 1983 nt for members of the genotypes 1, 2 and 3 and 2025 nt for members of genotype 4. This protein is highly immunogenic and is responsible for the functions in assembly and host interaction. It has a high nucleotide heterogeneity and has been subject of both diagnostic tests and vaccine development (Engle *et al*., 2002)

ORF 3 has a size of 369 nt and encodes a small phosphorylated protein which binds to the hepatocellular cytoskeleton and forms a complex together with the capsid protein. Other possible ORF 3 functions are related to the regulation of cellular signs (Jiménez de Oya *et al*., 2007).

1.2.5 Transmissions

The main route of human HEV transmission is fecal-oral. The first reported outbreak pointed already towards an association between ingestion of water or food contaminated with HEV (Aye *et al*., 1992)

The meat products from HEV-infected reservoir animal species are capable of transmitting HEV to humans and are a public health concern. HEV primarily replicates in the liver of infected animals; however, extra-hepatic sites of HEV replication have also been demonstrated in the gastrointestinal tissues, mesenteric and hepatic lymph nodes, and spleen. Uncooked and undercooked meat products of these infected organs of HEV reservoir animals like rat, wild boar, swine etc. has been linked to the numerous cases of HEV worldwide (Pavio *et al*., 2010; Miyashita *et al*.,2012)

It has also been suggested that HEV can be transmitted from one farm to another by fecal contamination or the movement of people and animals (Yan *et al*., 2008)

Other less common routes are vertical transmission (transplacental) as well as horizontal via blood transfusion or organ transplantation. Seroprevalence of Hepatitis E has been found in Renal Transplant Recipients (Rostamzadeh *et al*., 2011). The first molecular evidence for transfusion-transmitted HEV came in 2004 from a 67-year-old Japanese patient. The HEV sequence was highly similar to that of one donor sample (Matsubayashi *et al*., 2004)

Research suggested that HEV can also be transferred from mothers to baby that is transplacental transmission (Khuroo *et al*., 2003)

1.2.6 Epidemiology

1.2.6.1 HEV Scenario in World

No single data-source for hepatitis E is available. No international agency or national government requires cases or outbreaks of hepatitis E to be notified to a central

authority. Hepatitis E virus (HEV) is a significant international public health problem and it is estimated that 2.3 billion people are infected globally (WHO, 2012)

Until 1997, hepatitis E was thought to occur only in developing countries including Africa, central Asian republics of the former Soviet Union, Afghanistan , Bangladesh , Borneo, Burma , China , India , Mexico , Mongolia , Nepal , Pakistan , Thailand , Vietnam , and some parts of the Middle East. In these countries, the disease is a significant public health concern and is both endemic and epidemic, with human outbreaks generally associated with fecal contamination of drinking water (Meng *et al*., 1999)

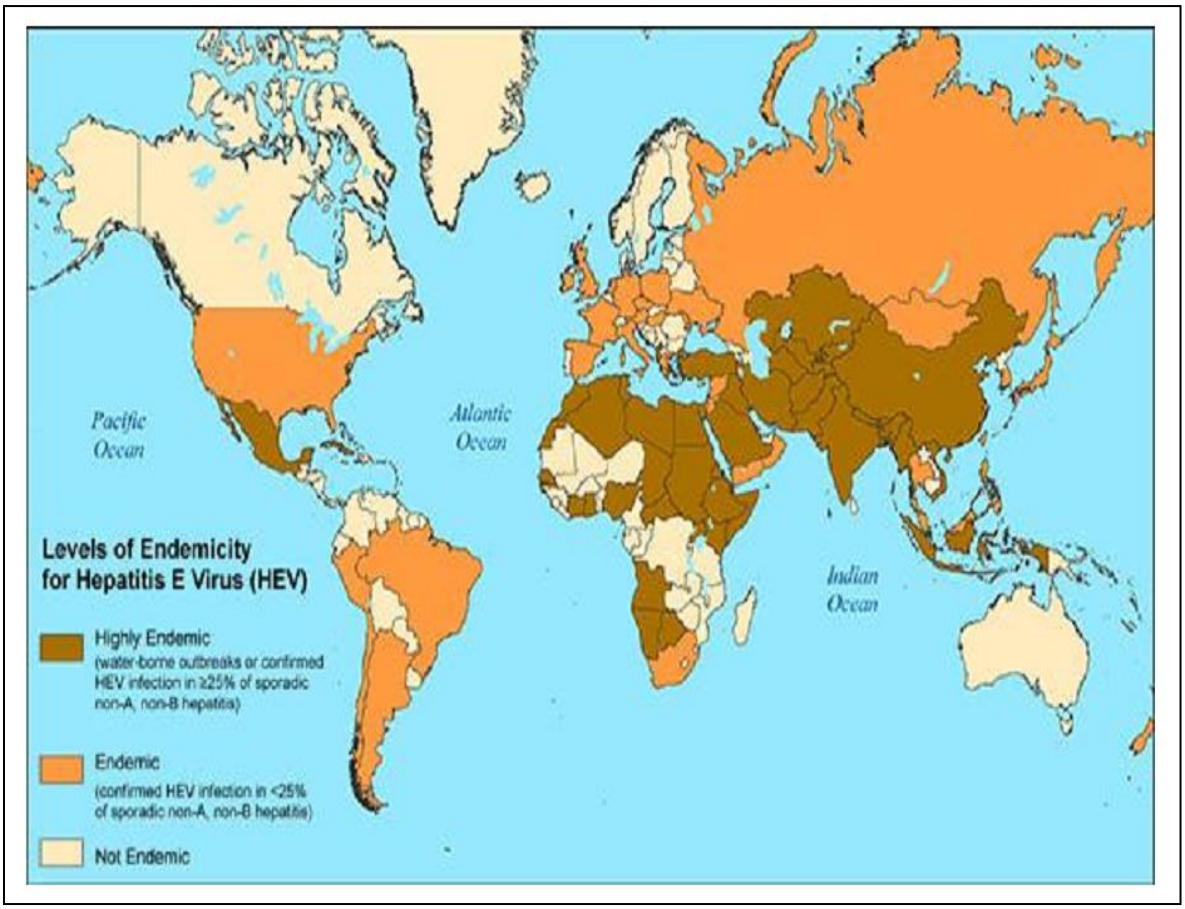

Fig 1.8 Worldwide Epidemiology of HEV (CDC)

The earliest well-documented report of the disease was a large epidemic of water-borne hepatitis occurring in New Delhi, India in 1955 (Vishwanathan, 1957). HEV is transmitted primarily by the fecal–oral route, and has been reported to occur as large waterborne epidemics and small outbreaks. Sporadic cases of HEV infection have also been reported

in non endemic, developed countries, where its occurrence is usually associated with travel to endemic areas (Ishikawa *et al*., 1995; Donati *et al*, 1997)

HEV-associated hepatitis also occurs among individuals in industrialized countries who have no history of travel to endemic areas (Purcell *et al*., 2001). Hepatitis E generally results in asymptomatic or mild illness similar to Hepatitis A, except in pregnant women who experience up to 20 percent mortality (Chin, 2000)

1.2.6.1.1 Geographical Distribution

The four HEV genotypes which are distributed worldwide and prevalence ranges between the different continents and between different socioeconomic situations.

The Genotype 1 was initially found in Asian countries such as Bangladesh and Myanmar and then in African countries such as Chad and Morocco (Sugitani *et al*., 2009; Yin *et al*., 1994).

Genotype 2 sequences have been found in Mexico and Nigeria (Huang *et al*., 1992; Lu *et al*., 2006).

Genotype 3 have been observed in the US, Japan, Argentina, Brazil and in European countries such as Belgium, France, Germany, Hungary, Italy, the Netherlands, the United Kingdom (Banks *et al*., 2004; dos Santos *et al*., 2009; Fukuda *et al*., 2007).

Genotype 4 sequences have been observed in China, Taiwan and Japan (Inoue *et al*., 2009; Liu *et al*., 2012). Recently, genotype 4 has been also found in swine from Belgium being the first report of genotype 4 in pigs in Europe; however it remains unclear how the genotype 4 strain was introduced into the European swine population (Hakze-van der Honing *et al*., 2011).

Multiple genotypes might occur in the same country, population or even in the same individual (human or animal). The distribution of the various HEV genotypes in both human and animal populations in China (where genotypes 1, 3 and 4 are present)

is a very good example of how complex the geographical distribution can be (Li *et al*., 2009).

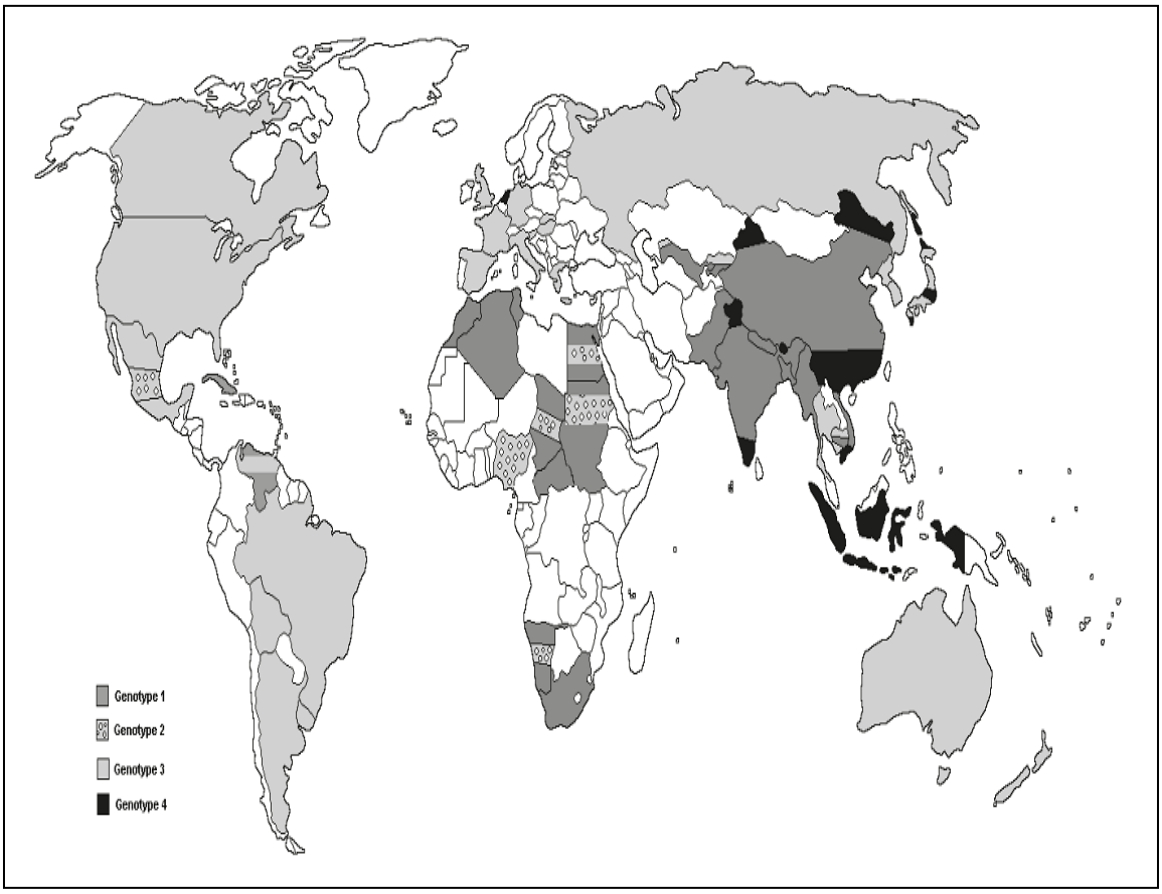

Fig 1.9: Geographical distribution of HEV genotypes. Genotype 1 and 2 consist of epidemic strains, exclusively found in humans. Genotypes 3 and 4 comprise zoonotic strains and have been isolated from sporadic cases of acute human HEV infection and animals reservoirs, particularly domestic and wild pigs. (Genotypes 1 and 2 HEV strains are restricted to the human population, while Genotypes 3 and 4 HEV strains infect both humans and other animals with zoonotic transmission routes) (Santiago, 2012)

1.2.6.2 HEV Scenario in Nepal

The first documented epidemic of hepatitis E in Nepal occurred in the Kathmandu valley in 1973 which affected more than 10,000 people, mostly young adults in the age group of 16 to 35 years age. This epidemic was spread over nearly 10 months period from

January to October, and reached a sharp peak during July and August monsoon rains. Nearly 2.4% population was affected, and caused more than 10,000 cases of acute hepatitis, of which 70% were among young adults in the age group 16 to 35 years. At the peak of epidemic, 118 pregnant woman were Hospitalized due to acute hepatitis, of which 41 developed acute hepatic failure (AHF).Mortality rate among infected Pregnant women were 25.4% (Hillis *et al*., 1973; Shrestha *et al*., 1975)

Five years later another epidemic of HEV occurred in the Kathmandu valley in 1981. This outbreak began in May 1981 and continued till September 1982, and had peaks in the rainy season of both years. As in previous epidemic, it predominantly affected young adults in the age group 16-35 years (70%) of which 70% were male. Twenty-five out of 119 pregnant women admitted to the hospital died of acute hepatic failure (mortality 21%).The epidemic was labeled as non-A, non-B hepatitis based on negative serology for acute hepatitis A and hepatitis B (Shrestha SM and Malla DS , 1883)

Another Five years later next epidemic occurred again in Kathmandu in 1987.As previous epidemic sharp during July and August. Serological tests done in 393 patients showed that 10 (2.3%) had hepatitis A, 13 (3.1%) hepatitis B, and the reminder 370 (88.7%) non-A, non-B hepatitis. Spherical 32 nm VLP was recovered from stool samples in 3 (Xue-Yi *et al*., 1991)

1.2.6.2.1 Sera-epidemiology of HEV in Nepal

Presence of anti-HEV IgG in the absence of anti-HEV IgM indicates past exposure to HEV. The seroepidemiology study done in 1999-2000 showed that the average prevalence of anti-HEV IgG among normal population in Nepal is 38%.

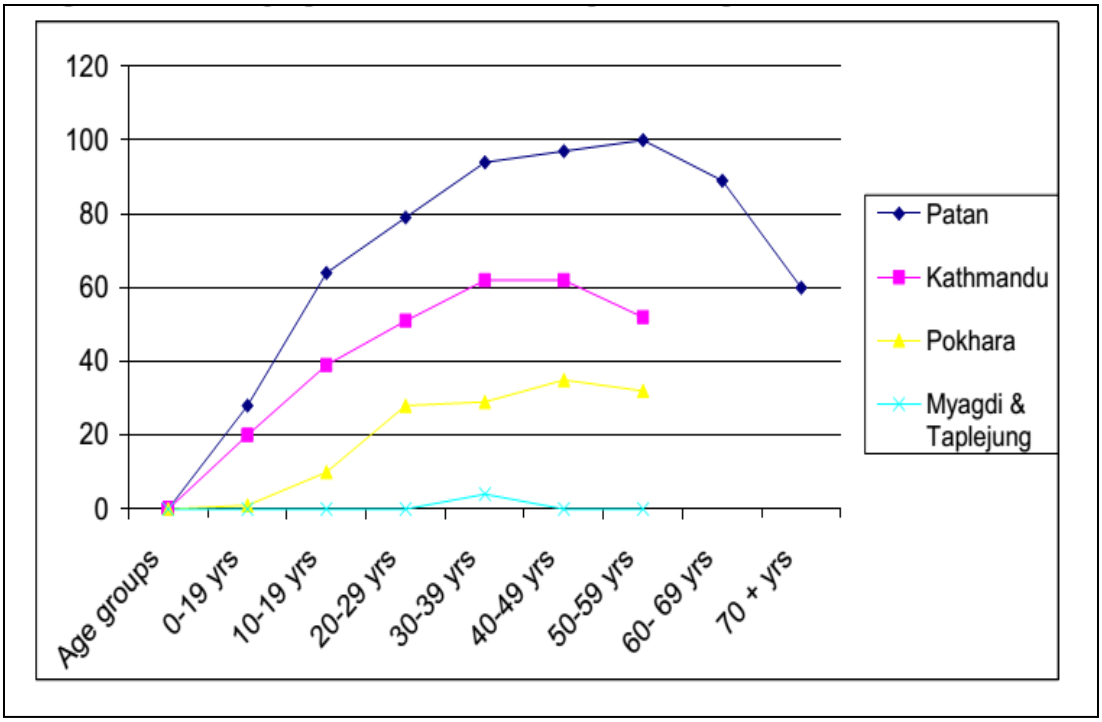

Fig 1.10 Anti-HEV IgG prevalence in different parts of Nepal (Shrestha SM, 2006)

It is about 16% in the age group of 1-9 years and increases to 42% at 40-49 years and then decline. This Prevalence is different according to geographical locations. It is more common in Kathmandu valley (38%), intermediate in major urban areas like Pokhara and Birganj (23%) and very low in rural areas, almost none in remote hilly areas like Myagdi and Taplejung (0%).Based on the seroepidemiology study, Kathmandu valley is designated as the hyper-endemic area, other urban areas endemic and rural areas as non-endemic area for hepatitis E.

