EPIDEMIOLOGICAL, SEROLOGICAL, IMMUNOLOGICAL AND MOLECULAR PROFILES OF HEPATITIS B VIRUS (HBV) IN NEPAL.

M.Sc. Thesis

2018

Submitted To:

CENTRAL DEPARTMENT OF BIOTECHNOLOGY

Tribhuvan University

Kirtipur, Kathmandu, Nepal.

Supervised By:

Dr. Smita Shrestha

CENTRAL DEPARTMENT OF BIOTECHNOLOGY

Kirtipur, Kathmandu, Nepal

A thesis report submitted in partial fulfillment of the requirement of the M Sc. degree in Biotechnology

Submitted By:

Sila Mahatara

BT 217/ 071

Redg no: 5-2-8-33-2010

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Recommendation:

This is to certify that the research work entiled **"EPIDEMIOLOGICAL, SEROLOGICAL, IMMUNOLOGICAL AND MOLECULAR PROFILES OF HEPATITIS B IN NEPAL**" has been carried out by **Ms. Sila Mahatara** under my supervision.

This thesis work was performed for the partial fulfillment of the Master of Science in Biotechnology under the course code BT 621. The result presented here is his/her original findings. I/we, hereby, recommend this thesis for final evaluation.

…….

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Certificate of Evaluation

This is to certify that this entitled "**EPIDEMIOLOGICAL, SEROLOGICAL, IMMUNOLOGICAL AND MOLECULAR PROFILES OF HEPATITIS B IN NEPAL"** presented to evaluation committed by **Ms. Sila Mahatara** is found satisfactory for the partial fulfillment of master of Science and Technology.

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Sila Mahatara

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Abbreviations:

Ab:	Antibody
ACHBV:	African cichlid hepatitis virus
Ag:	Antigen
AIDS:	Acquired Immuno Deficiency Syndrome
ALP:	Alkaline phosphatase
ALT:	Alanine transferase
Anti-HBc:	Antibody to hepatitis B core antigen
ART:	Antiretroviral Therapy
AST:	Aspartate transferase
BCP:	Basal Core Promoter
Bili (D):	Bilirubin direct
Bili (T):	Bilirubin total
^{o}C:	Degree
cccDNA:	Covalently closed circular Deoxy-ribonucleic acid
CD:	Cluster of Differentiation
CDC:	Centers for Disease Control and Prevention
cDNA:	Complementary Deoxy-ribonucleic Acid
CHB:	Chronic Hepatitis B
CSF:	Cerebral Spinal Fluid
CTL:	Cytotoxic T Lymphocyte
CO:	Cut-off Value
DNA:	Deoxy-ribonucleic Acid
dsDNA:	Double stranded DNA

ELISA:	Enzyme-Linked Immunosorbent Assay
ER:	Endoplasmic Reticulum
FAM:	6-Carboxyfluorescein
FCV:	Famciclovir
FDA:	Food and Drug Administration
FHF:	Fulminate Hepatic Failure
GSHV:	Ground Squirrel hepatitis virus
HAV:	Hepatitis A Virus
HBcAg:	Hepatitis B core Antigen
HBeAg:	Hepatitis B e Antigen
HBIG:	Heatitis B Immune Globulin
HBsAg:	Hepatitis B surface Antigen
HBV:	Hepatitis B Virus
HCC:	Hepatocellular Carcinoma
HCW:	Health Care Workers
HDV:	Hepatitis Delta Virus
HEV:	Hepatitis E Virus
HEX:	Hexachloro 6-carboxyfluorescein
HIV:	Human Immunodeficiency Virus
IgG:	Immunoglobulin G
IgM:	Immunoglobulin M
IFN:	Interferon
LC:	Liver Cancer
NASH:	Non-alcoholic steatohepatitis
NC:	Negative Control

NRTI:	Nucleosides Reverse Transcriptase Inhibitors
NUCs:	Nucleotide Analogues
OIs:	Opportunistic Infections
ORF:	Open Reading Frame
PCR:	Polymerase Chain Reaction
pgRNA:	Pre-genomic ribonucleic acid
QC:	Quality Control
RBC:	Red Blood Cell
RDT:	Rapid Diagnostics Test
RH:	RNase H
RNA:	Ribonucleic Acid
rpm:	Revolution per Minute
RT:	Reverse transcriptase
RT-PCR:	Real Time PCR
RUQ:	Right Upper Quadrant
SGPT:	Serum Glutamic Pyruvate Transferase
SGOT:	Serum Glutamic Oxaloacetic Transferase
SP:	Spacer
SS:	Single stranded
STD:	Sexually Transmitted Disease
Tm:	Melting temperature
TP:	Terminal Protein
WHO:	World Health Organization
WHV:	Woodchucks hepatitis virus
µl:	Microliter

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ABSTRACT

Hepatitis B virus (HBV) is a common cause of liver disease and hepatocellular carcinoma. The most common route of transmission being spreading through the blood transfusion and organ transplants. It infects more than 350 million people worldwide. It is estimated that 260,000 individuals are chronically infected with HBV in Nepal and majority of them are unaware of their infection.

In this study, epidemiological study was conducted by direct interviewing with all suspected patients. The serological study for different antigens and antibody was conducted by Rapid Diagnostic strip test (RDT). Enzymes Linked Immunosorbent Assay (ELISA) was used to confirm this test. Moreover, the molecular test was done by using Real Time PCR for the quantification and genotyping of HBV. Sequence analysis was done by using Sequence analysis software V 5.2 and phylogenetic tree was constructed by neighbor joining method.

A total of 500 Nepalese suspected HBV patients were enrolled where 64 % (n=320) were found to be HBV positive and 36 % (n=180) were HBV negative. This study was done based on the HBsAg positivity. HBV infection was higher in males (n=213) as compared with female (n=106) with male and female ratio of 2.01:1. The most productive age groups of 20-40 years followed by 40-60 years were found to be associated with the hepatitis B infection. The sero-prevalence rate was higher in province 2 followed by province 3.

All the samples recorded positive for HBsAg from ELISA were assayed for Liver function tests (LFT). The level of total bilirubin was found higher in 25.31 % (n=81) patients indicating liver damage. However, the result obtained from the estimation of ALT in HBsAg positive cases showed that 54.69 % (n=197) have elevated level of ALT indicating that these samples seem to have carrier state of the infection. Thrombocytopenia is a common feature of chronic liver disease and was reported in 0.94 % (n=3) of total HBV infected patients.

The viral load was found to be highest in chronic HBV patients with HBeAg positive. Genotype D was found to be common among the Nepalese population. HBV sub- genotypes A1, C1, D1, CD and D4 were detected in samples after sequence analysis. Phylogenetic tree showed that genotype-A1 was very closely related to isolates from France and Belgium, Genotype-C1 from Japan, Genotype-D1 & D4 from India. CD-recombinant genotype indicated probable divergence of Genotype C to Genotype D or recombinant event might have occurred in S-gene as S-gene determines Genotype.

Key Words: Hepatitis B virus (HBV)**,** seroprevalence, LFT, Genotype, Viral load, DNA.

CHAPTER ONE

INTRODUCTION

1.1) Early History:

The understanding of Hepatitis B virus (HBV) came in 1963 by when Dr. Baruch Blumberg discovered an antigen that detected in the presence of HBV in blood samples. He discovered this virus while he was researching the genetics of disease susceptibility from the thousands of blood samples and then study whether these differences were associated with a particular disease or not. He discovered an unusual antigen from the blood sample of Australian Aborigine, later called "Australian antigen" which is responsible for the cause of Hepatitis B infection. Now a day this Australian antigen is known as Hepatitis B surface antigen (HBsAg). Prince and Okochi in 1968 found that Australian antigen was found excessively in hepatitis B patients. The HBsAg is the hallmark of infection and appears in the serum after some days or weeks before clinical symptoms manifest.