1.2.7 HEV and the immune system

1.2.7.1 Humoral immune response

Immune responses, both humoral and cellular, can play a role in the pathogenesis of viral infections. The humoral response consists of antibodies that may neutralize the virus and play an important role in recovery from acute HBV infection. In HEV infection, both IgM and IgG antibodies appear concomitantly with the development of jaundice,

and persist for different periods of time. Although these antibodies appear to have neutralizing activity and their longevity and exact role in protection against HEV reinfection remains unclear (Schofield *et al*., 2000; Khuroo *et al*., 1993).

An immunoglobulin or antibody refers to proteins that bind to antigens in specific cases. Both IgM and IgG refer to a class of immunoglobulin. Antibodies are produced by the immune system to fight antigens like bacteria and viruses. IgM refers to those antibodies that are produced immediately after an exposure to the disease, while IgG refers to a later response. IgG is found throughout the body, mainly in most of the bodily fluids, while IgM is found mainly in the blood and lymphatic fluids**.** IgM antibodies are usually found in a human body after it has been exposed to a disease, IgG is the long term response of the body to a disease**.** It means IgM shows the recent infections while IgG shows the past infections of diseases. IgM is temporary and disappears after a few weeks then it is replaced by IgG but IgM is larger in size compared to IgG.

The concentration of IgM antibodies gradually decline after 3-6 months of the onset of an infection. As a result, the quantity of IgM antibodies in blood helps doctors diagnose the stage of an infection. IgG antibodies is the most abundant antibody, can cross the wall of blood vessels to fight viruses and persist for 2 to 13 years after the onset of an infection. To diagnose hepatitis E in a patient, doctors firstly measure the quantity of IgM HEV antibodies or significant increases of IgG antibodies in a patient blood. Presence of IgM antibodies in the blood of patients infected with HEV represents current or recent infection with HEV whereas presence of IgG antibodies in the blood of patients infected with HEV represents past infection with HEV.

1.2.7.2 Cellular Immune Response

In cellular immune responses, both innate and adaptive immunity, are important for viral clearance. Natural killer (NK) cells, natural killer-like T (NKT) cells and antigen-specific MHC class I-restricted $CD8^+$ cells have been shown to lyse virus-infected targets in several Hepatitis viral infections (Szomolanyi-Tsuda *et al.*, 2002). These immune

responses sometimes may be important in the pathogenesis of hepatotropic viral infections, because NK, NKT, and $CD8^+$ T cells are known to undergo preferential concentration in the liver compared with the peripheral blood (Doherty *et al*., 2003). In addition, infection with HBV or HCV is associated with massive recruitment of antigen-nonspecific inflammatory cells into the liver, which helps in viral clearance through the production of proinflammatory cytokines such as tumor necrosis factor-*α* (TNF-*α*) and interferon-*γ* (IFN-*γ*) (Guidotti *et al*.,2006). In contrast to HBV and HCV infection, there is little information about immune responses in HEV infection. In the peripheral blood of patients with acute hepatitis E, immune reactive cells that responded to a mixture of peptides representing parts of HEV proteins and to recombinant HEV ORF2 protein.(Aggarwal *et al*.,2006; Naik *et al*.,2002).

1.2.8 HEV Pathogenesis

1.2.8.1 HEV Infections in Human

HEV infection can cause acute liver disease which is mild and self-limited in the majority of cases. However, in some cases it can induce the so-called "Fulminant Hepatic Failure" (FHF) which is a severe acute hepatic disease with low chances of recovery. The non-specificity and diversity of the clinical symptoms may lead to misdiagnosed cases (Sherman *et al*., 2011).

In most cases the HEV is asymptomatic; in which patients typically clears the virus rapidly, while the symptomatic patient experiences clinical signs including anorexia, hepatomegaly, myalgia, jaundice and sometimes abdominal discomfort, nausea, vomiting, and fever.Clinical signs and symptoms including the incubation period can range from 15 to 60 days (Meng X.J, 2010).

1.2.8.2 Fulminant hepatic failure

Fulminant hepatic failure (FHF) or acute liver failure (ALF) which is acute hepatitis followed by encephalopathy within four weeks of the first symptoms. There is a loss of function of 80-90 % of the liver cells (O'Grady *et al*., 1993).

The mechanism of FHF pathogenicity related to HEV is not completely understood. The complications associated with FHF are hepatic encephalopathy, cerebral edema, coagulopathy, hepatic parenchyma necrosis, renal failure, pulmonary edema, cardiovascular disorders and coma (Alam *et al*.,2009).

1.2.8.3 HEV infection in pregnant woman

Hepatitis E in pregnant women is an explosive disease with elevated fatality rates. In comparison with other hepatitis viruses, HEV is most frequently associated with severe complications in pregnant women (Beniwal *et al*.,2003).

Fulminant hepatic failure was more common among HEV-infected women (55%) who were many times at higher risk than non-HEV infected women (20%); maternal mortality was also higher to fulminant hepatic failure in the HEV infected group (41%) vs. 7% in the non-HEV group (Patra *et al*.,2007).

The relationship between pregnancy and increased mortality rates up to 20% in HEV endemic regions; however, this relationship appears to be geographically dependent and may be associated with other underlying factors such as virus genotype or concurrent infections with other pathogens. Complications with concurrent HEV infection during pregnancy include death of both the mother and fetus, abortion, premature birth, and death of the baby shortly after birth (Hussaini *et al*., 1997).

Vertical transmission from the mother to fetus was reported in 33% of cases and HEV RNA was reportedly detected in human colostrum as well (Balayan *et al*., 1990). Unfortunately it is not understood well why pregnancy resulted in severe hepatitis E manifestation (Khuroo *et al*., 1995).

1.2.8.4 HEV in Animals

In the mid-nineties there was a search for an animal reservoir of HEV. After experimental infection swine feces, observed presences of HEV particles in the feces swine (Balayan *et al*., 1990).

In 1997, partial genomic HEV RNA fragments infecting swine were reported for the first time and phylogenetic analysis confirmed that both swine and human sequences were closely related (Meng *et al*., 1997b).

Domestic pigs and wild boars are now considered as the main reservoir for HEV genotypes 3 and 4 .However HEV RNA has been found in other animal species also such as deer, mongoose, rabbit, rat and chicken (Meng, 2010). In addition, anti-HEV antibodies have been found in various other animal species such as wild rodents, dogs, cats, cattle, sheep, goats and horses (Vitral *et al*., 2005).

The first evidence for zoonotic transmission of HEV was reported in association with the ingestion of deer meat. Genomic sequences of the viruses found in frozen deer meat matched 100 % to the ones recovered from HEV patients (Tei *et al*., 2003).

1.2.8.5 HEV Infections in immunocompromised individuals

HEV infections may prolong or chronic, it has been observed primarily among the patients with in compromised immune systems and often, organ transplant recipients and cancer patients like lymphoma and leukemia receiving immunosuppressive drugs. The course of disease also may progress to a chronic state with cirrhosis of the liver and persistence of viral shedding

1.2.9 Prevention and Control

In the developing countries good sanitation conditions such as access to clean water and sewerage systems are fundamental in the control of hepatitis E

outbreaks. For instance, the use of chlorination reduces the amount of fecal coli forms and contributes to the control of hepatitis E (Naik *et al*.,1992).

In developed countries the consumption of raw or undercooked meat and meat products of Virus reservoirs animals like swine, wild boar and deer should be avoided.

Few measures can be applied in order to prevent vertical transmission of HEV. The presence of HEV RNA and Anti-HEV IgG has been reported in colostrum, but HEV infected mothers can safely breastfeed. Close contact (mother-baby) should be avoided only if acute disease (with viremia) is present (Kumar *et al*., 2001).

At least two distinct recombinant HEV vaccines went to clinical trials. One vaccine is based on a recombinant capsid protein expressed via the baculovirus system using Spodoptera frugiperda (Fall armyworm) cells and produced by GlaxoSmithKline®(Shrestha *et al*.,2007)

The apparently most promising vaccine is called "HEV 239" and is based on a recombinant peptide corresponding to aa 368 to 606 of the capsid protein of a genotype 1 isolate. It is expressed in bacterial cells (E. coli) and produced by Wantai Biological Pharmaceutical®, China (Li *et al*., 2005a)

1.2.10 Diagnosis

Due to its clinical and epidemiological characteristics the diagnosis of HEV is may be difficult. The first assay for the detection of HEV was based on immune electron microscopy (Balayan *et al*., 1983). Afterwards different serological and molecular assays (RT -PCR and qRT-PCR) were invented. A proper diagnose of hepatitis E in humans should combine markers for liver function, the appropriate serological test and molecular detection. The results from serological tests should consider the epidemiological condition. For instance a positive antibody titer in an endemic region may be meaningless.

In general the Serological diagnosis includes the detection of IgG and IgM antibodies against HEV as well as HEV RNA in serum and feces by ELISA (Teshale E.H and Hu D.J, 2011). Sometimes, disease is diagnosed by Western Blotting techniques by detecting different proteins in HEV.

RT-PCR and qRT-PCR has also been employed for diagnosis of HEV. The first amplification of a HEV genome has taken place together with the first isolation of HEV cDNA from bile of an experimentally infected macaque using a random primer strategy (Reyes *et al*., 1990). Afterwards different RT-PCR setups with a number of primers were used in order to detect different regions of the HEV genome.

1.3 Research Design

1.3.1 Research Hypothesis

Hepatitis E virus infection is the one of leading causes of hepatitis worldwide and is associated with a high mortality rate in pregnant women. It is a major cause of food and water associated death in Southeast Asia, causing both sporadic disease and major epidemics. In Nepal especially capital city i.e. Kathmandu, there were 5 major outbreak of HEV affecting more than 40,000 individual and one HEV outbreak in Biratnagar affecting more than 6000 individual in 2014. Besides that, HEV infection has been observed frequently affecting people with immunocompromised. Although, there are few report of prevalence of HEV among PLHIV is higher that non HIV infected individual, we plan to study the prevalence and molecular details of Hepatitis E in the HIV infected individual. If the prevalence is higher in HIV infected individual; we might need to study the pathogenesis including immunological response and associated factor which contribute for the high prevalence in the PLHIV. At the end of the study we can suggest concern Government authority for vaccination to the HIV infected individual against HEV. This study can also give massage about geographical distribution of circulating

genotype and subtype in Nepal and its correlation with different variable like age, sex, CD4 count, viral load etc.

1.3.2 Research Objectives

General Objective

To study the seroprevalence and genetic characterization of HEV in HIV infected individual.

Specific Objective

- To study the immunological response (CD4 count) of HIV infected individuals
- To study the HIV viral load suppression by combination of ART regimen
- To study the prevalence of HEV among HIV infected individual
- To differentiate acute and chronicity of HEV among HIV infected individual
- Conformation of HEV ORF-1 and ORF-2 by RT-PCR
- Sequencing and phylogenetic analysis of HEV isolate

1.3.3 Research Plan

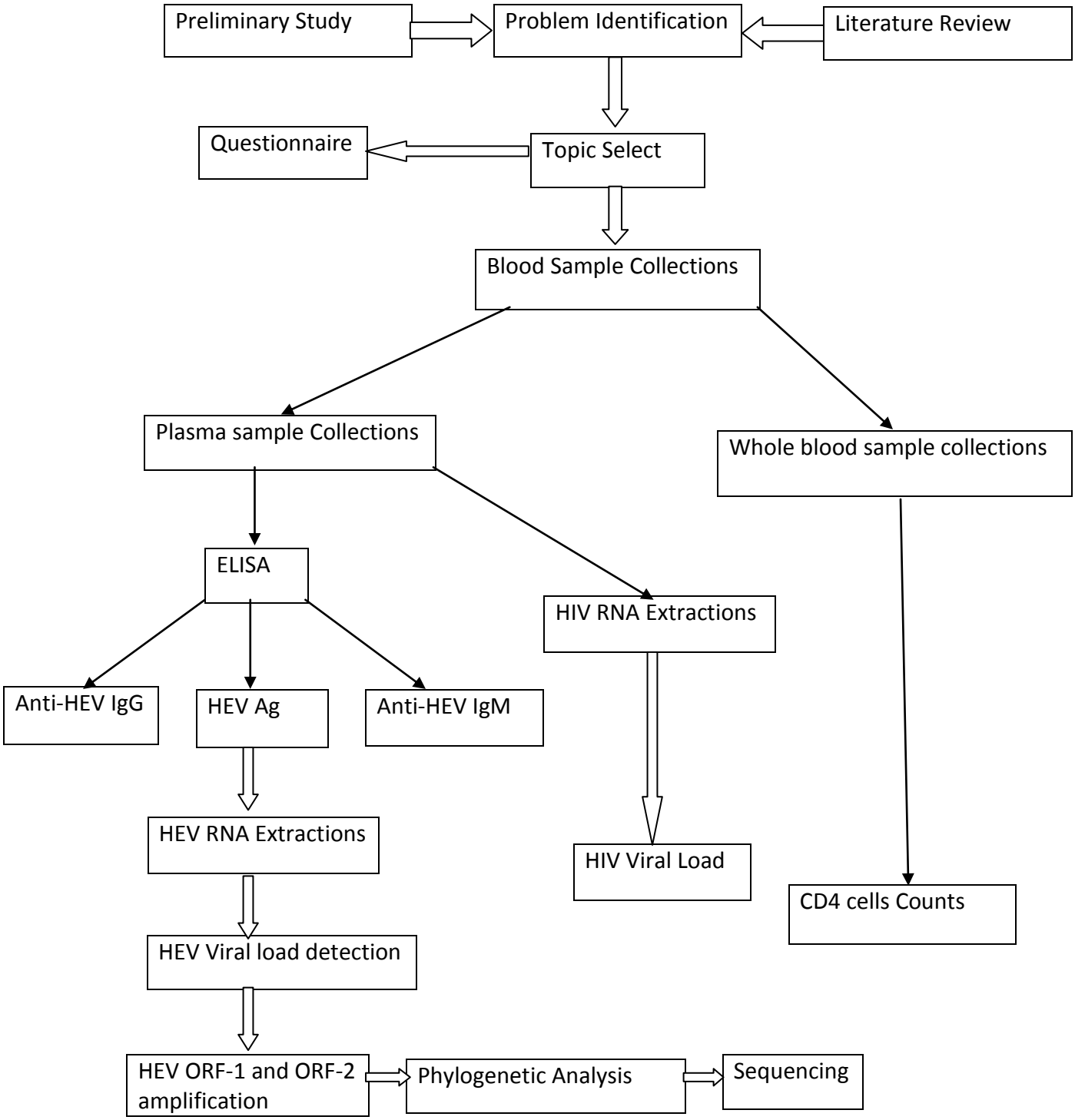

Figure 1.11 Research Plan

1.3.4 Rationale

The research is as based on the Nepalese blood samples; NPHL is the place where HIV infected patients all over the Nepal is visiting. This is the first study conducted in Nepal. Different studies show that HEV were more prevalence in developing countries than developed countries. Nepal is also the developing countries and poor sanitation problems leads to higher anti-HEV seroprevalence among the general population and population infected with HIV. HEV causes a major public health issue in Nepal and reported HEV as the most important cause of all the clinical types of hepatitis commonly found in Nepal.