The first commercial Hepatitis B vaccine is Heptavax, which was invented in 1981. This was plasma-derived vaccine and contained the collection of blood from HBsAg positive patients. The vaccine was subjected in three dose series to inactive the viral particles that included formaldehyde and pasteurization (John, 2000). This vaccine is no longer in use since 1990. A second generation of genetically engineered HBsAg vaccine was introduced in 1986. These new approved vaccines are synthetically prepared and don't contain blood products. Hepatitis B vaccine is used as first anti-cancer vaccine as it helps to prevent liver cancer. The development of safe and effective vaccines constitutes one of the remarkable scientific achievements of the 20th century. In 1992, World Health Organization (WHO) recommends that childhood hepatitis B vaccination schedules should be included in immunization programs of all countries. It also recommends providing the first hepatitis B vaccine dose within 24 hours of birth. This approach prevents HBV transmission from HBsAg-positive mothers to their offspring in more than 90% of cases. However, many countries with high burden of disease had not introduced vaccination schedules into their national immunization programs. In addition, the coverage of complete doses of vaccination is low in many countries that had introduced the vaccine.

Therefore, HBV infection has become major public health problem as new infections continue to occur.

1.2) Burden of disease:

Viral hepatitis is a major public health problem, with more deaths annually attributable to this group of diseases than to HIV, malaria or tuberculosis. It is now tenth leading cause of death worldwide. According to WHO, approximately 257 million people are living with HBsAg positivity and 350 million are suffering from chronic HBV infection. In 2015, HBV infection result 887,000 deaths caused by chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC). HBV infection results for 600,000 death per year (Ott, Stevens, Groeger, & Wiersma, 2012).

About 87-90 % of patient develops immunity and clears the infection or become chronic carriers after infection with HBV. The risk of chronicity is determined by the age at which the infection is acquired (Hyams, 1995). The infection becomes chronic in 90 % among the neonates and children up to 1 year of age, 30 % for the age of children between 1-5 years of old. In addition, it is only about 2% when the infection is acquired after 5 years of age or in adulthood (Lai, Ratziu, Yuen, & Poynard, 2003).

Many individuals eventually achieve a state of nonreplicative infection. The prolonged immunologic response to infection may lead to the development of cirrhosis, liver failure, or HCC in up to 40 % of patients (Goldstein et al., 2002). HCC incidence has increased worldwide, and the disease is now the third most frequent cancer, killing 300,000–500,000 people each year (Sung et al., 2012).

According to Centers for Disease Control and Prevention (CDC), HBV infection is common among adults of age group 25–44 years. The prevalence is highest in male as compared to female. The prevalence of HBV infection is generally high in Asia and Africa. In highly endemic areas as well as in areas with low endemicity, perinatal or infancy mode of transmission is predominant where more than one third are chronic HBV carrier (Yi, Chen, Huang, Zhou, & Fan, 2016).

1.3) Systemic Classification:

Group: Group VII (ds DNA –RT)

Order: Unassigned

Family: *Hepadnaviridae*

Genus: Orthohepadnavirus

Species: Hepatitis B virus

Hepatitis B virus (HBV) is a small DNA virus, which belongs to the family Hepadnaviridae and genus *Orthohepadnavirus.* It is only one virus of Hepadnaviridae that is pathogenic to human. However, similar viruses *are* found in woodchucks, ground squirrels, tree squirrels, Peking ducks, and herons (Liang, 2009). HBV infection can induce diseases, such as acute and chronic hepatitis, liver cirrhosis, liver fibrosis and HCC, and thus severely threats global human health. It can survive outside the body for at least 7 days and can be easily transmitted through blood and bloody fluids. It is an enveloped, hepatotropic, non-cytopathic virus and has unusual features similar to retroviruses. It has a complex life cycle and replicates through RNA intermediates. HBV is highly detectable in serum of HBV positive patients and electron microscopy shows two spherical and filamentous structures of 22 nm diameters and one double-shelled virion of 42 nm diameters. The genome is highly organized with partially double stranded DNA and encodes four open reading frames for surface, core, polymerase, and X genes. adr, adw, ayr, ayw are the four serotypes of HBV which are divided on the basis of antigenic epitopes present on its envelope proteins. a is common determinant and d/y and w/r are two mutually exclusive determinants of serotypes. These serotypes are predominant in different regions of the world. Based on nucleotide sequence variation, HBV is divided into ten (A-J) genotypes and forty subtypes. This classification is based on more than 8% nucleotide divergence for genotypes and 4-8 % for subtypes, which plays a key role in understanding of HBV geographical distribution, evolution and transmission of virus. Differences among genotypes are also related to disease severity, clinical progression, response to antiviral treatment and prognosis.

1.4) Structure of hepatitis B virus:

Three morphologically distinct forms of viral particles are seen in serum of HBV positive patient under electron microscope. They are Dane particle called virion, spherical and filamentous particles. Dane particle is a complex, spherical and infectious double-shelled structure having diameter of 42 nm and consists of outer lipid envelope and an icosahedral nucleocapsid core (Hruska & Robinson, 1977). The outer envelope has embedded proteins, which play roles in viral binding and entry into susceptible cells. These three enveloped proteins are LHBs, MHBs and SHBs, which are in the ratio of 1:1:3. SHBs is the most abundant enveloped protein and is composed of S region, the middle protein MHBs is about 5-15% and composed of pre-S2 and S region. The large protein, LHBs is only 1-2 % and contains pre-S1, pre-S2 and S regions. The nucleocapsid encloses viral DNA and DNA polymerase, which has reverse transcriptase similar to retroviruses. Spherical structure and filamentous particle are non-infectious particles having diameter of 20 nm and 22 nm respectively. These are made up of lipid and proteins that form parts of surface antigens (HBsAg). Sometimes, the filamentous particles consist of all these three enveloped proteins. However, the spherical particles have only SHBs or SHBs and MHBs but not LHBs. Spherical and filamentous particles are “empty” envelope, as they don’t contain any core proteins or nucleic acids. These are present in blood at least 100 folds more over than virions and used as primary antigenic particles in original vaccine. The components of HBV are:

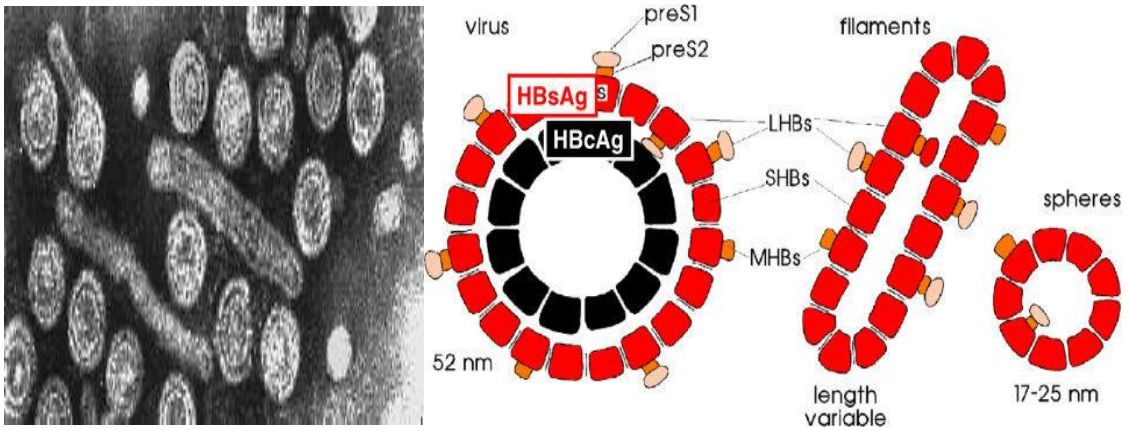

Figure 1.1 Electron micrograph of circulating forms of HBV particles in the blood is shown at the left side and a Dane particle, filamentous and spherical particles of HBV are shown at the right side of the figure (Liang, 2009).2202

1.4.1) Hepatitis B surface antigen (HBsAg):

HBsAg is the surface antigen of hepatitis B virus. It is also known as Australian antigen and is first serological marker for HBV infection. It can be detected in 1-2 weeks or as last as 11-12 weeks after the exposure to infection. It appears on the blood before the symptoms of HBV infection occurs. The presence of HBsAg for less than six months indicates an acute infection while more than six months indicates as chronic infection. HBsAg forms a small lipid particle in which many antigen proteins are present. There are three domains; S, Pre-S1 and Pre-S2 in each antigen protein. All three domains are present in L-type antigen, S- and Pre-S2 are present in M-type antigen and only S domains are present in S-type antigen (Brunetto, 2010). Pre-S1 domain is important for host or hepatic cell recognition and Pre-S2 domain for cell penetration during HBV infection. A lot of non-infectious S-type HBsAg are produced by the hepatic cells in infected patients (Cornberg et al., 2017).

1.4.2) Hepatitis B core antigen (HBcAg):

Hepatitis B core antigen (HBcAg) is a core antigen found on the surface of nucleocapsid of HBV. The presence of HBcAg indicates an active viral replication; it means it can be easily transmit from infected person to other person. It is detected only in liver cells after liver biopsy but not in the bloodstream so called “particulate”. Therefore, there is no test for the detection of HBcAg. This antigens disappears in the early stage of infection but sometimes can be detected by the IgM specific HBcAg patient’s blood.

1.4.3) Hepatitis B e Antigen (HBeAg):

HBeAg is non-structural and accessory peptide, which is expressed from C open reading frame (ORF). It is non-particulate which can be detected in the bloodstream when the virus is actively reproducing. It indicates the ongoing viral replication in liver cells but it is not essential for the viral replication. It appears in the bloodstream with HBsAg and disappears before the HBsAg disappears. The function of HBeAg is unknown but it is believe that it acts as “immune tolerogen” and suppress the immune system thus resulting in persistence chronic liver damage. Although HBeAg and HBcAg share a significant portion of their constituent amino acid residues but they exhibit characteristically distinct antigenicity.

1.4.4) HBV DNA polymerase:

HBV DNA is 95 kD and encoded by polymerase ORF. It covers about 80 % of the whole HBV genome. It is 833-846 amino acids long and its length depends upon different genotype. The process by which polymerase is translated in HBV is still in contentious (Kann, 2002). It was recorded that translation of the polymerase protein is started from start codon ATG of the polymerase ORF (Roychoudhury & Shih, 1990) while others showed that polymerase synthesis may involve leaky scanning that does not result in core protein translation (Kozak, 1989). HBV consists of four domains in which two reverse transcriptase (RT) and RNase H are conversed domains and other two are terminal protein (TP) and spacer domain (SP) (Beck & Nassal, 2007).

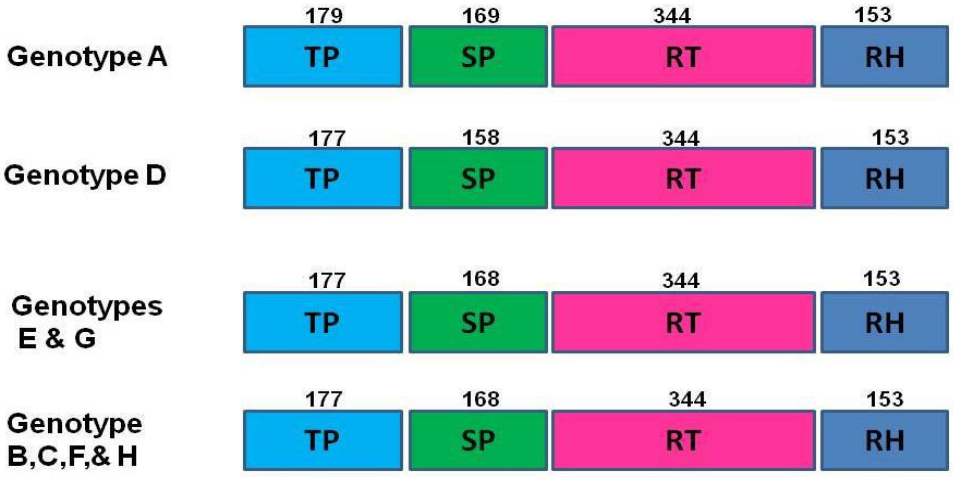

Figure 1.2: A schematic representation of HBV polymerase showing the different lengths of each polymerase domain in amino acids for the different genotypes. TP: Terminal protein, SP: Spacer, RT: Reverse transcriptase and RH: RNase H

1.4.5) HBx:

HBx, a viral protein is the smallest among all the four partially open reading frames (ORF) found in the HBV. It is 154 amino acids with molecular mass of 17 kDa. HBx is present only in mammalian virus. HBx may modulate protein degradation pathways, apoptosis, transcription, signal transduction, cell cycle progress, and genetic stability by directly or indirectly interacting with host factors (Ajiro & Zheng, 2014). This protein also suppresses the host antiviral defenses and promotes the viral replications. This protein will mainly attack the nucleus and mitochondria of host cell. It forms a heterodimeric

complex with its cellular target protein, and this interaction dysregulates centrosome dynamics and mitotic spindle formation. It interacts with damaged DNA Binding Protein (DDB1) redirecting the ubiquitin ligase activity of the CUL4 DDB1 E3 complexes, which are intimately involved in the intracellular regulation of DNA replication and repair, transcription and signal transduction. The HBx-induced transcripts fall into three major categories, including genes that encode ribosomal proteins, transcription factors with zinc-finger motifs, and proteins associated with polypeptide degradation pathways (Wu et al. 2001, 2002).

1.5) Genomic Organization of virus:

The genome of HBV is partially doubled stranded DNA of around 3.2 Kb. It has the smallest genome among all the double stranded DNA viruses, which are capable of independent replication and infect human. It forms relax circular genome with the circularity maintained by 5'cohesive ends, attached to the viral DNA polymerase. It has a long, complete negative DNA strand and a short and incomplete positive DNA strand with 5' end capped with oligonucleotides. The mRNA transcription takes place from the negative DNA strand. The genome has 48 % of guanine and cytosine content. The genome of HBV is organized in such a complex way so that all the ORFs are partially overlapped and encodes the P (polymerase), C (core), S (surface) and X proteins (Cento et al., 2013).

The possibility for overlapping of genes is due to translation of a single strand of nucleic acid into three unique proteins. This occurs due to translation of genetic code to specify amino acids or termination signals by reading triplets of nucleotides resulting in formation of protein in first, second and third nucleotide of gene sequence. The P ORF contains alternate overlapping ORFs in over 63% of its length. The surface ORF is contained completely within the polymerase ORF, whereas the core and X gene ORFs overlap the polymerase ORF over 23 and 39% of their sequence lengths, respectively. The consequence of this genetic arrangement is that HBV can encode approximately 50% more unique protein (ie. proteins that do not share any amino acid sequences in common) by mass than viruses without overlapping genes. Beginning from the first initiation codon of each ORF, HBV can encode four proteins with a combined molecular mass of approximately 180 kD. This figure is approximately 1.5 times, or 50% greater, than the theoretical upper limit of approximately 120 kD of total protein encoded by a genome of 3,200

nucleotides without overlapping ORFs. Thus, hepadnaviruses produce substantially more unique protein per genome unit than other viruses (Miller, Chung, Girones, & Purcell, 1989).

S-gene: The S gene encodes a long ORF with three in-frame start (ATG) codons that divide the gene into three sections, pre-S1, pre-S2 and S. Two others envelope proteins M and L are encoded by preS2 and S gene and pre-S1, pre-S2 and S regions respectively (Siegler & Bruss, 2013). The preS1 and preS2 characterize two or more immunogenic fragments of HBsAg. 'a' determinant and many other specific antigenic determinants is common to all HBsAg and the d, y, w, and r determinants are mainly of epidemiologic importance. The development of cellular and humoral immunity to HBsAg is protective, and recombinant HBsAg provides the basis for the HBV vaccines currently available (Lee, 1997).

C gene: It consists of precore and core regions. S and C genes both have multiple in-frame translation initiation codons and give rise to related but functionally distinct proteins. The *C* ORF encodes HBcAg or HBeAg depending on whether translation is initiated from the core or precore regions, respectively. The precore and core proteins initiates from the first and second initiation site respectively. Therefore, the precore proteins have all the sequences of core proteins as well as additional 29 amino acids at its N-terminus. Core protein has intrinsic property, self-assembles into a capsid-like structure, and contains a highly basic cluster of amino acids at its N terminus.