Similarly, various researches showed that there is maximum probability of co-infection of HEV in patients infected with HIV. Previous massive outbreaks of HEV from adjacent areas of Nepal have been reported in 1981, 1982, and 2014.

Chapter II

LITERATURE REVIEW

Since the initial description of the Human Immunodeficiency Virus type I (HIV-1) in 1983 (Barré-Sinoussi *et al.*, 1983) and HIV-2 in 1986 (Clavel *et al*., 1986), these two viruses have been identified as the primary cause of Acquired Immunodeficiency Syndrome (AIDS).

Most replication competent HIV depend on three genes: group antigen (gag), polymerase (pol) and envelope (env) (Wong-Staal F, 1991).

Hepatitis E virus possess a positive strand RNA genome with a size of approximately 7.2 kb with three partly overlapping open reading frames (ORFs), a capped 5' end and polyadenylated 3' end (Mushahwar, 2008).

The association between HEV and human immunodeficiency virus (HIV) infections had been debated from 1990s based on serological studies (Montella *et al*., 1994).

HEV infections may prolong or chronic, it has been observed primarily among the patients with in compromised immune systems. Course of disease also may progress to a chronic state with cirrhosis of the liver and persistence of viral shedding. Currently, chronic HEV infection in immunocompromised individuals is an emerging and significant clinical problem (Kamar *et al*., 2008; Dalton *et al*., 2009).

Among immunosuppressed persons in industrialized countries, hepatitis E virus (HEV) is a cause of sporadic acute viral hepatitis and chronic hepatitis (Kuniholm *et al*., 2009).

Immunoglobulin (Ig) G antibody (anti-HEV IgG) prevalence in persons infected with HIV (32/452 [6.6%]) than in blood donors (27/1,473 [1.8%]) in Argentina (Thomas *et al*., 1997). However, in other studies, IgG anti-HEV prevalence did not differ between persons infected and non-infected with HIV (Bissuel *et al*., 1996).

Different Seroprevalence data of anti-HEV IgM in the patients infected with HIV seems to be controversial and differs significantly in various countries, ranging from 1.5% to 9.4% in france and the United Kingdom respectively (Maylin *et al*., 2012; Keane *et al*., 2012).

Between 2009-2011, five prevalence studies conducted in Europe in 93–735 persons infected with HIV reported prevalence rates of antibodies to HEV and/or HEV RNA ranging from 0% to 11.3% (Madejon *et al*., 2009;Sellier *et al*., 2011). However, HEV markers that were assessed as well as the clinical and immunological characteristics of patients infected with HIV differed in these studies.

In another studies, there was a 71% prevalence of HEV infection among HIV-positive adults, compared to a prevalence of 24% in HIV-negative adults (Michael, 2013). Additionally, hepatitis E cases have been reported since 2008 in patients infected with HIV and progression toward chronic hepatitis E was observed (Colson *et al*., 2009)

The ELISA is based on recombinant proteins from the ORF2 gene, which encodes the major capsid protein, and the ORF3 gene, which encodes a short protein of unknown function; from genotype 1 and 2 HEV strains expressed in *Escherichia coli* (colak *et al*., 2002; Favorov *et al*., 1992*).*

Viral nucleic acids were purified from 200µL of serum plus internal-control bacteriophage MS2 (Dreier J *et al*., 2005; Rolfe KJ *et al*., 2007).

Two sets of primers to amplify segments of ORF1 and ORF2 of HEV (as described by Wang et al., 1999).

Sequencing of PCR product of ORF-1 (550bp) and ORF-2 (191bp) based on the Sanger method (Sanger & Coulson, 1975; Sanger *et al*., 1977).

Assemble a 530 nucleotide (nt) ORF1 sequence and a 191nt ORF2 sequence corresponding to nt 80-609 and 6369-6559, respectively, of the Myanmar (B1) strain of HEV (GenBank Accession #M73218)(Tam A.W *et al*.,1991)*.*

Chapter III

MATERIALS AND METHODS

3.1 Study and sample collection site

National Public Health laboratory, Teku, Kathmandu, the centrally located diagnostic research laboratory in the capital city of Nepal and only one reference lab of Nepal Government was the study site for this research work. All the suspected patients from all over the country visit this research center for diagnosis of HIV. Blood samples were collected from those patients visiting NPHL. The whole laboratory work was conducted in the National Public Health Laboratory, Teku, Kathmandu, Nepal.

3.2 Sample size and Ethical Approval

Study was carried out from December 2014 to July 2015 and total 270 Blood samples were collected during the regular routine examination for this study having HIV positive were enrolled for this study. Random sampling procedure was followed during blood sample collection. The study was jointly reviewed and approved by the Research Committee of National public health laboratory and Nepal Health Research Council.

3.3 Inclusion criteria and Exclusion criteria

HIV patients confirmed with HIV rapid diagnostic tests and CD4 counts (<500/ml) at the National public health laboratory prospectively were enrolled in the study. The identity of the participants was kept blind. Patients' CD4 counts above 500 cells/ml were excluded in this research work.

3.4 Blood Sample Collections

3.4.1 Whole blood Sample Collection

Whole blood around 5 mL samples was collected into sterile EDTA blood collection tube in Sample Collection Ward in NPHL, Nepal. Random sampling was carried out for the sampling procedure based on their health complains. During sample collection, the clinical background information of the patients was kept along with their referral form. The samples were used for plasma separation and this whole blood was taken directly for CD4 counts by Flow cytometer.

3.4.1.1 CD4+ T-lymphocytes Assay

Human immunodeficiency virus (HIV) infection characterized by decrease and eventually depletion of $CD4^+$ T-lymphocytes (helper T cells) was studied by calculation of proportion of CD4+ T cells (Helper T cells) and CD8+ T cells (Inducer T cells)using immunophenotyping. The TruCount method having TriTEST 3-color (Becton, Dickinson and Company, California, USA) in TruCount tubes with a lyophilized pellet containing a known quantity of fluorescent beads like CD3 Fluorescein isothiocyanate (FITC)/CD4, phycoerythrin (PE)/CD45 and peridinin chlorophyll protein (PerCP) was used for cell population identification. The anti-coagulated whole blood was incubated with the monoclonal antibodies present in TruCount tubes (20 µL). The antibodies were targeted to the various cellular antigens (CD4, CD8 and CD45) that identify specific cell populations (phenotypes). Lysing buffer to the blood was used to remove red blood cells. The monoclonal antibodies were pre conjugated to fluorescent tags (Present in Trucount Tube) that emit light of a certain frequency when excited by a laser beam. The specimens were analyzed on a flow cytometer, Becton Dickinson (BD). A precise quantity of whole blood (50µL) was added to the tubes, and the lymphocytes were stained with TriTEST monoclonal antibodies (mAb) as instructed by manufacturer.

For each patient's sample, a BD Trucount tube with the sample identification Number was labeled. 20µl of BD Tritest CD3/CD4/CD45 reagent was pipetted in to the bottom of

the tube and ultimately 50 µl of well-mixed, anti-coagulated whole blood was pipetted in to the bottom of the tube. Cap the tube and vortex gently to mix and Incubate for 15 min in the dark at RT (20 °C to 25 °C).After incubation, 450 µl 1X BD FACS lysing solution to the tube was added. Cap the tube and vortex gently to mix and again Incubate for 15 min in the dark at RT. Finally, the sample was analyzed on the flow Cytometer.

3.4.2 Plasma Sample collection

The anticoagulated blood samples in blood collection tube were centrifuged at 3000 rpm for 5 min. After centrifugation, the upper supernatant clear layer, the blood plasma was transferred to labeled eppendrop tube. The collected plasma sample was taken in cold chain and was stored at -20^{o}C in a deep freeze until further procedure. This blood plasma was used for HIV viral RNA extractions for the Viral load by Real Time PCR and anti-HEV IgG, IgM and HEV Ag by ELISA.

3.4.2.1 HIV RNA Extraction

After collection and centrifugation, 1mL plasma (treated with anticoagulants) was stored at 2–8°C for up to 6 hours but for long-term storage, freezing was done at –20°C to –40°C. RNA was isolated from 140 µL of plasma sample using QIAamp Viral RNA Mini Kit according to the manufacture instructions. Frozen plasma samples werer not thawed more than once because repeated freezing and thawing leads to denaturation and precipitation of proteins, causing reduced viral titers and subsequently reduced yields of the isolated viral RNA. In addition, cryoprecipitate formed by freeze–thawing will cause clogging of the QIAamp membrane. Internal Control was supplied along with sample for the efficiency of sample preparation and downstream assay.

The sample was first lysed under highly denaturing conditions to inactivate RNases and to ensure isolation of intact viral RNA. Ethanol and Buffering conditions were then adjusted to provide optimum binding of the RNA to the QIAamp membrane, and the sample was loaded onto the QIAamp Mini spin column. The RNA binds to the membrane, and contaminants were efficiently washed away in two steps using two different wash buffers. High-quality RNA was eluted in a special RNase-free buffer, ready

for direct use or safe storage. The purified RNA is free of protein, nucleases, and other contaminants and inhibitors.

The 630 µl of prepared Buffer AVL containing carrier RNA was pipetted into a 1.5 ml microcentrifuge tube. 140 µl of Plasma was added in the microcentrifuge tube and mix by pulse-vortexing for 15 s. Incubate at room temperature (15–25°C) for 10 min and centrifuge the tube. After incubation, 560 µl of ethanol (96–100%) to the sample was added and vortexing for 15 s and Centrifuge. Transferred 630 µl of the solution from microcentrifuge tube to the QIAamp Mini column and centrifuge at 8000 rpm for 1 min. Place the QIAamp Mini column into a clean 2 ml collection tube, and discard the tube containing the filtrate. Carefully open the QIAamp Mini column, and repeated above process. For washing, 500 µl of Buffer AW1 was added and centrifuge at 8000 rpm for 1 min and the ultimately 500 µl of Buffer AW2 was added and centrifuge at full speed 14,000 rpm for 3 min. After washing, dry centrifuge at full speed for 1 min was done. Place the QIAamp Mini column in a clean 1.5 ml microcentrifuge tube and then discard the old collection tube containing the filtrate and 60 µl of Buffer AVE was added. Incubate at room temperature for 1 min and Centrifuge at 8000 rpm for 1 min. Eluted 60 µl of pure viral nucleic acid thus extracted was stored at -20°C in a deep freeze until further procedure.

3.4.2.1.1 HIV RNA amplification by Real Time PCR

For the viral RNA amplification and cDNA preparation, artus HI Virus-1 RG PCR Kit (QIAGEN, Germany) was used. During amplification uses the primers SK145 and SKCC1B to define a sequence of 155 nucleotides within the highly conserved region of the HIV-1 gag gene. The gag region encodes the group specific antigens or core structural proteins of the virion. The HIV-1 gag genes are generally about 1500 nucleotides in length and are located at the approximate positions 789-2290 in the HIV genome. The nucleotide sequence of the primers has been optimized to yield equivalent amplification of Group M subtypes of HIV-1.The HI Virus-1 RG Master A and B containing reagents and enzymes for the reverse transcription and specific amplification of a 155 bp conserved region of Gag gene of the HIV-1 genome was used as the process

was one step Real Time PCR. The amplified region was later subjected for direct detection in fluorescence channel green. Additionally, internal control was also used as supplied in kit to identify possible PCR inhibition which was detected by fluorescence channel orange in Rotor gene Q. External control was used to determine the amount of viral RNA.

The work was carried as mentioned by the manufacturer. First, there was preparation of Master Mix and then preparation of PCR assay.

Table: 3.1 Primers for HIV-1 viral load

Primers	**Sequence**
Forward SK145	5'-AGTGGGGGGACATCAAGCAGCCATGCAAAT-3'
Reverse SKCC1B	5'-TACTAGTAGTTCCTGCTATGTCACTTCC-3'

Table: 3.2 Preparation of master mix for HIV RNA amplification

Particular	**µl/Sample**
HIV-1 RG Master Mix A	12 µl
HIV-1 RG Master Mix B	18 µl
HIV-1 RG IC	2 µl
Total Volume	32 µl

Table: 3.3 Preparation of PCR assay for HIV RNA amplification

Particular	**µl/Sample**
Master Mix	30 µl
Sample(Template)	20 µl
Total Volume	50 µl

Table: 3.4 Standards positive controls for HIV RNA amplification

Particular	**Copies/ml**
HIV-1 RG OS 1	10
HIV-1 RG OS 2	100
HIV-1 RG OS 3	1000
HIV-1 RG OS 4	10000

The 12 µl of master mix A, 18 µl of master mix B and 2 µl of internal control were pipetted into each 200 µl PCR tube. Then 20 µl of the eluted sample RNA was added to each tube and mix well by pipetting up and down several times. Corresponding, 20 µl of the Quantitation Standards (HIV-1 RG QS 1-4) were used as positive controls and 20 µl of water used as a negative control and then carefully close the tubes. Set up the PCR cycle and according to the numbering PCR tubes were carefully kept in Rotor Gene 6000 Real Time PCR was done.

Thermal cycle programmed for 90 seconds at 95°C as initial denaturation, followed by 35 cycles of 30 sec at 95°C for denaturation, 60 sec at 50 °C as annealing, 30 sec at 72 °C for extension, and final extension at 72 °C for 5 min.

3.5.3 Serology and Molecular Method

3.5.3.1 Enzyme Linked Immunosorbent Assay (ELISA)

We tested 270 HIV specimens using highly sensitive and specific anti-HEV IgG, anti-HEV IgM, and HEV Ag assay kits (Wantai Biological Pharmacy Enterprise, Beijing, China). The assays were carried out according to manufacturer instructions, except that we substituted two each of 1:16 and 1:32 dilutions of the manufacturer-provided positive control for the undiluted positive control, and we did not repeat the tests. Plates were

processed using an automated ELx50 plate washer (BioTek, Winooski, Vermont, USA) and optical densities were read using a VERSAmax ELISA microplate reader with SoftMax software (Molecular Devices, Sunnyvale, California, USA).

3.5.3.1.1 Detection of HEV antigen and antibodies against HEV

The Plasma samples were tested for the anti-HEV IgG, anti-HEV IgM, and HEV Ag by enzyme-linked immunosorbent assay (ELISA) using Wantai anti- HEV IgG, anti-HEV IgM and HEV ELISA kits (China), according manufacture instructions. The optical density (OD) of each sample was read at 450nm after blanking. The cut-off value used for the anti-HEV IgG assay was 0.17, that for the anti-HEV IgM assay was 0.21, and that for the anti-HEV Ag assay was 0.19. Samples with A/C.O value less than 1 for anti-HEV IgG, IgM and anti-HEV AG are negative or non reactive for the assay and sample with A/C.O value greater than 1 value are positive or reactive for the test. But samples with A value to Cut-off ratio between 0.9 and 1.1 are considered borderline and retesting of these samples in duplicates is required to confirm the initial results.

A = Optical density of each Sample

Calculation of Negative Control (Nc):

$$Nc = \frac{Nc1+Nc2+Nc3}{3}$$

Calculation of Cut-off Value (C.O.):

C.O. = Nc + 0.16

⇧

ELISA factor according to Kit

3.5.3.1.1.1 HEV Antigen

3 wells as Negative Control, 2 wells as Positive Control and 1 well as Blank were marked. 20µl of Specimen Diluents in each well except in Blank was added and 50µl of Sample in each well except in Blank was also added. Cover the plate with the plate cover and incubate for 60 min at 37°C. After incubation, 100µl of HRP-Conjugate into each well except in Blank was added. Cover the plate with plate cover Incubate for 30 minutes at 37°C. After incubation, washed each well 5 times with diluted Wash Buffer, after washing turn down the plate onto Blotting Paper and tap it to remove any reminders. After that, 50µl of Chromogen A and 50µl of Chromogen B solutions into each well was added. Incubate the plate at 37°C for 15 minutes avoiding light, produces blue color in Positive control and each sample positive wells. After producing color, 50µl of Stop Solution in to each well was added and mix gently. Intensive yellow color developed where blue color was developed. Calibrate the plate reader with the Blank well, read the absorbance at 450nm. The Cut-off value was calculated and evaluates the results. (Note: read the absorbance within 10 minutes after stopping the reaction)

3.5.3.1.1.2 Antibodies to HEV (IgG and IgM)

3 wells as Negative Control, 2 wells as Positive Control and 1 well as Blank were marked. 20µl of Specimen Diluents in each well except in Blank was added and 50µl of Sample in each well except in Blank was also added. Cover the plate with the plate cover and incubate for 30 min at 37°C. After incubation, washed each well 5 times with diluted Wash Buffer, after washing turn down the plate onto Blotting Paper and tap it to remove any reminders then added 100µl of HRP-Conjugate into each well except in Blank. Cover the plate with plate cover and Incubate for 30 minutes at 37°C. After incubation, washed each well 5 times with diluted Wash Buffer, after washing turn down the plate onto Blotting Paper and tap it to remove any reminders. After that, 50µl of Chromogen A and 50µl of Chromogen B solutions into each well was added. Incubate the plate at 37°C for 15 minutes avoiding light, produces blue color in Positive control and each sample positive wells. After producing color, 50µl of Stop Solution in to each

well was added and mix gently. Intensive yellow color developed where blue color was developed. Calibrate the plate reader with the Blank well, read the absorbance at 450nm. The Cut-off value was calculated and evaluates the results.

3.5.3.1.1.3 HEV Viral load detection

A subset of 12 HIV specimens with positive HEV antigen tests was selected for HEV viral detection by Real Time PCR .Viral nucleic acids were purified from 200µL of serum using the MagNA Pure LC 2.0 robotic instrument with the MagNA Pure LC Total Nucleic Acid Kit - High Performance (Roche Diagnostics, Indianapolis, Indiana, USA).HEV RNA was then amplified from total nucleic acid eluates using qRT-PCR.

3.5.3.2 HEV ORF-1 and ORF-2 detection by RT-PCR

A subset of 2 HIV specimens with positive HEV viral load was selected for confirmation of ORF-1 and ORF-2 because we sequenced ORF-1 and ORF-2 regions, so there must be amplification of ORF-1 and ORF-2. Formation of cDNA from extracted HEV RNA in the first step and amplification of cDNA in the second step of PCR. During second step PCR, we amplified segments of ORF1 and ORF2 using two sets of degenerate primers.

Amplicons were sent to the Johns Hopkins Genetic Resources Core Facility (GRCF), where they were sequenced on an Applied Biosystems 3730 DNA Analyzer (Life Technologies Corp., Grand Island, NY, USA) using the Sanger method. Contiguous sequences from ORF1 and ORF2 of the HEV genome were assembled from sequence fragments returned by the GRCF using Codon Code Aligner (CodonCode Corp., Centerville, MA, USA).

BLASTN v.2.3.0 (Altschul *et al*., 1997) was used to identify highly homologous sequences of other known HEV strains available in the GenBank entries. We used MEGA v.6 software (Tamura *et al*., 2013) to align the sequences and to carry out phylogenetic analyses using the maximum-likelihood method.

3.5.4 Nucleic acid Amplification

3.5.4.1 Oligonucleotides

For the HEV ORF-1 and ORF-2 amplification of HEV 100 pmol/µl, following primers were used. In ORF-1 and ORF-2 reason, we used two sets of degenerate primers.

Table: 3.5 Primers used in ORF-1 of HEV molecular Diagnostic

Primer Name	**Sequence (5'→ 3')**	**Location**	**Amplicon size**
(np ex-fw+rev)	GGTGGTTTCTGGGGTGAC	nt3898–3917	550

Table: 3.6 Primers used in ORF-2 of HEV molecular Diagnostic

Primer Name	**Sequence (5'→ 3')**	**Location**	**Amplicon size**
Forward(gt3-OS)	TGATTCTCAGCCCTTCGC	nt3961–3980	191
Reverse (gt3-OAA)	AGGGGTTGGTTGGATGTA	nt4477-4497	191

3.5.4.2 HEV Viral cDNA preparation by reverse transcription

The cDNA were prepared from extracted viral RNA of plasma samples of HIV positive patients. In the reverse transcription process, the complimentary DNA was formed from RNA with the help of the enzyme reverse transcriptase. Initially, a pre-master mix was prepared using RNAse free water in the 0.2ml PCR tube. Reverse primer and RNA template was added to 13 µl of the pre-mix in the 0.2ml PCR tube, and placed in Thermocycler at 45ºC for 60 minutes. At the end of the reaction cycle the mixture was chilled at 4° C.

Reagents and protocol for reverse transcription.

Table: 3.7 Preparation of master mix for cDNA amplification

Pre-master Mix	
Reagents	**Single Reaction(µl)**
10X RT buffer	2
Nuclease free water	8.7
Multiscribe reverse transcriptase	1
25XdNTPs(100mM)	0.8
RNAse inhibitor	0.5
Total	13

Table: 3.8 Preparation of reverse transcription reaction mixture for cDNA amplification

ORF-1 cDNA		ORF-2 cDNA	
Reagents	**Single Reaction(µl)**	**Reagents**	**Single Reaction(µl)**
Pre-master mix	13	Pre-master mix	13
Template RNA	2.5	Template RNA	2.5
Primer(np-ext-rev)	2	Primer(gt3.OAA)	2
Total	17.5	Total	17.5

3.5.4.3 HEV viral cDNA amplification by Polymerase chain reaction (PCR)

The PCR is a molecular technique to amplify copies of a certain DNA fragment. It consists in several cycles of denaturation, annealing and elongation. Once DNA will separate in to single strand after denaturation, the primers will bind or anneal to a specific region of template DNA at a determined temperature, the Taq polymerase enzyme amplify number of copies from first template DNA. These three steps will be repeated several times (cycles) in the interest of increase the amount of DNA molecules.

After the reverse transcription was carried out, the PCR mix was added to the 0.2ml tube containing the cDNA. PCR was carried out on extracted RNA samples to identify HEV sequences using two sets of degenerate oligonucleotide primers in the segment of

open-reading frame 1 (ORF-1) and segment of open- reading frame 2(ORF-2) as described in Tables **(3.5 and 3.6).**

Reagents and protocol for RT PCR

PCR was carried out in a 25 µl reaction volume with 13 µl nuclease free water, 4 µl 5 x buffers, 25µM of 2.5 µl of each primer, 2.5 µl cDNA, 10mM of 0.4 µl dNTPs, and 5U of 0.1 µl Tag Polymerase. Thermal cycle programmed for 90 seconds at 95°C as initial denaturation, followed by 35 cycles of 30 sec at 95°C for denaturation, 30 sec at 55 °C as annealing, 90 sec at 72 °C for extension, and final extension at 72 °C for 5 min.

Table: 3.9 Preparation of PCR Mixture for ORF-1 segment

S.N.	**Reagents**	**For Single Reaction(** µl **)**
1	Nuclease free H2O	13
2	Primers (np ex-fw+rev)	5
3	5x Buffer	4.0
4	dNTPs	0.4
5	cDNA	2.5
6	Tag Pol	0.1
	Total Volume	25.0 µl

Table: 3.10 Preparation of PCR Mixture for ORF-2 segment

S.N.	**Reagents**	**For Single Reaction(**µl)
1	Nuclease free H2O	13
2	Forward Primer(gt3-OS)	2.5
3	Reverse Primer (gt3-OAA)	2.5
4	5x Buffer	4.0
5	dNTPs	0.4
6	cDNA	2.5
7	Tag Pol	0.1
	Total Volume	25.0 µl

Agarose gel electrophoresis and gel documentation

The amplified product was run in 1.5 % agarose gel made in 1X TAE buffer at 100 V using 400mA for 35 min and visualized under UVP-Gel Doc-It ™ imaging System. Electrophoresis chamber was filled with TAE buffer containing 0.01% ethidium bromide. 8 µl of PCR product was mixed with 2 µl of loading buffer, and 8 µl added in each slot.

3.5.4.4 Sequencing

PCR product which showed positive to HEV ORF-1 and ORF-2 amplification , were sent to the Johns Hopkins Genetic Resources Core Facility (GRCF), where they were sequenced on an Applied Biosystems 3730 DNA Analyzer (Life Technologies Corp., Grand Island, NY, USA) using the Sanger method. Before sequencing, the products obtained after PCR amplification were cleaned up in order to remove un-incorporated dNTPs and residual primers was done using MACHEREY-NAGEL PCR clean-up Gel extraction kit according to manufacturer's protocol.

Table: 3.11 Primers for amplify the ORF1/ORF2 region for sequencing.

Primer name	Sequence (5' – 3')
DAFGR1F	TCAATATGCTGAACGCGCGAGAAACCG
HEVmR2	GCNCCTTCDGMNGACATCC
ORF1 WANG	CTGGTTCCGTCTCAGTGATCCGGGGG
ORF1 ORT	AACGCCACAAGGGCCATGAACA
ORF2 OOTTS	TGCTGGTAACATCATCATGAGACAGAGCG
ORF2 OFT	CTCTGTTGTCTTAAACAAGAGAGGTC

Reagents and protocol for Sequencing

Table 3.12 Preparation of PCR mixture for sequencing

S.N.	Reagents	For Single Reaction
1	Nuclease free H2O	4.2 µl
2	Template	2.0 µl
3	5x Sequencing buffer	2.0 µl
4	310 Mix	1.0 µl
5	Primer	0.8 µl
	Total Volume	10 µl

Table: 3.13 PCR conditions employed for Sequencing

Step	temperature	duration	cycles
Initial Denaturation	94°C	5 min	1 cycle
Denaturation	94°C	15 sec	35 cycles
Annealing	50°C	10 sec	
Extension	60°C	2min	
Final Extension	60°C	4min	
Hold	4°C	∞	

BLASTN was used to identify highly homologous sequences to the epidemic strains in GenBank. We also searched GenBank using keywords to locate and obtain sequence data for other Nepali and South Asian HEV isolates of HEV, as well as more distant HEV isolates, including representatives of the four genotypes known to affect humans. Sequences were aligned using ClustalW.

3.5.4.5 Phylogenetic analysis

Phylogenetic analysis was performed using the MEGA 6.0. Phylogenetic distances were calculated (Kimura two-parameter method) and trees generated based on the neighbor-joining. Branch lengths are proportional to genetic distances

Chapter IV

RESULTS

In this research work, Nepalese population was found to be infected and/or asymptomatic carrier with HEV. The study subjects were analyzed on the basis of sex, Age, CD4 count, viral load, ART Regimen and opportunistic infections.

4.1 Geography, Sex and Age based Result

The patients enrolled were found of 24 districts of Nepal. They were of 166 Male and 104 female of different age group of 15 adolescence, 163 adult and 92 old as mentioned in the table below (Table **4.1** and **4.2**).

Table: 4.1 Geography, Sex and Age based study.

Parameter	Participants
Districts	24
Sex	
Male	166
Female	104
Age	
<20 Y	15
20-40 Y	163
>40 Y	92

Table 4.2 Geography location of Participants

S.N.	Districts	No. of Individuals	S.N.	Districts	No. of Individuals
1	Kathmandu	48	13	Gulmi	1
2	Nawalparasi	24	14	Janakpur	3
3	Rupandehi	20	15	Bardiya	2
4	Bhaktapur	18	16	Banke	3
5	Chitwan	30	17	Sunsari	9
6	Lalitpur	29	18	Palpa	1
7	Dang	4	19	Parsa	21
8	Gorkha	6	20	sarlahi	4
9	Arghakhanchi	2	21	Rautahat	6
10	Bara	7	22	Nuwakot	10
11	Dhanusa	4	23	Dhading	8
12	Mahottari	3	24	Kaski	7
Total				**24**	**270**

4.2 CD4 count

CD4 count detection was the inclusion criteria of our study. Only blood samples with CD4 counts<500cells/ml were included in the study. Total 0f 270 patients with CD4 counts less than 500cells/ml enrolled in the study, of which only 11 patients were detected CD4 counts<100 cells/ml, 97 patients detected CD4 counts100-300 cells/µL and 162 were detected CD4 counts 301-499 cells/ml.

Table: 4.3 CD4 counts of individuals

CD4 Counts	No. of Participants
<100 cells/ml	11
100-300 cells/ml	97
301-499 cells/ml	162

4.3 HIV Viral Load

Out of 270 patients enrolled in our study, of which only 100 patients followed-up the ART Regiment after doing Viral Load evaluation. During viral load evaluation, only 21 patients were showed viral load more than 400 copies/ml because the Real Time PCR used in our study detects viral load only >400copies/ml. However, 79 patients were not viral load at detectable level by PCR machine but they had must certain amount of viral load which were less than 400 copies/ml shown in following table(4.3).

Table: 4.4 HIV viral loads of the individual

Viral Load	Number of Participants
Not Detected(<400 copies/ml)	79
>400 copies/ml	21
Total	100

During viral load evaluation, HIV RNA was detected in the form of HIV RNA copies and then converted to HIV RNA copies/ml because viral load is in copies/ml. 36 well plate Real Time PCR machine was used, following result were obtained shown in table….We used four external controls; 10.100,1000 and 10000 RNA Copies. Out of 36 patients only

8 patients were viral load >400 copies/ml or detected by PCR machine with particular CT value was observed shown in following table (4.4). As like, such type of results were obtained for remaining 66 patients.

Table: 4.5 HIV viral loads with CT value

S.N.	Viral RNA Copies	RNA Copies/ml	CT Value
S1	10 (10)	4,286	27.70
S2	98 (100)	42,857	30.84
S3	1040 (1000)	428,570	34.14
S4	9709 (10000)	4,285,700	37.33
4	1	429	47.04
7	32	13,714	42.47
11	35	15,000	35.71
15	1	429	35.58
16	1	429	43.17
32	1	429	45.20
34	2	857	47.13
35	1	429	41.20
Remaining	ND	< 400	-

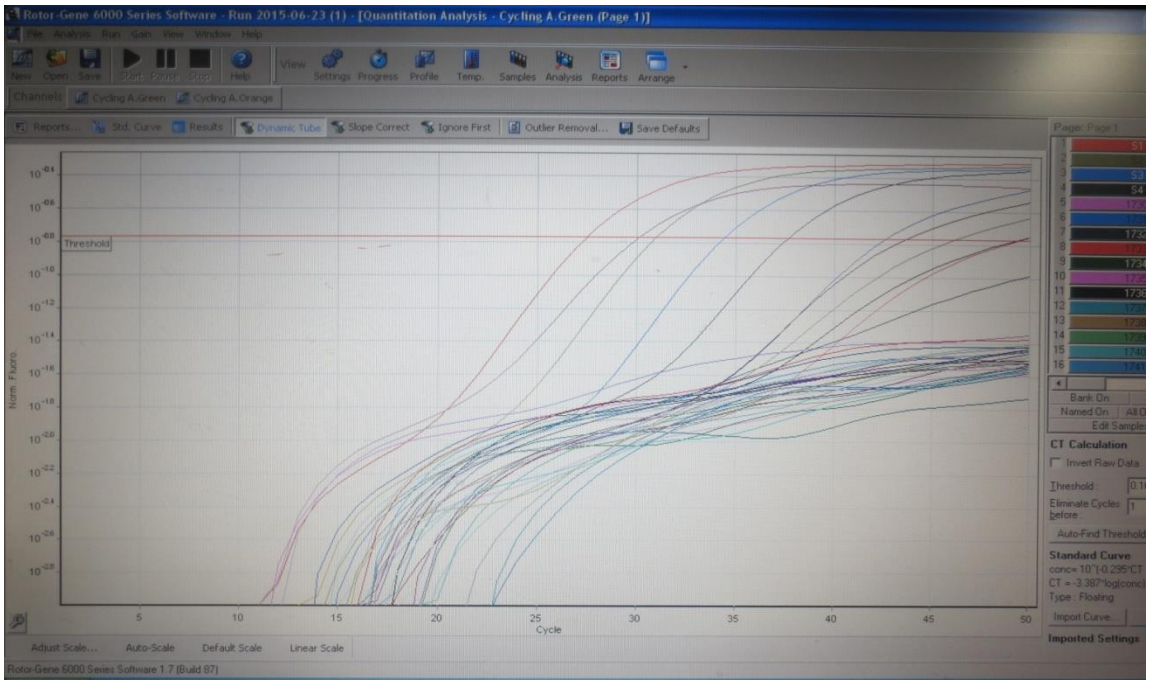

Fig 4.1 Fluorescence curve of viral load

The fluorescence curve drawn number of cycle Vs RNA Copies shown by Real Time PCR for these 36 patients viral load shown in Figure **4.1.** A patient whose viral load is high which have CT value is low and patients whose viral load is low then their CT value is high.