P gene: It covers 80% of the entire viral genome and encode DNA polymerase enzyme, which plays a vital role in DNA synthesis and encapsidation of RNA. It overlaps with HBcAg, HBsAg and X genes either fully or partially in different ORFs (Midori Kobayashi & Koike, 1984). It acts as a primer for reverse transcription of the pregenome by binding to the5'end of HBV DNA. It is somewhat immunogenic during both acute and chronic infection.

X gene: It is an enigmatic protein encoded by X ORFs. It is necessary for HBV infection and replication in vivo. It acts as a transcription transactivator for cellular and viral genes and functions in transcription of viral RNAs from cccDNA in infected hepatocytes. It lacks DNA binding activity so the transcription function is mediated through interaction with

cellular factors. It was known that HBx proteins play an important role in the HBV induced carcinogenesis. However, the function of specific mutations in X gene is still unknown. In some patients with HCC, mutation and deletion of X gene have been observed.

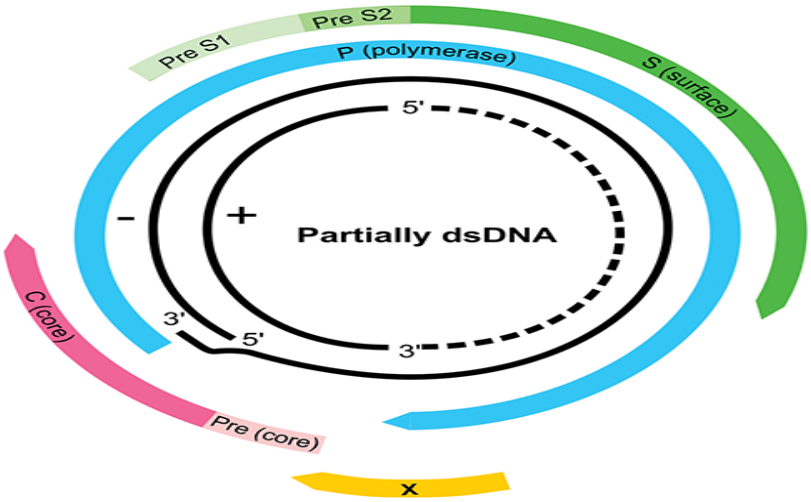

Figure 1.3: Structure and organization of HBV genome.

1.6) Replication cycle of HBV:

Hepatitis B virus has a complex and unique life cycle. It includes the activity of error-prone enzyme, reverse transcriptase for replication and a high production of virion per day. It can produced as many as 10^{12} viruses per day (Lazarevic, 2014). Virus replicates in liver and sheds into blood in high concentration. HBV life cycle starts with the attachement of virus to the host cell membrane via the pre-S1 domain of envelope proteins. Viral membrane fuses with cell membrane as a result genome is released into the cells. Various factors like hormones, growth factors and cytokines play an important role in the regulation of replication of HBV. In the nucleus, partial dsDNA (double stranded DNA) genome is repaired into fully dsDNA which is then super coiled to form covalently closed circular DNA (cccDNA) by the viral polymerase. Then, the cccDNA is transcripted by host RNA Pol-II to produce all viral RNAs necessary for the protein formation and viral replication. The viral RNAs are bifunctional pre-genomic RNA (pgRNA) and sub- genomic RNA. The former pre-genomic RNA function as template for viral DNA synthesis and as messenger for the translation of pre- C, C and P protein. Sub-genomic RNAs serves for the translation of X and envelope proteins. In the cytoplasm, all

the viral RNA are translated into viral envelope, core, polymerase proteins, HBx and HBcAg. The pgRNA form the complex with the core protein and polymerase via its epsilon stem loop structure and results in self assembled of RNA in the cytosol. Once the viral RNA is encapsidated, reverse transcription begins. The process of synthesis of both negative and positive viral DNA strands is sequential. The first negative strand is synthesized from the encapsidated RNA templated. During this process, pgRNA starts to decrease gradually by RNAse H activity of polymerase. Then, this negative DNA strand is used as template for synthesizing the second positive DNA strand until it reaches 50-70 % of the length of negative strand. Then the newly formed mature genome are either transport back their content to the nucleus or assembled within envelope proteins. In the nucleus, the genome is converted to cccDNA molecules and maintain a stable intranuclear pool of transcriptional template (Pourcel & Summers, 1986). While other genome assemble with envelope proteins are converted into mature virions either in cytosol or in the endoplasmic reticulum. In endoplasmic reticulum (ER), HBsAg are intially synthesized and polymerized and later on transported to post ER and pre-Golgi compartements. The detail process of assembly and released of virus is still unknown. But it was suggested that assembled HBV virions are exported to Golgi apparatus by endocytosis for modification of their glycans in the surface and then released out of the host cell to complete their life cycle(Seeger & Mason, 2000).

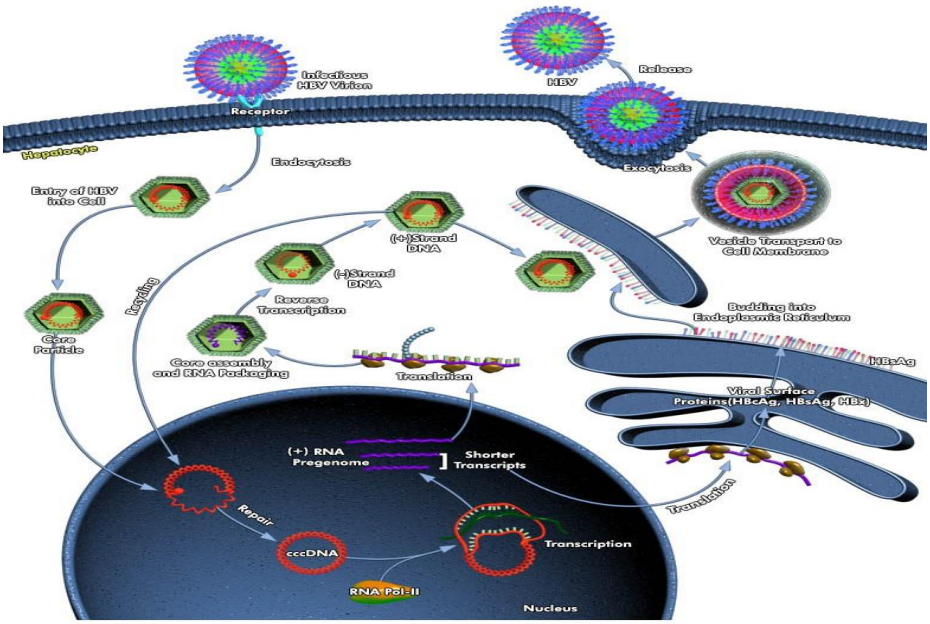

Figure 1.4: Replication cycle of HBV

1.7) Transmission of virus:

Hepatitis B is a highly contagious disease. It can easily transmit from one person to another as it can survive outside the body for at least 7 days. The ways of transmission of virus include intimate personal contact, parenterally and perinatally. Higher risk of individuals for hepatitis B include intravenous drug users, children of mothers with HBV, men who have sex with men, patients on hemodialysis, those exposed to blood or blood products and those who travel to countries with moderate to high rates of Hepatitis B. It is also reported that small quantities of the virus can be present in tears, saliva, and breast milk, but it isn't in enough quantities to transmit the virus. Hepatitis B virus is not spread by sharing eating utensils, breastfeeding, hugging, kissing, holding hands, coughing, or sneezing.

1.7.1) Sexual Contact:

Hepatitis B is also known as sexually transmitted disease (STD). Hepatitis B virus is easily transmitted through the semen, menstrual or vaginal fluids and can enter into the body through a break in the lining of the rectum, vagina and urethra. During acute infection, HBV DNA is present in free form as well as in integrated form in the seminal fluid and spermatozoa respectively (Hadchouel et al., 1985). Homosexual men are at higher risk of infection due to sexual contact. However, there are an increasing proportion of HBV infections in heterosexual men. The increased risk of HBV infection in heterosexuals include the factors like duration of sexual activity, number of sexual partners, history of sexual transmitted disease, and positive serology for syphilis. Sexual partners of prostitutes, clients of prostitutes, injection drug users, are at particularly high risk for infection.

1.7.2) Drug IV abusers:

HBV infection is highly common amongst injection drug abusers (IDUs) mainly through the sharing of contaminated injection equipment and unprotected sexual contact. They used different types of sharp instruments like sharp needles for injecting illegal drugs. It is also transmitted through the cottons and water used by them. 44%- 80% of IDUs who have been injecting drugs for more than 10 years are infected with HBV (Lum et al., 2003). The probability of becoming HBV infected is highest during the first years of injecting. Adult IDUs are at higher risk of HBV injection. IDUs usually have high prevalence

of anti-HCV and anti-HBc in many countries including the United States and have co-infection rates of HIV, HBV, and HCV (Murrill et al., 2002).

1.7.3) Health workers:

People who are regularly in contact with the HBV sample like workers of hospital, research lab are at the higher risk of HBV (Lewis, Enfield, & Sifri, 2015). Transmission occurs if they are accidently come in contact with a used needle or others sharp instruments which are used to draw blood of infected patients. This type of accidental inoculation is 6- 20%. The occurrence of transmission of HBV through needle stick injury was approximately 2 % with HBeAg negative blood and 19 % with HBeAg positive blood (Werner & Grady, 1982). Thus, healthcare workers (HCWs) are at risk for exposure to HBV from infected patients, and correspondingly, HBV-infected health workers may potentially transmit HBV to patients.

1.7.4) Perinatal or Vertical transmission:

Mother infected with HBV can easily transfer the virus to her baby. Two common mode of transmission of HBV from infected mothers to child occurs: transmission through transplacenta in utero: natal transmission during delivery when the baby encounters the mother's body fluids in the birth canal. However, breastfeeding doesn't spread the virus from a woman to her child. It was known that hepatitis B vaccine and hepatitis B Immune Globulin (HBIG) couldn't block the transplacental transmission. As this transmission occurs antenatally and follow two possible mechanisms: hemagenous route and cellular transfer. Through hemagenous route like threaten abortion, HBV can transmit into fetus through the breakage of placental microvascular (Navabakhsh, Mehrabi, Estakhri, Mohamadnejad, & Poustchi, 2011). The second mechanism includes the transmission of HBV into fetus through the villous capillary endothelial cells as the placental tissue of mother's have high titer of HBV (Hou, Liu, & Gu, 2005).

1.7.5) Body piercings and tattoos:

Body piercing is common practice of puncturing or cutting body part for the purpose of wearing jewelry. This procedure can be done by using a needle, indwelling cannula, the dermal punch, a piercing gun, cork and anesthesia to create a hole on any part of skin. It can be done almost anywhere like ears, nose, lip, tongue, nipple, and genital organs and

is getting famous across all age group throughout the world so that make it impossible to regulate (Laumann, Uk, & Derick, 2006). However, the potential health risk of this beauty behavior has been gradually noticed by the public but far more than enough. The virus can be spread when the piercing or tattooing instruments are in contact of infected blood and aren't sterilized. Some studies suggested that body piercing and tattooing are independent risk factor for spreading HBV and HCV (Ahmed, Irving, Anwar, Myles, & Neal, 2012). However, other studies argued that there is no significant correlation between body piercing and transmission of HBV while some revealed with an outcome indicating body piercing is a protective factor (Yang et al., 2015).

1.7.6) Toiletries:

Grooming items like toothbrushes and razors, equipment used by manicurists, estheticians, barbers and cosmetologists can also spread the virus if they carry blood from a person who is infected.

1.7.8) Others:

Serous exudates such as the cerebral spinal fluid (CSF) have been implicated as possible vehicles of transmission. The presence of HBV antigens has been reported in urine, faeces, bile, sweat and tears but has not been confirmed. There have been cases of family outbreaks of hepatitis B where no known exchange of blood has occurred. The entrance of the virus through the membranes of the eye or mouth must be a possible route of transmission. All biological fluids from a HBV infected individual must be treated as potentially infectious. Although HBsAg has been detected in mosquitoes and bed bugs, there is no convincing evidence for replication of the virus in these insects. The role of arthropod vectors in uncertain although mechanical transmission of infection must be a possibility.

1.8) Signs and Symptoms of hepatitis:

There is no sign, symptom of hepatitis B in acute and chronic cases, but serious, and life-threatening complications can still develop. People with suppressed immune system and children below five years of age don't show any signs and symptoms but become carriers and infect others people. According to the World Health Organization (WHO), up to 90 percent of infants infected with HBV within their first year of life will develop chronic

hepatitis B, and 30 to 50 percent of children infected before age six will develop it. Signs and symptoms of hepatitis B range widely from mild to severe, from asymptomatic subclinical infection to fulminant fatal disease. Acute symptoms of HBV appear from 60 to 120 days after exposure to the virus and last from several weeks to 6 months. The sign and symptoms of HBV may include abdominal pain, dark urine, fever, joint pain, loss of appetite, nausea and vomiting, weakness, fatigue and yellowing of your skin and the whites of your eyes (jaundice). An onset of symptoms like anorexia, nausea, fatigue and malaise, or flulike symptoms, such as cough, pharyngitis, coryza, headache, photophobia, and myalgias can precede the onset of jaundice. Fever is uncommon in HBV infection. Physical examination features in HBV patients are nonspecific but can have mild splenomegaly, mild enlargement and tenderness of the liver and posterior cervical lymphadenopathy in 15 percent to 20 percent of patients. Fulminant disease manifests with a change in mental status and coagulopathy.

1.9) Opportunistic infections of HBV:

1.9.1) HBV/HIV co-infection

Hepatitis B virus (HBV) and hepatitis C virus (HCV) are common among the people who are at the high risk of human immunodeficiency virus (HIV) (Liang, 2009). The reason behind this is as both are blood borne pathogens and transmitted through perinatal and horizontal mode and unsafe sexual contact. About 1% that is 2.7 million of people infected with HIV is co-infected with HBV. Globally, the prevalence of HBV infection in HIV-infected persons is 7.4%. Liver disease has been an emerging problem in HIV infected persons. It may bring complication in the delivery of antiretroviral therapy (ART) with increased risk of drug-related hepatoxicity and affecting the selection of specific agents (Sulkowski, 2008). HIV-infected persons also have increased rates of HBV replication and accelerated disease progression leading to cirrhosis, liver fibrosis, HCC, end-stage liver disease and liver-related deaths compared with HBV mono-infected persons. Therefore, WHO has recommended screening of HIV infected persons with infection of HBV regardless of the stage of diseases. HIV-infected patients have lower rates of anti-HBs seroconversion and anti-HBe than non-HIV-infected persons resulting in higher rate of chronic HBV. Tenofovir, which is included

in the treatment combinations recommended in first intention against HIV infection, is also active against HBV.

1.9.2) HBV/HDV co-infection:

Hepatitis delta virus (HDV) is a replication-defective HBV-dependent single-stranded (ss) RNA virus, which requires simultaneous presence of HBV to propagate. HDV is common among the HBV infected persons with low viral replication, rapid inflammation and progression of liver disease in spite of better response to anti-viral treatment. It was supposed that HBV replication is suppressed by HDV infection via the inhibition of host DNA-dependent RNA polymerase II by the large delta antigen of HDV (Huo et al., 1998). Globally 15 million individuals are infected with HDV, among them approximately 5 % are chronic HBV carriers (Sheldon et al., 2009). HBV-HDV co-infection is associated with an acute self-limited illness that leads to the development of fulminant hepatic failure, whereas HDV infection with chronic HBV carrier, term as superinfection causes an exacerbation of hepatic disease. HDV superinfection results in severe and active progression of liver damage and may increase the mortality due to end-stage liver disease (Huo et al., 1998). Liver disease usually developed due to the failure of immune system to clear hepatitis viruses imposed on the liver. However, the full mechanism of how these viruses trigger hepatocarcinogenesis is not understood yet. Different mechanisms have been proposed for the explaining development and progression of HCC by HCV, HBV and HDV infection. These include continuous antiviral inflammatory responses, ongoing immune clearance of infected cells and hepatocyte regeneration which all occur during chronic infection and lead to genetic and epigenetic changes that put patients at increased risk for HCC progression (Mesri, Feitelson, & Munger, 2014).