4.4 Enzyme Linked Immunoassay Sorbent (ELISA)

The study of total of 270 patients with HIV infection included in the study found that 32% (n=87) patients were positive to anti-HEV IgG, and 6% (n=17) patients with positive to anti-HEV IgM. However, only 4% twelve patients (4%) were observed HEV Ag Positive. The evaluation was done taking the cutoff value of IgG and IgM were 0.19 and Ag was 0.21 OD.

Table: 4.6 Calculation of Cut-off Value of IgG, IgM and Ag

Particular	Negative Control	Cut-off Value
IgG	0.03 (< 0.03)	0.03+0.16 = 0.19
IgM	0.05	0.05+0.16 = 0.21
Ag	0.03 (< 0.03)	0.03.0.16 = 0.19

4.4.1 Prevalence of anti– HEV IgG, IgM antibody and HEV Ag with Gender, Age Group and CD4 cell count

A total of 270 patients with HIV positive were enrolled in the study. Of these, 61 %(n=166) were male with a mean age of 38 years (range 4–69 years) and 39 %(n=104) were female with mean age of 37 years (range 4-75 years). The prevalence of HEV in patients infected with HIV by Sex, Age and CD4 cell count and demographic data are shown in table (4.6).

Table: 4.7 Prevalence of Anti–Hepatitis E Virus IgG, IgM and HEV Ag by Gender, Age Group and CD4 cell count

		← Positive →			← Negative →	
Parameter	**N=270**	**Anti-HEV IgG (%)**	**Anti-HEV IgM (%)**	**HEV Ag (%)**		**Mean Age**
No.of patients	270	87(32)	17(6)	12(4)	170(62)	37
Male	166(61)	58(35)	10(6)	6(4)	95(58)	38
Female	104(39)	29(28)	7(7)	6(6)	42(59)	36
CD4 cell Count						
<100 cells/µL	11(4)	4(36)	3(27)	3(27)	1(9)	34
100-300 cells/µL	97(36)	31(32)	6(6)	4(4)	58(59)	39
301-499 cells/µL	162(60)	52(32)	8(5)	5(4)	102(62)	36
Age						
<20 Y	15(6)	4(27)	0(0)	0(0)	11(73)	12
20-40 Y	163(60)	54(33)	13(8)	8(5)	92(56)	33
>40 Y	92(34)	29(32)	4(4)	4(4)	94(59)	48

4.4.1.1 Prevalence of Anti-HEV IgG, IgM and HEV Ag with Gender

In male, 35%(n=58) patients were positive to anti-HEV IgG,6%(n=10) were positive to anti-HEV IgM and 4%(n=6) patients were positive to HEV Ag whereas in female 28%(n=29) were positive to anti-HEV IgG, 7%(n=7) patients were positive to anti-HEV IgM and 6%(n=6) were positive to HEV Ag.

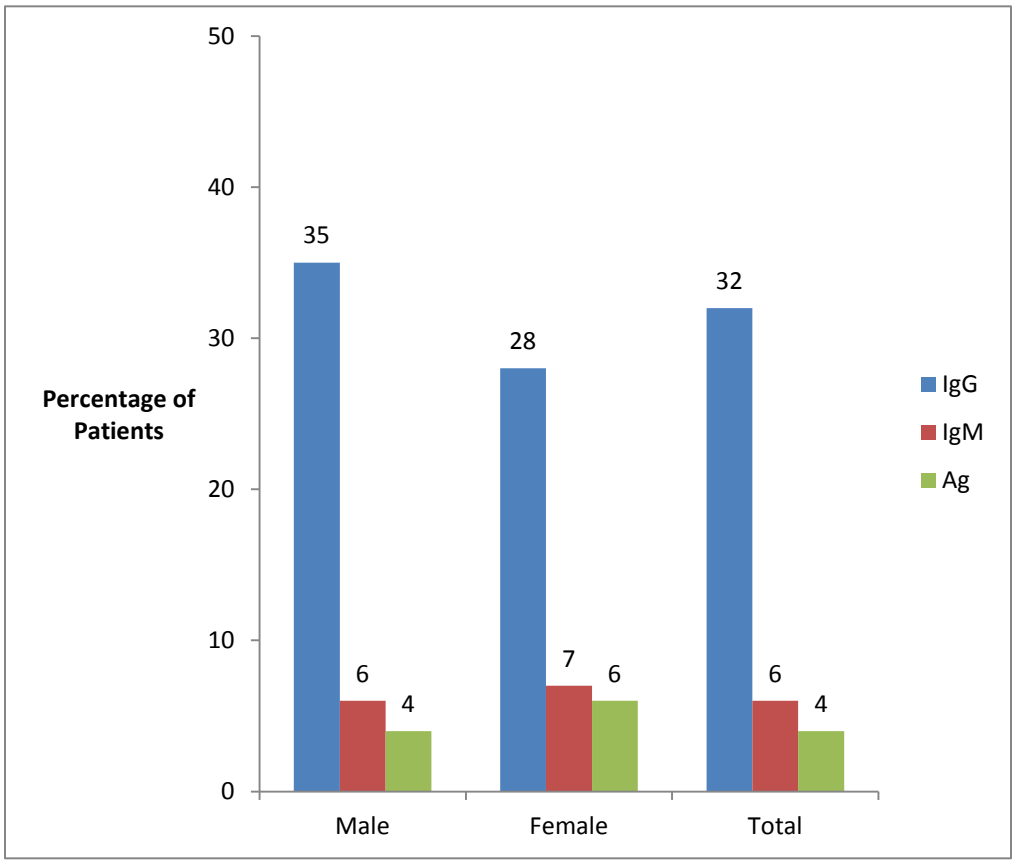

Fig 4.2 Prevalence of Anti–HEV IgG, IgM and HEV Ag with gender

4.4.1.2 Prevalence of Anti–HEV IgG, IgM and HEV Ag with CD4 count

CD4 count is the crucial marker in the HIV infected patients because it is the primary infected cells in HIV/AIDS. The CD4 count number <100 cells/mL was in 4 %(n=11) patients of 36 %(n=4) were positive to anti-HEV IgG, 27 %(n=3) were positive to anti-

HEV IgM and 27 %(n=3) were positive to HEV Ag. However, CD4 count 100-300 cells/mL was found in 36% (n=97) patients of 32%(n=31) were positive to anti-HEV IgG, 6%(n=6) were positive to anti-HEV IgM and 4%(n=4) were positive to HEV Ag .But, CD4 count more than 300 cells/mL was found in 60%(n=162) patients of 32%(n=52) were positive to anti-IgG, 5%(n=8) positive to anti-IgM and only 3%(n=5) were positive to HEV Ag .

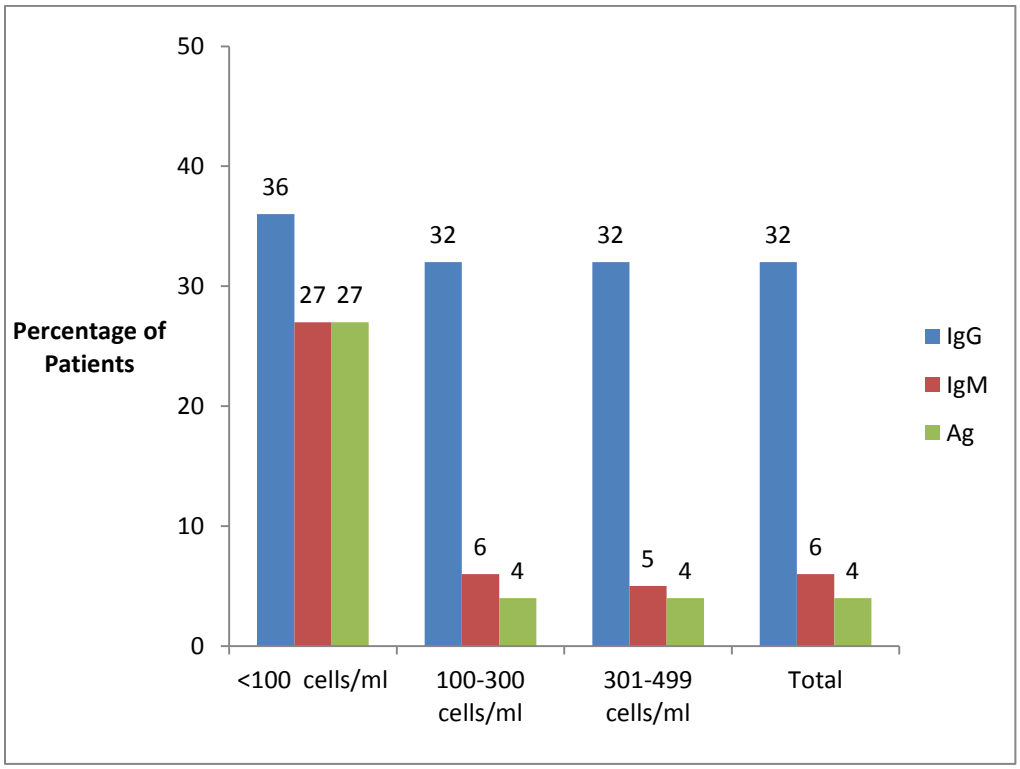

Fig 4.3 Prevalence of Anti–HEV IgG, IgM and HEV Ag with CD4 count

4.4.1.3 Prevalence of Anti–HEV IgG, IgM and HEV Ag with Age Group.

It has been observed that sero-prevalence of HEV also vary with different age groups and this is our most exciting result. In adolescence (age less than 20Y), 6 %(n=15) patients were present among which only 26 %(n=4) were positive to anti-HEV IgG but none of them was positive to anti-HEV IgM and HEV Ag. In adult (age ranges from 20-40), 60 %(n=163) patients were present of which 33 %(n=54) were positive to anti-HEV

IgG, 8 %(n=13) were positive to anti-HEV IgM and 5 %(n=8) were positive to HEV Ag. In higher age (age more than 40Y), 34% (n=92) patients were present among which 32 %(n=29) were positive to anti-HEV IgG, 4 %(n=4) were positive to anti-HEV IgM and 4 %(n=4) were positive to HEV Ag.

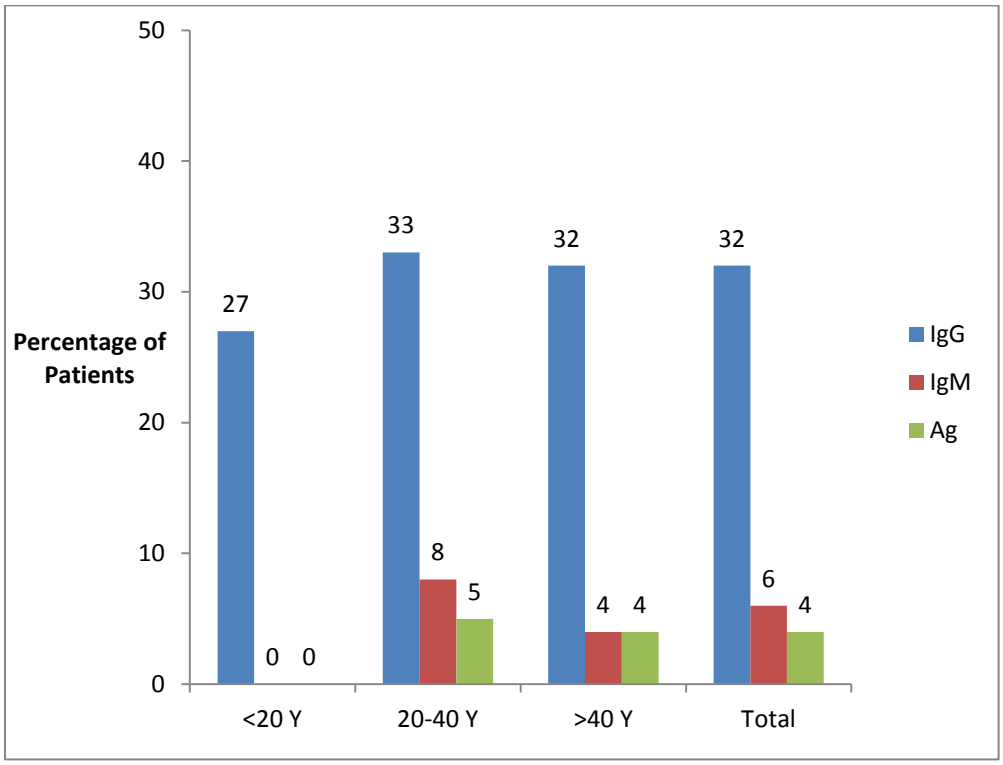

Fig 4.4 Prevalence of Anti–HEV IgG, IgM and HEV Ag with Age Group.

4.4.2 Prevalence of anti–HEV IgG, IgM and HEV Ag with Viral load, ART Regimen and Opportunistic infections

Out of 270 patients enrolled in our study, 100 patients were taken ART drugs as prescribed by Doctors. Of these 100 patients, most of them were taken 10 different combinations of first line drugs and few of 7 %(n=7) were taken five different combinations of second line drugs respectively. Maximum patients of 32 %(n=32) used the drugs combinations of azt/3tc/efv shown in following table **(4.8).**

Table: 4.8 Prevalence of Anti–HEV IgG, IgM and HEV Ag by Viral load, ART Regimen and Opportunistic Infections.

Parameter	N=100	Anti-HEV IgG(%)	Anti-HEV IgM(%)	HEV Ag(%)
Viral Load				
< 1000	87(87)	25(29)	4(5)	2(2)
> 1000	13(13)	5(38)	3(23)	2(15)
Opportunistic Infections	5(2)	1(20)	2(40)	1(20)
TB	3	1	0	0
CMV	1	0	1	0
Oral Candidiasis	1	0	1	1
ART Regimen				
1st Line	93(93)	27(29)	6(6)	4(4)
2nd Line	7(7)	2(29)	1(14)	0(0)

4.4.2.1 Prevalence of Anti–HEV IgG, IgM and HEV Ag with HIV Viral Load

The Real Time PCR (Corbet Rotter Gene) used during this study detects the viral copies number only more than 400 copies/ml. Out of 100 patients, 87%(n=87) patients were low viral load (Viral load less than 1000 copies/ml) among which 29%(n=25) were positive to anti-HEV IgG , 5%(n=4) were positive to anti-HEV IgM and only 2%(n=2) were positive to HEV Ag. However, only 13 %(n=13) patients were high viral load (Viral load more than 1000 copies/ml) of which 38 %(n=5) were positive to anti-HEV IgG, 23 %(n=3) were positive to anti-HEV IgM and 15 %(n=2) were positive to HEV Ag.

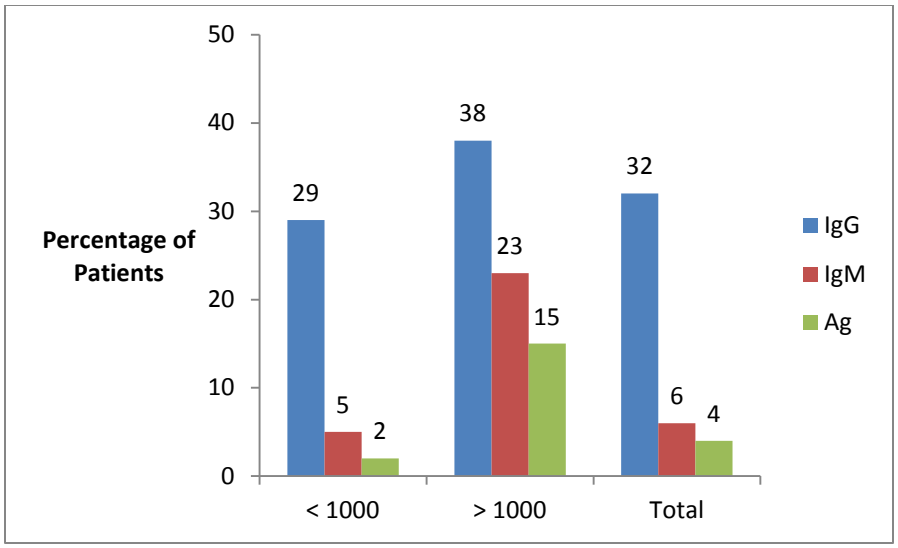

Fig 4.5 Prevalence of Anti–HEV IgG, IgM and HEV Ag with HIV Viral Load.

4.4.2.2 Prevalence of Anti–HEV IgG, IgM and HEV Ag with ART Regimen

Only 7%(n=7) patients of total enrolled in this study followed-up the second line drugs of which 29%(n=2) were positive to anti-HEV IgG, 14%(n=1) were positive to anti-HEV IgM and none of them were positive to HEV Ag. But, 93 %(n=93) patients followed-up first line drugs among which 29 %(n=27) were positive to anti-HEV IgG, 6 %(n=6) were positive to anti-HEV IgM and only 4 %(n=4) were positive to HEV Ag positive was found.

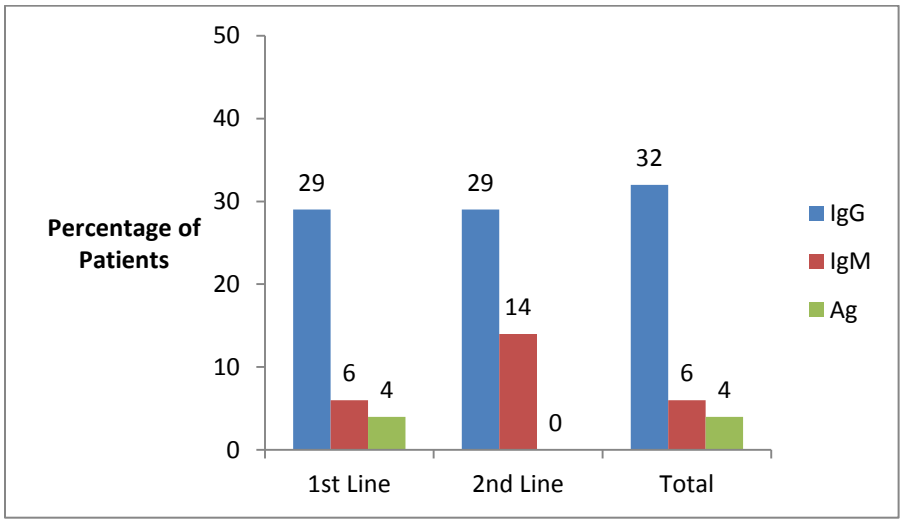

Fig 4.6 Prevalence of Anti–HEV IgG, IgM and HEV Ag with ART Regimen

4.4.2.3 Prevalence of Anti–HEV IgG, IgM and HEV Ag with Opportunistic infections

100 patients were using ART drugs, of these only in 2 %(n=5) patients were detected in three different kinds of opportunistic infections; they are TB, CMV and oral candidiasis. Among these 5 patients, 20 %(n=1) was positive to anti-HEV IgG, 40 %(n=2) were positive to anti-HEV IgM and 20 %(n=1) was positive to HEV Ag was observed. Co - infection with different diseases in these patients, seroprevalence of HEV occurs differently. In patient co-infected with Oral Candidiasis, there was prevalence of both IgM and Ag but prevalence of IgG was absent whereas the patients co-infected with CMV and TB only prevalence of IgM and IgG respectively.

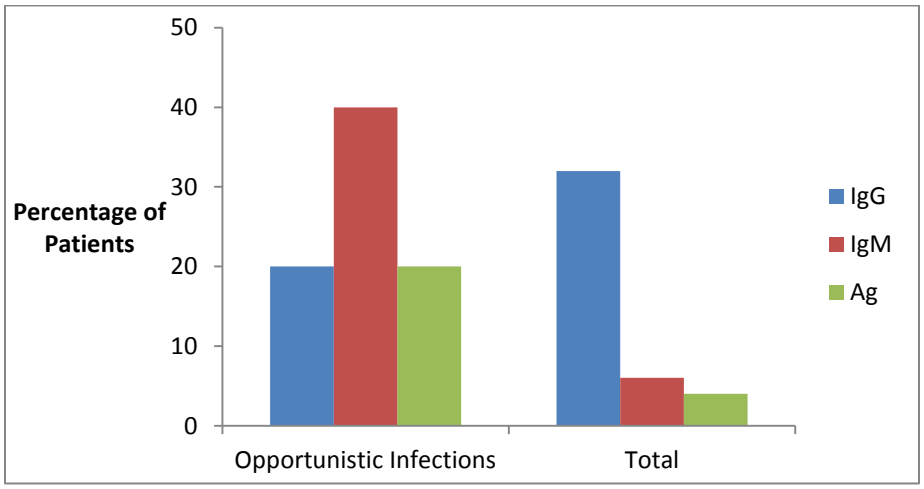

Fig 4.7 Prevalence of Anti–HEV IgG, IgM and HEV Ag with Opportunistic infections

4.5 Acute and Chronic Infection

Prevalence of anti-HEV IgG indicates the past infection, or chronic infection of HEV whereas prevalence of anti-HEV IgM and HEV Ag indicate the recent infection, or acute infection of HEV.

Table 4.9: Acute and Chronic infection of HEV

	N=270	Anti-HEV IgG (%)	Anti-HEV IgM (%)	HEV Ag (%)	HEV RNA(%)
No.of patients	270	87(32)	17(6)	12(4)	2(0.74)

4.6 ORF-1 and ORF-2 amplification

Of 12 HIV positive specimens with HEV antigen, 2 had detectable HEV viral load. Interestingly, these 2 specimens had sufficient RNA to attempt to isolate material for sequencing, ultimately which yielded expected products of ORF1 primer and expected products with ORF2 primer.

The successful amplification of Methyl transferase gene in ORF-1 and structural capsid protein gene in ORF-2 respectively, using two sets of primers, which was confirmed by agarose gel electrophoresis. Fragment size of approximately 530bp of Methyl transferase gene **(fig4.8**) and 191 bp of structural capsid protein gene **(fig4.9)** was amplified and verified by 100bp DNA ladder run along with it.

A single band at about 530 bp fragment in ORF-1 and 191bp fragment in ORF-2 indicated positive PCR amplicons. Remaining ones which showed non-specific bands were discard.

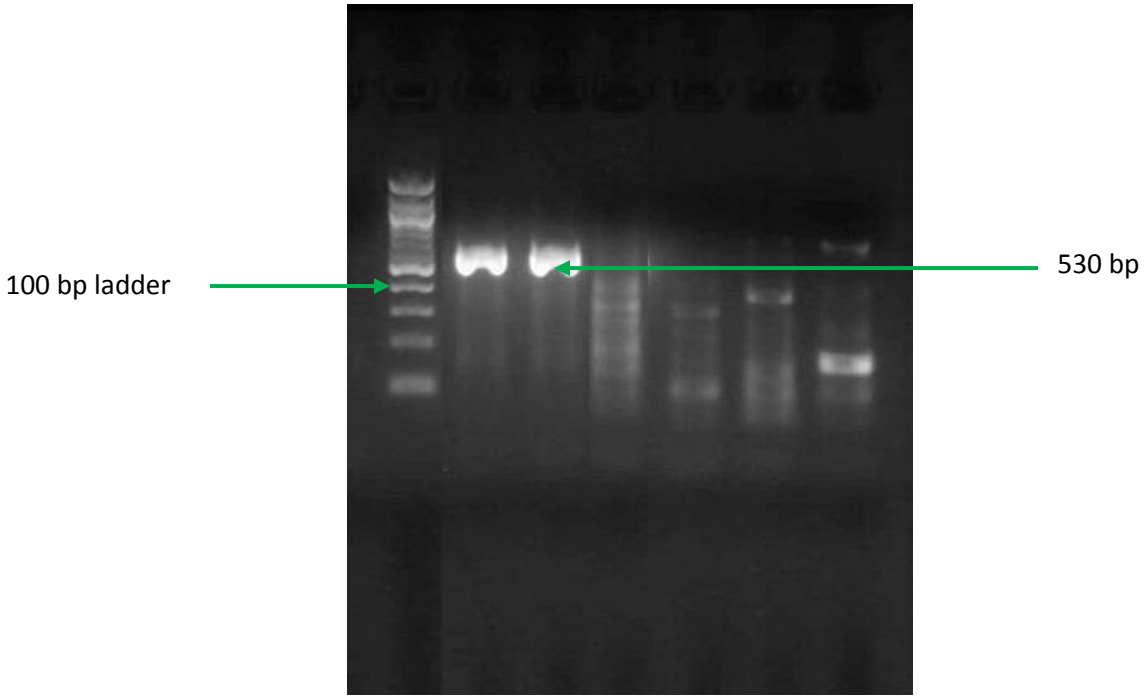

Figure 4.8 Agarose gel Electrophoresis (1.5%) of PCR product of Methyl transferase gene in ORF-1 fragment showing 100bp of Ladder.

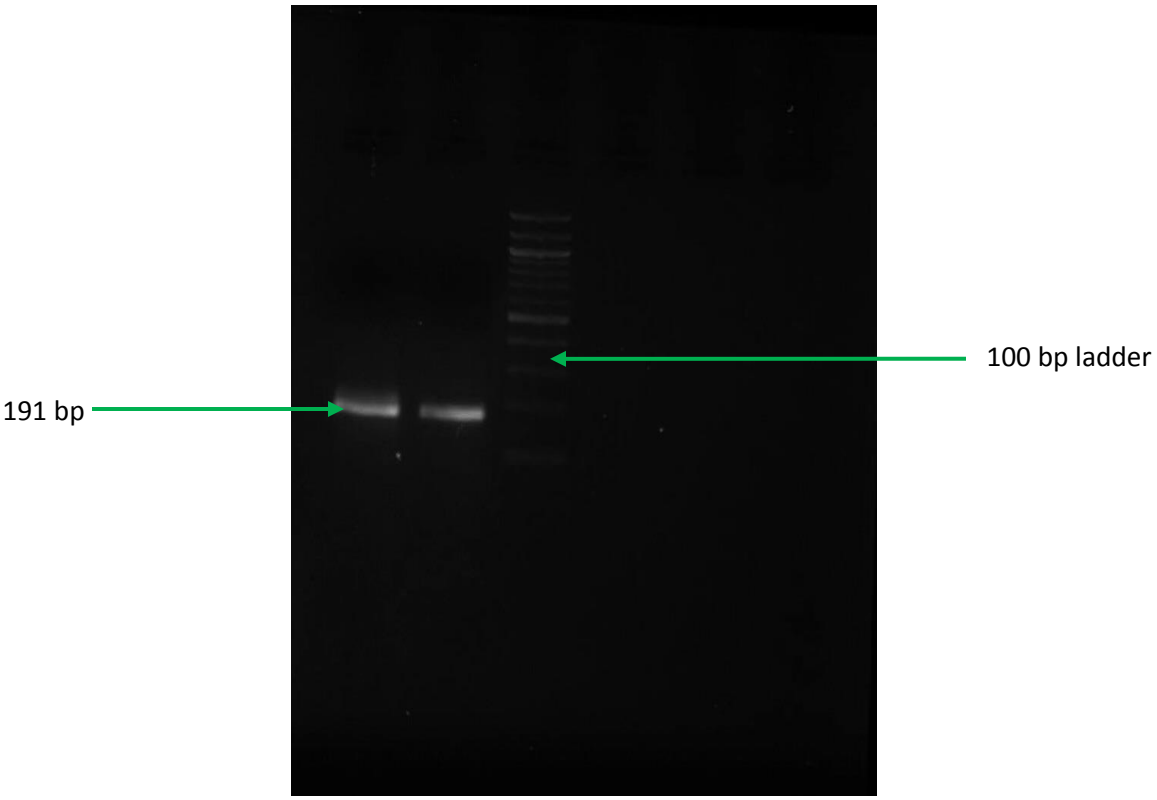

Figure 4.9 Agarose gel Electrophoresis (1.5%) of PCR product of structural capsid protein gene in ORF-2 fragment showing 100bp of Ladder.

4.6.1 HEV Sequence Identification

In additional to morphological character, Nucleotide based identification was performed by online library BLAST (www.ncbi.nlm.nih.gov/BLAST).We performed partial sequences of HEV ORF1 (methyl transferase gene, partial cds) and ORF2 (structural capsid protein gene, partial cds). Our sequence of 530 nucleotides in ORF-1 and 191 nucleotides in ORF-2 obtained after sequencing and was found to match more closely Indian Hepatitis E virus strain isolates than others. Also, isolates are closely related to Myanmar, Bangladesh, Pakistan, and China other than Nepali isolate .In ORF 1, Nepal isolates were maximum (94%) in sample 1 and (95%) in sample 2 similarity with Indian isolates respectively. Similarly, in ORF 2, our Nepal isolates were maximum (96%) similarity with Indian isolates in both samples.

4.6.2 Phylogenetic analysis

The PCR fragments of 530nt (bp) in ORF-1 (80 to 609) and 191nt (bp) in ORF-2 (Capsid position: 6369 to 6559) obtained from HEV positive samples after HEV molecular diagnosis were sequenced and phylogenetically analyzed. The level of divergence between the obtained sequences with other sequences is given in the following figures **(4.9).**Phylogenetic distances were calculated using the Kimura-2 parameter method. The phylogenetic tree was calculated using the neighbor-joining method. Branch lengths are proportional to the genetic distances.

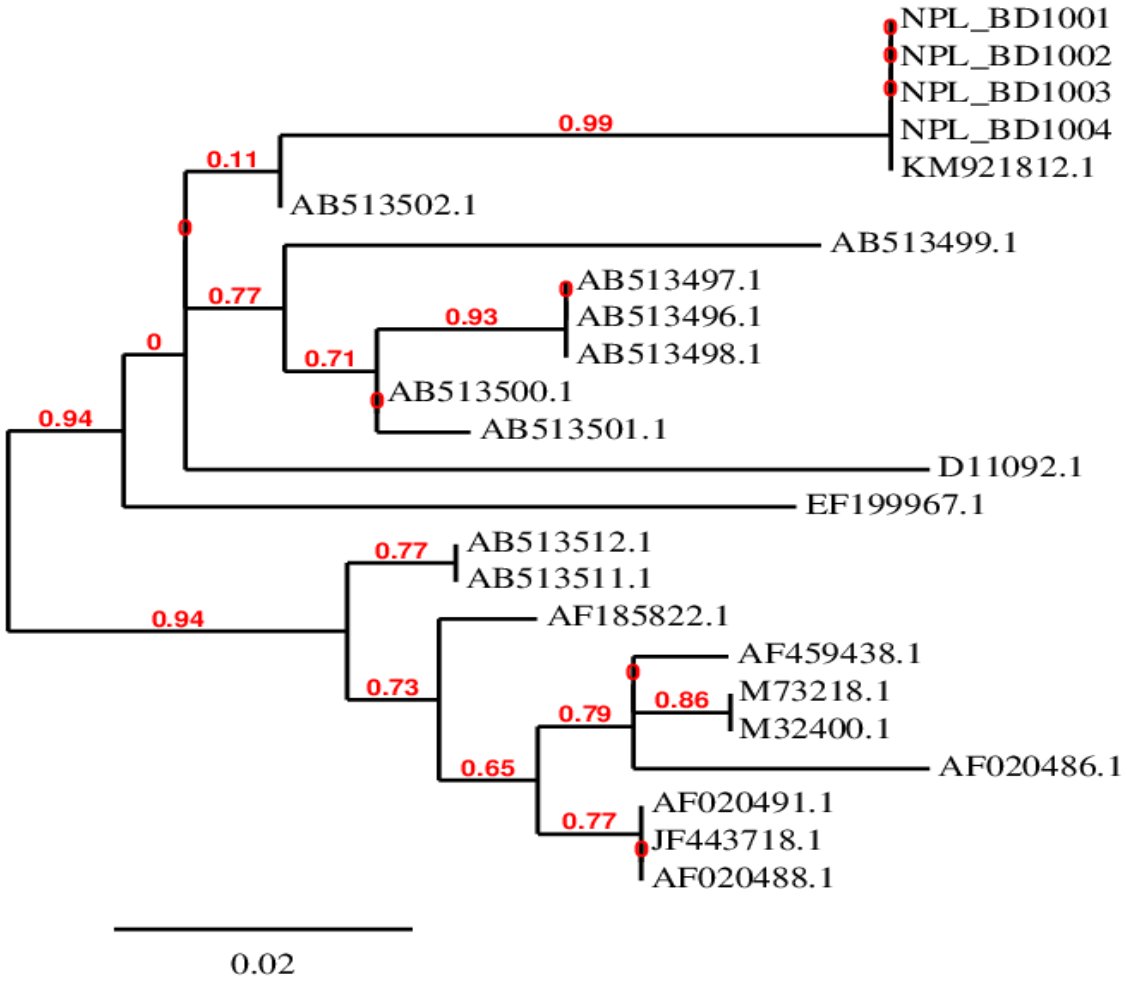

Figure 4.10 (Tree Figure): Phylogenetic analysis based on 550bp of the Methyl transferase gene in ORF-1 and 191bp of structural capsid protein gene in ORF-2

In both ORF 1 and ORF 2, the obtained HEV isolates sequence were closely located with Previous Nepalese and Indian HEV Isolates but only Previous Nepalese isolate were grouped together within same branch of our isolate, not with the any other isolates including Indian isolates. The closely linked isolate was HEV genotype 1a isolated from Nepalese and Indian Population; it was found 94% to 96% similarity.