HDV infection can also be protected by vaccination against HBV infection. HDV is known to have an eccentric propagation process and uses the host cellular polymerase. Because of which, HDV has become a challenging target for antiviral therapy. Treatment of chronic HDV infections is currently limited to extensive alpha-interferon therapy or in extreme situations, to liver transplantation (Farci, 2003). Lamivudine an antiviral nucleosides analog that is used for the reduction of HBV replication have not been successful in reducing HDV replication (Taylor, 2018). Similarly, treatment with famciclovir (FCV) was not effective against HDV infection. Clevudine is used for rapid reduction of serum

HBV DNA, HBsAg and cccDNA levels. In addition, it inhibits HDV RNA and also reduces HDV replication (Sheldon et al., 2008).

1.9.3) HBV/HCV co-infection:

HBV/HCV co-infection indicates the progression of liver fibrosis and development of cirrhosis. It is also the main factor for the development of HCC. HBV/HCV co-infection results 80 % of death from liver cancer and was the third most common cause of cancer mortality (Lazarus et al., 2018). In HBV/HCV, co-infection there is interplay between two viruses. HCV core protein can act as a gene-regulatory protein and negatively influence the expression of HBsAg. Indeed, there is still controversy in development of occult hepatitis B infection (OBI) in HCV co-infected patients. The emergence of HBV mutation can be limited by inhibitory effect of HCV because the decreased replication would produce less mutant genomes. This was, so far, confirmed for BCP/precore mutations, which were found less frequently in HBV/HCV co-infected than in HBV monoinfected patients

1.10) Natural history of HBV infection:

1.10.1) Acute hepatitis B infection:

The persistence of HBsAg in serum for less than six months is known as acute infection. It is also detected by the presence of IgM core antibody and anti-HBc in the window period. HBsAg is first detectable serological marker in the serum followed by the presence of HBeAg and HBV DNA. HBsAg can be detected from 1-2 weeks up to 11-12 weeks after exposure. HBeAg indicates the high replicative and infectivity stage of virus (Dna & Dna, 2002). Acute HBV infection can be detected approximately in one-third of patients with the development of clinical signs and symptoms while in two-thirds it remains undetectable with asymptomatic and subclinical illness. Acute hepatitis has the incubation period of averages of 8-12 weeks and can lasts for 4-24 weeks. Incubation period can be extent depending upon the titer of virus exposure and followed by short pre-icteric period. In this period, the patient has the symptoms like fever, anorexia, vomiting, fatigue, nausea, alterations in taste, anthralgias, malaise, urticoria and pruritus with onsets of jaundice and dark urine. Biochemically, during this period serum ALT and AST level begins to rise in between 500- 5000U/L, bilirubin above 10 mg/dL, ALP level and prothrombin time are normal while albumin level is normal or slightly decreases. Also high titer of HBV DNA

and HBsAg are detected in the serum of the patients. The pre-icteric period is followed by averaging 1-2 weeks of icteric period. During this period, HBsAg and HBV DNA are still present in serum but levels of virus decreases. The patient experiences predominant gastrointestinal symptoms, malaise and development of right upper quadrant (RUQ) pain with hematomegaly. Antibody to HBcAg (anti-HBc) and Antibody to HBeAg (anti-HBeAg) start to appear before the onset of clinical illness and after the clearance of HBeAg respectively. At the same time, level of anti-IgM decreases and anti-IgG starts to increase. Thus, the development of antibodies and loss of HBeAg indicates the initiation of recovery of acute infection. In convalescence period, icterus as well as jaundice resolves. HBsAg and HBV DNA are cleared from the serum followed by the appearance of antibody to HBsAg (anti-HBs) which indicates the development of immunity against HBV infection. Anti-HBs is not detected in 10-15 % of patients after the recovery but detection of anti-HBc indicates previous infection (Navabakhsh et al., 2011).

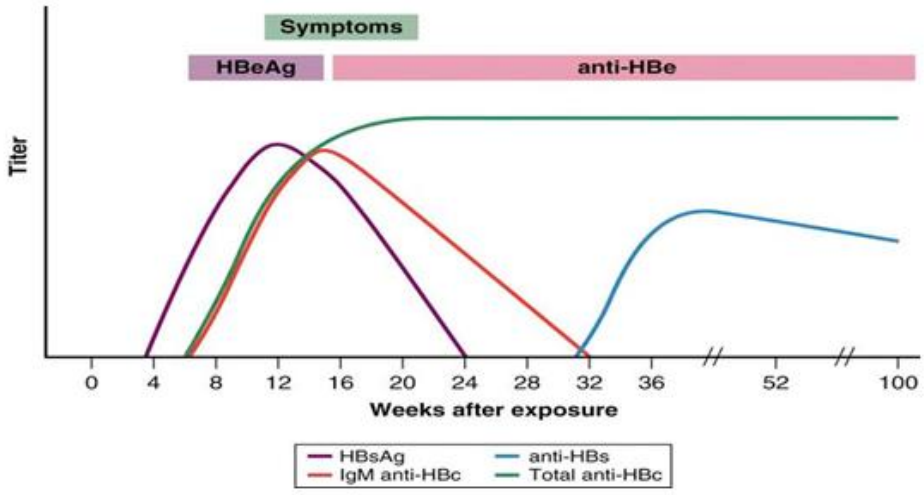

Figure 1.5: Characteristics of acute hepatitis B with recovery.

1.10.2) Chronic hepatitis B infection:

The presence of HBsAg in serum for more than 6 months is known as chronic hepatitis B infection. Chronic hepatitis infection follows the similar incipient pattern of serological markers as that of an acute hepatitis B infection. The appearance of HBsAg, HBeAg, anti-HBc and HBV DNA are also similar to an acute infection but are detectable in high titer in serum of chronic patients. Chronic hepatitis B infection is divided into five phase: im-

mune tolerant phase, immune reactive HBeAg-positive phase, inactive HBV carrier state, HBeAg-negative CHB and HBsAg-negative phase.

In immune tolerant phase, viral replication persists and has a high titer of HBV DNA (> 10^5 copies/ml). The patient is highly contagious, has normal or low level of ALT and AST, mild liver necroinflammation, and slow liver fibrosis (B J McMahon, 2005). The immune response is limited to anti-HBc antibody production primary IgM and then secondary IgG, but this does not act to neutralize infection.

Immune reactive HBeAg positive phase or chronic hepatitis B phase is associated with elevated ALT, presence of HBeAg and anti-HBe. The patient has moderate to severe liver necroinflammation with rapid progression of liver fibrosis. During this phase, the immune response reduces HBV replication and begins to clear HBeAg and HBsAg that occurs at a rate of 10–15% and 0.5–1% per year respectively) (Giacchino & Cappelli, 2010).

This phase ends with the seroconversion of HBeAg to Anti-HBe called inactive HBV carrier phase. It is characterized by normalization of ALT level, fall of HBV DNA to < 10^5 copies/ml, inactive liver inflammation and disappearance of HBsAg at the rate of 0.5 % per year (Hoofnagle, Doo, Liang, Fleischer, & Lok, 2007).

The inactive carrier state can be reactivated either spontaneously or by immune suppression. This stage is followed by the loss of HBeAg, high HBV DNA level and increased ALT called HBeAg –negative CHB. This may developed a year or many after inactive carrier state. This stage is more common in patients having genotype B, C, D than with other genotypes (Hoofnagle et al., 2007).

Progression to clearance of both HBsAg and HBeAg is known as the 'HBsAg-negative phase'. HBV viral replication may persist but is unlikely to be detectable in serum. Once in the HBsAg-negative phase, there is an improved outcome and a reduced risk of liver complications, although HBV may reactivate in individuals receiving immunosuppressive therapy and still represents a risk for organ donation.

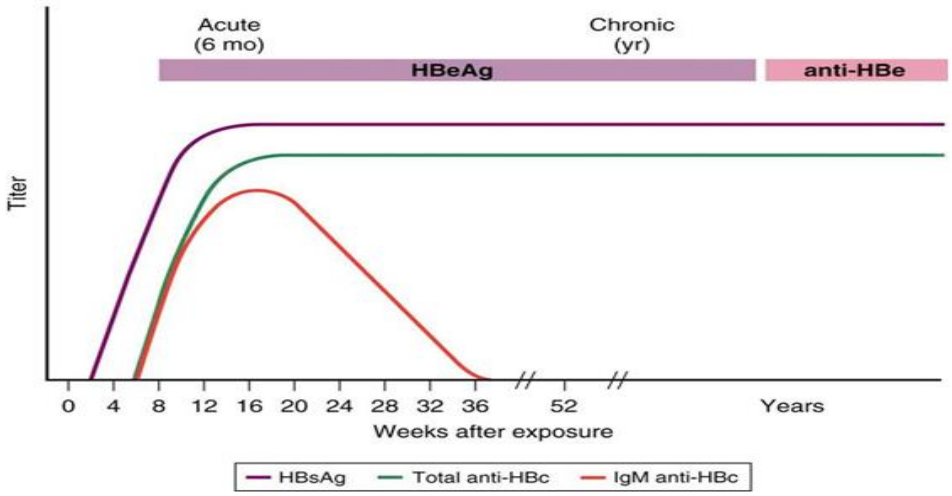

Figure 1.6: Characteristics of progression to chronic infection.

1.11) Occult hepatitis B infection:

Occult HBV infection (OBI) is defined as the presence of HBV DNA in liver with an absence of detectable HBsAg in the serum. The condition in which the amount of detectable HBV DNA in serum of OBI is relatively low below 200 IU/ml is term as true OBI. Similarly, the condition in which HBV DNA levels are similar to HBsAg but are HBsAg negative is termed as false OBI. Seropositive OBI is characterized by the presence of anti-HBc and anti-HBs, while neither anti-HBc nor anti-HBs is detected in seronegative OBI (Raimondo et al., 2008).

OBI is common among HIV and HCV patients, blood donor, cryptogenic liver disease and *dialysis patients. The molecular mechanisms involved in OBI are mutations in the "a" determinant of HBsAg, treatment-associated mutations, RNA splicing, and mutations in the pre-S region (Samal, Kandpal, & Vivekanandan, 2012).*

1.12) Diagnosis of HBV:

1.12.1) Serological test:

*HBV has different standard serological markers like HBsAg, HBeAg, Anti-HBc, IgM anti-*HBc, Anti-HBs, IgM Anti-HBs that are used for the measurement of HBV specific antigens or antibodies. These markers or combinations of markers are the mainstay for the diagnosis of different phases of HBV infection, to illuminate the natural course of chronic hepatitis B (CHB) and to monitor the antiviral therapy. HBsAg is the most reliable marker

of HBV carriage and HBeAg is the secondary marker used for the indication of high levels of virus. Anti-HBs indicates the recovery and immunity from HBV infection while Anti-HBc indicates previous or ongoing infection with HBV infection in an undefined period.

Table 1.1: Interpretation of hepatitis B serologic test results; Centers for Disease Control and Prevention (CDC).

Serological markers	Result	Interpretation
HBsAg Anti-HBc Anti-HBs	Negative Negative Negative	Susceptible
HBsAg Anti-HBc Anti-HBs	Negative Positive Positive	Immune to natural infection
HBsAg Anti-HBc Anti-HBs	Negative Negative Positive	Immune due to hepatitis B vaccination.
HBsAg Anti-HBc IgM anti-HBc Anti-HBs	Positive Positive Positive Negative	Acutely infected
HBsAg Anti-HBc IgM anti HBs Anti-HBs	Positive Positive Negative Negative	Chronically infected
HBsAg Anti-HBc Anti-HBs	Negative positive Negative	Interpretation unclear; four possibilities 1..Resolved infection 2.False positive anti-HBc 3".Low level" chronic infection 4. Resolving acute infection

1.12.2) Hepatitis B DNA Viral Load:

In the recent year, the level of HBV DNA in serum has been measured by molecular diagnostic methods using polymerase chain reaction technology. HBV DNA viral load is the markers for the replication activity of viruses and can be detected at the early stage of HBV infection. It reaches to peak level after three months of infection and gradually decreases in chronic infection. The detection and quantification of HBV DNA viral load has been a key part of the evaluation of patients with acute or chronic HBV infections. Quantification of the HBV DNA level provides prognostic information and useful in assessing or determining whether to initiate antiviral therapy. There is increased risk of progression to cirrhosis and HCC with individuals having high HBV DNA level. HBV DNA levels tend to be higher in HBeAg-positive patients compared with HBeAg-negative patients. A number of hybridization methodologies like real time PCR have been developed for monitoring HBV DNA levels. A wide dynamic range of viral load is detected by real –time PCR with lower range of 10-15 IU/ml and upper range of 10^7 -10^8 IU/ml. Therefore, it has become the standard and sensitive methods to monitor and quantify HBV DNA in clinical setting (Song & Kim, 2016). However, real –time PCR also helps to identify occult HBV infection in many cases.

Table 1.2: Different types of methods used for the quantification of HBV DNA (Source: WHO).

Methods	Commercial assay name	Measurable range (IU/ml)	Limits of detection (IU/ml) Using WHO HBV standard
Semi-automated qPCR	COBAS Ampliprep/COBAS TaqMan HBV test v2.0	20-1.7×10^7	20
Semi-automated real-time PCR	COBAS TaqMan HBV test for use with high pure system	29-1.1× 10^7	06
Automated real –time PCR	Abbott Real Time HBV	10-1 × 10^9	10
Branched DNA	VERSANT HBV 3.0 Assay	2,000-1 ×10^8	2,000

1.12.3) HBV Genotyping:

HBV genotype shows wide variation throughout the world due to geographical and ethical distribution. The pathogenesis of different HBV genotype and sub- genotype isn't fully understood. However, many data indicates that HBV genotype may affect the disease progression, risk for HCC and response to antiviral therapy. It has been suggested that HBV genotypes A and D are associated with a lower levels of both intracellular and extracellular HBV DNA and HBeAg as compared with genotype B and C. As a result, there is higher frequency of hepatocytes damage in patients with genotype B and C. In addition, genotype C cause more severe hepatic damage as compared to genotype B due to high replication capacity of virus. Further, genotype C and D show less respond to interferon (IFN) - α therapy than genotype A and B. In addition, it was reported that in patients with genotype C there is high level of ALT as compared to genotype B (Xibing, Xiaojuan, & Juanhua, 2013).

The main laboratory diagnoses of HBV genotype are molecular biological methods based on DNA detection. This includes techniques like reverse hybridization, INNO-LiPA, genotype-specific PCR assays, real-time PCR, restriction fragment-length polymorphism (RFLP), whole genome sequence analysis, microarray (DNA Chip), reverse dot blots and fluorescence polarization assay.

1.12.4) Liver Biopsy

Liver biopsy is a gold-stranded method, which gives useful information regarding HBV disease activity, hepatic pathology and initiation of anti-viral therapy. It is used to identify pathology that is associated with liver disease such as steatohepatitis, iron overload, autoimmune hepatitis, and drug-induced injury. It is not necessary in every patient diagnosed with HBV infection. As it is recommend only in selected patients based on age, HBV viral levels, and serological status like HBeAg negative and patients with mutation on HBV genome (Mani & Kleiner, 2009). In the case of chronic HBV, liver biopsy is necessary to assess serological and biochemical status of the HBV infection. These patients can be identified based on hepatic biochemical tests. In active chronic patients, there is usually high level of serum aminotransferase and HBV DNA greater than 10,000 IU/ml. On liver biopsy, they show inconsistent degrees of fibrosis and may be HBeAg positive or negative based on infection with wild type HBV or the precore mutant. As a result these

patients should be considered for antiviral therapy. Similarly, patients with inactive chronic infection have normal level of aminotransferase and undetectable HBV DNA lower than 10,000 IU/ml with HBeAg negative and anti-HBe positive. These patients lack inflammation and fibrosis on liver biopsy. Therefore, these patients shouldn't be considered for antiviral therapy (Campbell & Reddy, 2004).