Chapter V

DISCUSSION

Hepatitis E virus is a major cause of liver disease worldwide but its relationship to HIV has not been much attention so far, although several case reports indicate the clinical picture of HEV can be worsened and prolonged in HIV-infected patients (Dalton *et al*., 2009). The study shows relatively high anti-HEV IgG sero-prevalence in patients with HIV infection (Balayan *et al*., 1997; Fainboim *et al*., 1999).

We retrospectively analyzed the HEV sero-prevalence in a population of 270 HIV infected patients and found the HEV sero-prevalence of 32% anti-HEV IgG, with 6% anti-HEV IgM positive and 3% HEV-Antigen positive cases. The HEV seropositivity are higher compared to observations in a study conducted in Nepalese blood donors (Unpublished data) and blood donors in southwest Switzerland (4.9%) (Annatine, 2011). We found similar result with other reports in HIV infected groups from developed countries with substantial prevalence of anti-HEV IgM(6.6%) and HEV Ag(2.2%) but somewhat contrasting with anti-HEV IgG(10.4%) in Spain(Maria N, 2014),and same kind of result was observed in UK as well (FE Keane *et al*.,2010). Besides this, (Shrestha *et al., 2006)* reported 94% sero-prevalence of HEV during Biratnagar epidemic in 2014, which is far higher than our result. It is likely that this reflects differences in selection criteria; our patient population was non symptomatic, and we did not limit our sample to individuals with frank jaundice.

Our data clearly showed that there is no significant difference of prevalence of HEV in between male and female; prevalence of anti-HEV IgG in male slightly higher than in female but in the case of anti-HEV IgM and HEV Ag result is totally opposite whereas this is a low sero-prevalence completely different to that observed in pregnant women, which were not infected with HIV in Ghana (Andrew, 2009). The most striking finding of our study is the high HEV sero-prevalence observed in patients with adult age (33%) and which is strongly supported by the HIV infected patients in Africa, prevalence of anti-

HEV IgG in adult HIV infected Ghanese (45.3%) and Camerounese (14.2%) patients (Torsten Feldt *et al* .,2013). Similarly, the high (33%) sero-prevalence of anti-HEV antibodies in an adult HIV individuals in Nepal stands in marked support to the higher sero-prevalence observed in adults in Kathmandu (24.6%-66%)(Clayson ET *et al*.,1997; Izopet J *et al*.,2015) and elsewhere in South Asia (33.7%) in pregnant women in New Delhi (Begum N *et al.,*2009), 25-40% in Pune (Arankalle VA *et al*.,1995), 29.5% and 14.9% in urban and rural Vellore, and 22.5% in rural Bangladesh (Labrique AB *et al*.,2009). It has also observed that the prevalence of anti-HEV IgM and HEV Ag also higher in adult HIV infected patients as compared to adolescence and old. There was a suggestion that plasma samples positive for HEV were more likely to come from patients with CD4 counts <100 cells/µL of prevalence of anti-HEV IgG(36%), anti-HEV IgM(27%) and HEV Ag(27%) as suggested 45.3% prevalence of anti-HEV IgG in patients infected with HIV in Ghanese with low CD4 count (Torsten Feldt *et al* .,2013) and also Swiss data suggested the same(Kenfak-Foguena *et al*., 2011). In most endemic areas, an association has been observed between higher anti-HEV sero-prevalence and lower CD4 cell count (Kumar *et al*., 2004). Our data strongly suggested that the patients infected with HIV with low CD4 counts and patients in the age of adult have maximum chances of co-infection with HEV.

We designed the study as Prevalence of Anti–Hepatitis E Virus IgG, IgM and HEV Ag by Viral load, ART Regimen and Opportunistic infections. Our data did not demonstrate any strong relationship between HEV seropositivity of anti-HEV IgG and anti-HEV IgM with ART regimen used by HIV infected patients but it has show high prevalence of HEV Ag(5%) in patients who follow up the first line drugs, which totally absent in patients who follow up the second line drugs but our sample size would have to be higher to ascertain with confidence whether HEV Ag risk increases in patients who were using first line drugs Whereas in the case of viral load, our data have shown somewhat association of HEV seropositivity with HIV viral copies numbers in plasma of patients. In only 13 patients were high HIV viral load (Viral load more than 1000 copies/ml) of which 5(38%) were anti-HEV IgG positive, 3(23%) were anti-HEV IgM positive and 2(15%) were HEV Ag

positive, means if there is high quantity of HIV viral copies are present then there is high risk of co-infection of HEV and HIV.

The novel finding of our research showed that there was strong support for the highly chances of co-infection of HEV and HIV, where there was already occurrence of opportunistic infections by other diseases like TB, CMV etc in HIV infected patients. In the patients where other opportunistic infections occurred, highly prevalence of anti-HEV IgM(40%) and HEV Ag(20%) as comparison to patients where opportunistic infections were absent, which shows the recent infection of HEV. But the prevalence of anti-HEV IgG (20%) was somewhat lower than the other; strong support by this results as well because prevalence of anti-HEV IgG shows the past infection of HEV.

Our findings strongly propelled that the prevalence of anti-HEV IgG in all groups is comparatively higher. This might be due to Nepal is developing countries and highly endemic area for HEV because many of significant outbreaks of HEV were occurred in Nepal and these sero-prevalence data on HEV confirm that HEV should be included in the differential diagnose of patients with signs of (acute) hepatitis..

Plasma samples from two individuals were positive for HEV Ag, allowed amplification of both the ORF1 and ORF2 regions. The HEV sequence amplified from Nepalese HIV individuals shared a high degree of identity with HEV isolates from India and also similarity with HEV isolates from Myanmar, Bangladesh, Pakistan and China.

Phylogenetic analysis of the 530nt ORF1 sequence and 191nt ORF2 sequence suggested that the Nepal HEV isolates share a common ancestor with HEV strains from India identified as genotype 1a, including the Myanmar strain and a strain isolated during an outbreak in Madras, India (GenBank accession #X99441)(Lu L *et al*.,2006).

These sequences were approximately 95% to 96% identical to isolates from India, with the exception of 98% identity to the Indian isolate (Donati *et al*., 1997). Approximately 91% - 94% identity was shared with the Myanmar, Bangladesh, Pakistan, and Chinese isolates.

Cases of HEV in Nepal have historically been attributed to subgenotypes 1a (Shrestha SM *et al*., 2003). Molecular analysis of the HEV strain in HIV individuals in Nepal could help and clarify the strain by comparing with whole genome sequencing of HEV strain isolate from Nepal (Lhomme S *et al*., 2014; Wang H *et al*., 2010).

Limitation

This research work tried to imply for removal of utmost possible hindrances. Despite all our endeavors, few parameters remain as limitation to this work. The time constraint is the major factor to limit my ambitious study. Due to lack of chemical components like ALT, AST, we did not measure ALT or AST and ultimately we could not measure the function of liver.

Other limitation of our study is we had chosen the patient whose CD4 count is less than 500cells/ml and could therefore easily have missed patients with HEV with CD4 count >500.

The major limitation of the research work is the lack of time and budget due to which other research works related to HEV of the test samples could not be performed.

Chapter VI

CONCLUSION

Hepatitis E is the self limiting viral disease caused by Hepatitis E Virus, where poor sanitation in developing countries and eating the uncooked meat of different animals also the cause of HEV.

High sero-prevalence of HEV in immunocompromised individuals is comparatively higher than normal population. Our findings clearly showed that HEV infection is common in people's living with HIV/AIDS and a high prevalence of HEV in patients infected with HIV. In overall, it was found that out of 270 HIV patients; 32 %(n=87) patients were anti-HEV IgG positive and 6 %(n=17) were anti-HEV IgM positive but only 4 %(n=12) were observed HEV Ag Positive. There was highly seroprevalence of anti-HEV IgG, IgM and HEV Ag in patients infected with HIV with adults, low CD4 count and high HIV viral load; it is strongly associated with HEV sero-prevalence.

Most of the HEV infected individuals in Nepal was protected by mostly anti-HEV IgG antibody.

The HEV sequence amplified in this study from Nepalese HIV individulas shared a high degree of identity with Previous Nepalese and Indian HEV isolates found in Nepal and Indian of genotype 1a.

Recommendation

- Further immunological features of HIV patients with HEV infection should be analyzed to determine the risk factors for such co-infection.
- To study the sero-prevalence and genetic characterization of HEV in HIV infected patients with CD4 counts less 500cells/ml.
- Based upon the indicative strong association between HEV and HIV co-infection, the Nepalese government authoritative body should give cheap, reliable and easily accessible HEV vaccine to HIV infected individuals.
- We also recommend the Tribhuvan University and other research institutions to facilitate the lab for the research of different infectious viral diseases in Nepal like HIV, Hepatitis, etc.

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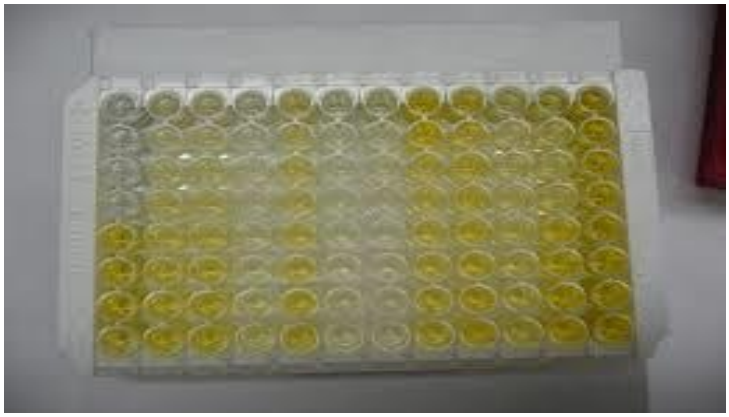
Appendices

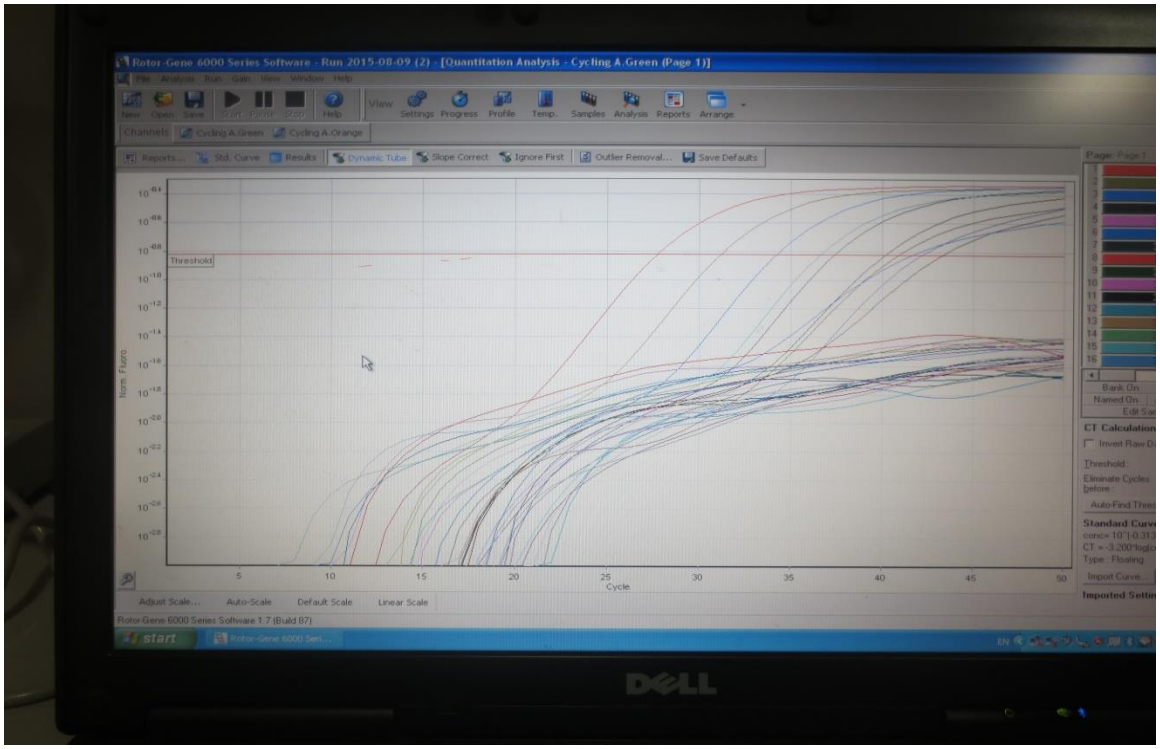
Appendix 1

ELISA Picture



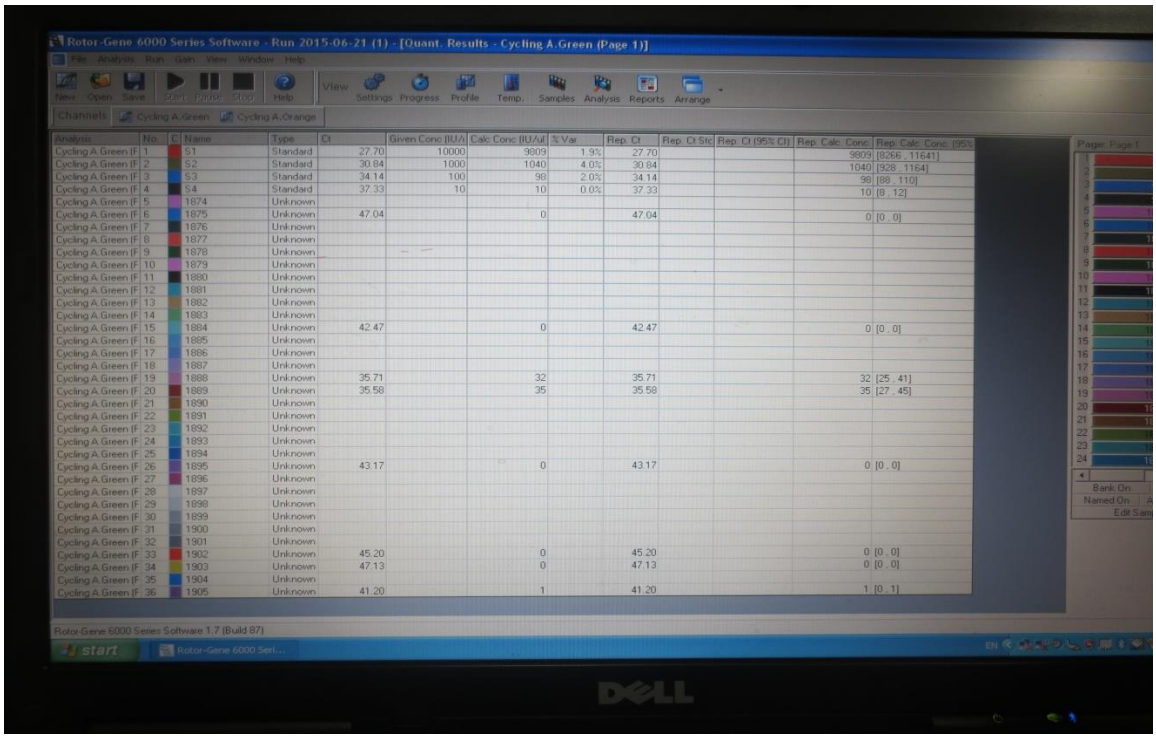
Appendix 2

Florescence Picture of HIV Viral Load



Appendix 3

HIV Viral Load Display Picture



Appendix 4

Table: Data of Age, Sex, CD4 count, Viral load, ART Regimen, and District

S.N.	Age	Sex	CD4	IgG	IgM	Ag	Current ART Regimen	ARV Currently Use	Viral Load(copies/ml)	Current District
1	36	M	264	Negative	Positive	Negative	1st	zdv/3tc/nvp	<400	Chitawan
2	30	F	204	positive	Negative	Negative	1st	zdv/3tc/nvp		Kathmandu
3	42	F	487	Negative	Negative	Positive	1st	efv+azt+3tc	<400	Rautahat
4	50	M	278	positive	Negative	Negative	1st	zdv/3tc/nvp	<400	Kathmandu
5	40	M	487	Negative	Negative	Negative	1st	zdv/3tc/nvp	<400	Nawalparasi
6	46	M	314	Negative	Negative	Negative	1st	zdv/3tc/nvp	1,169,996	Kathmandu
7	27	F	368	Negative	Positive	Positive	2nd	TDF+3TC+NVP	<400	Bara
8	30	F	359	positive	Negative	Negative	1st	zdv/3tc/nvp	857	Sarlahi
9	40	M	171	positive	Negative	Negative	1st	zdv/3tc/nvp	<400	Bara
10	10	M	285	Negative	Negative	Negative	2nd	3TC+ERV+TDF	<400	Chitwan
11	50	M	179	Negative	Negative	Negative	1st	zdv/3tc/nvp	<400	Nuwakot

12	40	M	488	positive	Negative	Negative	1st	zdv/3tc/nvp	<400		Nawalparasi
13	32	F	346	Negative	Negative	Positive	1st	zdv/3tc/nvp	<400		Nawalparasi
14	46	M	441	positive	Negative	Negative	1st	zdv/3tc/nvp	<400		Parsa
15	23	F	468	Negative	Negative	Negative	1st	zdv/3tc/nvp	<400		Rautahat
16	30	F	79	Negative	Negative	Positive	1st	azt/3tc/nvp	<400		lalitpur
17	32	M	436	Negative	Negative	Negative	1st	azt/3tc/nvp	<400		lalitpur
18	62	M	404	Negative	Negative	Negative	1st	azt/3tc/nvp	<400		Kathmandu
19	64	M	354	Negative	Negative	Negative	1st	azt/3tc/nvp	<400		Kathmandu
20	20	F	379	Negative	Negative	Negative	1st	azt/3tc/nvp		457	lalitpur
21	41	F	315	Negative	Negative	Negative	1st	azt/3tc/nvp		429	Chitwan
22	42	F	362	Negative	Negative	Negative	1st	zdv/3tc/nvp	<400		Kathmandu
23	24	F	367	Negative	Negative	Negative	1st	3TC+ERV+TDF			Bhaktapur
24	28	M	281	Negative	Negative	Negative	1st	azt/3tc/nvp	<400		Bhaktapur
25	24	F	288	Negative	Negative	Negative	1st	azt/3tc/nvp		18,857	Chitwan
26	40	F	409	Negative	Negative	Negative	1st	azt/3tc/nvp	<400		Bara
27	40	M	469	positive	Negative	Negative	1st	azt/3tc/nvp	<400		Chitwan
28	32	M	429	Negative	Negative	Negative	1st	azt/3tc/nvp	<400		Kathmandu
29	34	M	236	positive	Negative	Negative	1st	azt/3tc/nvp		1,169,996	Kathmandu
30	35	F	291	positive	Negative	Negative	1st	zdv/3tc/nvp	<400		Kathmandu
31	4	M	311	Negative	Negative	Negative	1st	zdv/3tc/nvp		457	Nuwakot
32	32	F	497	Negative	Negative	Negative	1st	zdv/3tc/nvp	<400		Nawalparasi
33	35	F	429	Negative	Negative	Negative	1st	efv+azt+3tc			Bhaktapur
34	30	F	423	Negative	Negative	Negative	1st	azt/3tc/nvp	<400		Chitwan
35	40	F	325	positive	Negative	Negative	1st	azt/3tc/nvp	<400		Kathmandu
36	36	F	6	Negative	Negative	Negative	1st	azt/3tc/nvp		857	Kathmandu
37	43	M	288	positive	Negative	Negative	1st	azt/3tc/nvp	<400		Bhaktapur
38	50	M	292	Negative	Negative	Negative	1st	azt/3tc/nvp	<400		Sarlahi
39	44	F	275	Negative	Negative	Negative	1st	azt/3tc/nvp	<400		Rupandehi
40	14	F	487	Negative	Negative	Negative	1st	azt/3tc/nvp	<400		Gulmi
41	36	F	434	Negative	Negative	Negative	1st	azt/3tc/nvp	<400		Rupandehi
42	29	M	226	Negative	Negative	Negative	1st	azt/3tc/nvp		429	Rupandehi
43	40	F	443	Negative	Negative	Negative	1st	azt/3tc/nvp			Kathmandu
44	37	M	328	positive	Negative	Negative	1st	azt/3tc/nvp	<400		Gorkha
45	36	M	137	Negative	Negative	Negative	1st	azt/3tc/nvp	<400		Rupandehi
46	69	M	204	Negative	Negative	Negative	1st	azt/3tc/nvp	<400		Nawalparasi
47	38	M	215	Negative	Negative	Negative	1st	azt/3tc/nvp	<400		Dang
48	33	M	381	Negative	Negative	Negative	1st	azt/3tc/nvp	<400		Chitwan
49	40	M	274	positive	Negative	Negative	2nd	Lopinavir/TDF /3TC	<400		Sarlahi
50	40	M	240	Negative	Negative	Negative	1st	azt/3tc/nvp	<400		Arghakhanchi
51	37	M	241	Negative	Negative	Negative	1st	azt/3tc/nvp	<400		Palpa
52	40	M	243	positive	Negative	Negative	1st	azt/3tc/nvp	<400		Chitwan
53	11	M	370	Negative	Negative	Negative	1st	zdv/3tc/nvp	<400		Nawalparasi

54	33	F	339	Negative	Negative	Negative	1st	zdv/3tc/nvp		429	Nawalparasi
55	35	M	481	Negative	Negative	Negative	1st	zdv/3tc/nvp	<400		Chitwan
56	37	M	46	positive	Negative	Negative	1st	zdv/3tc/nvp	<400		Kathmandu
57	25	F	419	Negative	Negative	Negative	1st	zdv/3tc/nvp	<400		kathmandu
58	44	F	446	Negative	Negative	Negative	1st	zdv/3tc/nvp	<400		Chitwan
59	37	M	337	Negative	Negative	Negative	1st	3TC+ERV+TDF			Dang
60	34	M	482	Negative	Positive	Negative	1st	zdv/3tc/nvp			Parsa
61	32	F	487	Negative	Negative	Negative	1st	zdv/3tc/nvp	<400		Bara
62	51	M	218	Negative	Negative	Negative	1st	tdf/3tc+ lpv/r		429	Kathmandu
63	64	M	184	Negative	Negative	Negative	1st	azt/3tc/nvp	<400		Chitwan
64	42	M	443	positive	Negative	Negative	1st	azt/3tc/nvp	<400		Kaski
65	19	M	465	Negative	Negative	Negative	1st	azt/3tc/nvp	<400		Parsa
66	35	M	227	positive	Negative	Negative	1st	azt/3tc/nvp	<400		Kaski
67	54	M	475	Negative	Negative	Negative	1st	azt/3tc/nvp	<400		Rupandehi
68	49	F	484	positive	Negative	Negative	1st	azt/3tc/nvp	<400		Nawalparasi
69	24	F	428	Negative	Negative	Negative	1st	azt/3tc/nvp		429	Nawalparasi
70	56	F	376	positive	Negative	Negative	1st	azt/3tc/nvp	<400		Dang
71	42	M	467	Negative	Negative	Negative	1st	azt/3tc/nvp	<400		lalitpur
72	11	F	372	Negative	Negative	Negative	1st	azt/3tc/nvp	<400		Dhading
73	32	M	497	Negative	Negative	Negative	1st	azt/3tc/nvp		17,571	Banke
74	35	F	455	positive	Negative	Negative	1st	azt/3tc/nvp	<400		Chitwan
75	35	F	464	Negative	Negative	Negative	1st	azt/3tc/nvp	<400		Nuwakot
76	10	F	369	Negative	Negative	Negative	1st	azt/3tc/nvp	<400		Jankpur
77	42	M	261	Negative	Negative	Negative	1st	azt/3tc/nvp	<400		Mahottori
78	75	F	424	Negative	Negative	Negative	1st	azt/3tc/nvp	<400		Dhanusa
79	30	F	294	Negative	Negative	Negative	1st	azt/3tc/nvp	<400		Bardiya
80	53	M	169	positive	Negative	Negative	1st	azt/3tc/nvp	<400		Banke
81	33	M	303	Negative	Negative	Negative	1st	efv+azt+3tc	<400		Dhanusa
82	33	M	498	Negative	Negative	Negative	1st	efv+azt+3tc	<400		Sarlahi
83	36	M	477	positive	Negative	Negative	1st	zdv/3tc/nvp	<400		Parsa
84	32	F	296	Negative	Positive	Positive	1st	zdv/3tc/nvp	<400		Kathmandu
85	53	M	247	Negative	Negative	Negative	1st	zdv/3tc/nvp		429	Rautahat
86	25	M	406	Negative	Negative	Negative	1st	zdv/3tc/nvp	<400		Nawalparasi
87	30	F	3	Negative	Negative	Positive	1st	azt/3tc/nvp	<400		Kathmandu
88	17	F	291	Negative	Negative	Negative	2nd	Lopinavir/TDF /3TC	<400		Rupandehi
89	50	M	116	Negative	Negative	Negative	1st	azt/3tc/nvp	<400		Dhading
90	33	M	215	Negative	Negative	Negative	1st	zdv/3tc/nvp	<400		Nuwakot
91	68	M	368	Negative	Negative	Negative	1st	azt/3tc/nvp	<400		Chitwan
92	28	F	226	Negative	Negative	Negative	1st	azt/3tc/nvp	<400		Parsa
93	27	M	332	Negative	Negative	Negative	1st	azt/3tc/nvp	<400		Bara
94	37	M	237	Negative	Negative	Negative	1st	azt/3tc/nvp	<400		Nuwakot
95	31	M	345	positive	Negative	Negative	2nd	TDF+3TC+NVP	<400		Kathmandu
96	30	M	268	Negative	Negative	Negative	1st	azt/3tc/nvp	<400		lalitpur

97	34	M	261	positive	Negative	Negative	1st	azt/3tc/nvp	457	Rupandehi
98	51	M	284	Negative	Negative	Negative	1st	azt/3tc/nvp	<400	Jankpur
99	35	M	440	Negative	Negative	Negative	1st	azt/3tc/nvp	<400	Dhanusa
100	35	F	362	Negative	Negative	Negative	1st	azt/3tc/nvp	<400	Mahottori

Appendix 1

HEV Sequence

>D|ORF1|s4|530nt

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AGGCTGCTCTAGCAGCGGCCAACTCTGCCCTGGCGAATGCTGTGGTAGTTAGGCCTTTTCTCTCTCACCAGCAGATT
GAGATCCTTATTAACCTGATGCAACCTCGCCAGCTTGTTTTCCGCCCCGAGGTTTTCTGGAACCATCCCATCCAGCGC
GTCATCCACAATGAGCTGGAGCTTTACTGCCGCGCCCGCTCCGGCCGCTGCATTGAAATTGGTGCCCATCCCCGCT
CAATAAATGATAATCCTAATGTGGTTCACCGCTGTTTCCTCCGCCCTGTCGGGCGTGATGTCCAGCGCTGGTATACTG
CTCCCACTCGCGGTCCGGCTGCCAATTGCCGACGTTCCGCGCTGCGCGGGCTCCCTGCTGCCGACCGCACTTACTG
CTTCGACGGGTTTTCCGGCTGTAACTTTCCCGCCGAGACGGGCATCGCTCTCTATTCCCTCCATGACATGTCACCAC
CTGACGTCGCCGAAGCCATGTTTCGCCACGGTATGACGCGGCTTTATGCTGCCCTCCATCTTCCACCT

>D1|ORF1|500nt

TGGCGaATGCTGTGGTAGTTAGGCCTTTTCTCTCTCACCAGCAGATTGAGATCCTTATTAACCTGATGCAACCTCGCC
AGCTTGTTTTCCGCCCCGAGGTTTTCTGGAACCATCCCATCCAGCGCGTCATCCACAATGAGCTGGAGCTTTACTGC
CGCGCCCGCTCCGGCCGCTGCATTGAAATTGGTGCCCATCCCCGCTCAATAAATGATAATCCTAATGTGGTTCACCG
CTGTTTCCTCCGCCCTGTCGGGCGTGATGTCCAGCGCTGGTATACTGCTCCCACTCGCGGTCCGGCTGCCAATTGC
CGACGTTCCGCGCTGCGCGGGCTCCCTGCTGCCGACCGCACTTACTGCTTCGACGGGTTTTCCGGCTGTAACTTTC
CCGCCGAGACGGGCATCGCTCTCTATTCCCTCCATGACATGTCACCACCTGACGTCGCCGAAGCCATGTTTCGCCAC
GGTATGACGCGGCTTTATGCTGCCCTCCATCTTCCACCT

>D1|ORF1|504t

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AGGCTGCTCTAGCAGCGGCCAACTCTGCCCTGGCGAATGCTGTGGTAGTTAGGCCTTTCTCTCTCACCAGCAGATTG
AGATCCTTATTAACCTGATGCAACCTCGCCAGCTTGTTTTCCGCCCCGAGGTTTTCTGGAACCATCCCATCCAGCGCG
TCATCCACAATGAGCTGGAGCTTTACTGCCGCGCCCGCTCCGGCCGCTGCATTGAAATTGGTGCCCATCCCCGCTCA
ATAAATGATAATCCTAATGTGGTTCACCGCTGTTTCCTCCGCCCTGTCGGGCGTGATGTCCAGCGCTGGTATACTGCT
CCCACTCGCGGTCCGGCTGCCAATTGCCGACGTTCCGCGCTGCGCGGGCTCCCTGCTGCCGACCGCACTTACTGCT
TCGACGGGTTTTCCGGCTGTAACTTTCCCGCCGAGACGGGCATCGCTCTCTATTCCCTCCATGACATGTCACCACCT
GACGTCGCCGAAGCCATG-TTCGCCACGGTAtGACGCGGCT----------

>D|ORF2|s4|191nt

-
CGACTGTTAAGCTGTATACATCTGTAGAGAATGCTCAGCAGGATAAGGGTATTGCAATCCCACATGACATTGACCTCG
GAGAATCCCGTGTGGTTATTCAGGATTATGATAATCAACATGAACAAGATCGGCCGACGCCTTCCCCGGCCCCATCG
CGCCCTTTCTCTGTCCTTCGAGCTAACGATGTGCTT

>D4|ORF2iS|170nt

----------TCTGTAGAGA-
TGCTCAGCAGGATAAGGGTATTGCAATCCCACATGACATTGACCTCGGAGAATCCCGTGTGGTTATTCAGGATTATGA
TAATCAACATGAACAAGATCGGCCGACGCCTTCCCCGGCCCCATCGCGCCCTTTCTCTGTCCTTCGAGCAACGATGT
GCTT

>D4|ORF2iAA|157nt

-
GACTGTTAAGCTGTATACATCTGTAGAGAATGCTCAGCAGGATAAGGGTATTGCAATCCCACATGACATTGACCTCGG
AGAATCCCGTGTGGTTATTCAGGATTATGATAATCAACATGAACAAGATCGGCCGACGCCTTCCCCGGCCCCATCGC
GC----------