1.12.5) Liver Function Test:

The most commonly used liver function tests are alanine transaminase (ALT) or serum glutamic pyruvate transaminase test (SGPT), aspartate aminotransferase (AST) or serum glutamic oxaloacetic transaminase test (SGOT), alkaline phosphatase (ALP), bilirubin total, bilirubin direct and albumin test. These tests are done in order to check the condition and overall function of liver. ALT and AST tests are done to measure enzymes released by the liver while albumin and bilirubin tests measures the protein, albumin and bilirubin.

1.12.5.1) Serum glutamic pyruvate transaminase:

Serum glutamic-pyruvate transaminase or Alanine transaminase test (SGPT/ALT) is blood test used for the detection of liver damage. It is present in high concentration in liver and to a lesser concentration in skeletal muscles, pancreas, heart, kidney, spleen and lung. It is used to metabolize protein in our body. It converts an amino acid called alanine found in proteins into pyruvate, an important intermediate in cellular energy production. Its level increases, if the liver is damaged or not functioning properly associated with some degree of hepatic necrosis such as viral or toxic hepatitis, non-alcoholic fatty acid (NAFLD), cirrhosis and obstructive jaundice. According to Mayo Clinic, the normal range of ALT is 7-55 units per liter (U/L). It has been suggested that gender-specific thresholds be applied because male have slightly more normal ALT levels than female. A study conducted in the U.S. states that an ALT upper limit of 29 IU/L for men and 22 IU/L for women (Ruhl & Everhart, 2012). However, the upper limit threshold of ALT level should be lowered because people who have slightly raised ALT levels that are within the upper limit of normal (35-40 IU/L) are at an increased risk of mortality from liver disease (Ruhl & Everhart, 2012). Characteristically ALT/SGPT is generally higher than AST/SGPT in acute viral or toxic hepatitis whereas for the most patients with chronic hepatic disease, ALT/SGPT levels are generally lower than AST/SGPT levels. Elevated

ALT/SGPT levels have also been found in extensive trauma and muscle disease, circulatory failure with shock, hypoxia, myocardial infraction and haemolytic disease. ALT, AST level and their ratio (ALT/AST) is clinically used as biomarkers for liver health.

1.12.5.2) Serum glutamic oxaloacetic transaminase:

Serum glutamic oxaloacetic transaminase or Aspartate transaminase (SGOT/ AST) is an enzyme found in the liver, brain, heart, muscles, kidneys and red blood cells. It makes bile that help to digest food and removes waste products and toxins from the body. It produces proteins and other substances that help to clot blood. The level of AST is low in our body. High levels of AST indicate the liver damage as well as heart and kidneys.

1.12.5.3)Alkaline phosphatase (ALP):

ALP is an enzyme which hydrolysis phosphatase at an alkaline pH. It exists in different form depending on its origination. It is found in all tissues throughout the body but in high concentrations in the liver, osteoblasts of bone, placenta of pregnant woman, kidney, intestinal walls and lactating mammary glands. It plays an important role in metabolism of phosphatase in liver and development within the skeleton. Its concentration in blood is used as biomarkers to diagnosis hepatitis and osteomalacia. The normal range of ALP varies within a person and depends on the gender, blood types and age. At 37˚C, the normal range of ALP for females within the age group of 4-15 years is 54-369 U/ L, 20-50 years is 42-98 U/L and above 60 years is 53-141 U/L. Similarly for males of 1-12 years is 54-369 U/L, 20-50 years is 53-128 U/L and above 60 years is 56-119 U/L (BUTTERWORTH, 1980). The normal range is higher in children of age group 4-15 years and decrease with age. It is also two to four times more during pregnancy. An abnormal level of ALP indicates a disease condition with liver, gallbladder and bones. However, they may also indicate malnutrition, kidney cancer tumors, intestinal issues, a pancreas problem, or a serious infection. ALP levels can be greatly increased in liver or bones diseases like cirrhosis, liver cancer, hepatitis, cholecystitis, Paget's disease, osteomalacia, rickets, primary hyperparathyroidism, osteogenic sarcoma. It is decreased in pernicious anemia, chronic myelogenous leukemia, Aplastic anemia, hypothyroidism and hypophosphatasia.

1.12.5.4) Bilirubin Total and Direct:

Bilirubin is the reddish yellow bile pigment formed by the breakdown of hemoglobin from senescent red blood cells (RBC) and myoglobin from muscle breakdown. It is excreted in urine and bile and is responsible for the yellow discoloration in jaundice and yellow color of bruises. It is carried to liver by the albumin. In the liver, bilirubin is conjugated with glucuronic acid and form direct bilirubin. It means it becomes water-soluble and can be excreted. It travels from liver into small intestine and a very small amount passes into kidney, which is then excreted in urine. Therefore, the amount of direct bilirubin present in serum in healthy individuals is trivial that is more than 10% of measured total bilirubin. Direct bilirubin gives urine its distinctive yellowish color. Bilirubin that is bound to the albumin in the blood is called unconjugated or indirect bilirubin. Conjugated or direct bilirubin is non-toxic while unconjugated or indirect bilirubin is toxic to the body, as it is not excreted throughout the body. All these direct and indirect bilirubin in the blood form the total bilirubin. The normal range of direct bilirubin is 0-0.4 mg/dL; total bilirubin is 0.3-1.0 mg/dL. An elevated level of direct bilirubin implies liver disease. In addition, it is important to note that only direct bilirubin appears in urine. The presence of bilirubin in urine usually implies liver disease. High level of bilirubin is due to medical conditions like hepatitis, cirrhosis, gallstones, Gilbert's syndrome, cancer of gallbladder, hemolytic anemia, transfusion reaction and drug toxicity. But the level of bilirubin is high in newly born baby due to stress of birth. Low level of bilirubin is due to theophylline, phenobarbital and increased vitamin C levels.

Elevations of the indirect bilirubin level when the direct bilirubin level remains normal may also indicate an increased load of bilirubin caused by hemolysis. Anemia and an elevated reticulocyte count are usually present in such cases as shown in below table 1.3

Table 1.3: Common Causes of Isolated Bilirubin Elevation

Causes	Direct bilirubin	Indirect-bilirubin	Associated features
Liver diseases	Elevated	Elevated or normal	Liver enzyme levels often elevated

Hemolysis	Normal	Elevation represents more than 90% of total bilirubin.	Anemia usual increased reticulocyte count, normal liver enzymes level (although LDH may be elevated.
Gilbert's syndrome	Normal	Elevation represents more than 90% of total bilirubin (common).	No abnormal liver tests; no anemia; onset in late adolescence; fasting makes bilirubin rise

1.13) Treatment:

No specific treatment has been recommended for acute hepatitis B infection. While chronic infection can be treated with oral antiviral drugs like Tenofovir and entecavir. These drugs are the most known potent drugs for the suppression of HBV infection and also delay the progression of cirrhosis, liver cancer, and enhance the long-term survival. These drugs show few side effects and rarely lead to drug resistances as compared with other drugs. The most common side effects in HBV patients who use Tenofovir are nausea, rash, pain, diarrhea, headache, depression and weakness. Others common side effects in patients with advanced HBV infection include vomiting, itching, sleeping problems, dizziness and fever.

Tenofovir is nucleoside reverse transcriptase inhibitors (NRTIs) drug, which is approved by US Food and Drug Administration (FDA) for the HIV treatment. It is also hepatitis B reverse transcriptase inhibitors (RTI) and recommended by WHO for the treatment of HBV infection in people 12 years old or older. Tenofovir works in similar ways for both HIV and chronic HBV infection. It blocks reverse transcriptase and prevents both HIV and HBV from multiplying, and can lower the amount of HIV and HBV in the body. Tenofovir can increase the amount of CD4 cells in patients. As this drug doesn't reduce the risk of passing HBV infection to others. Tenofovir is highly restricted to patients having kidney diseases, pregnant woman, women who are breast-feeding and older people above 65 years. It is because Tenofovir is filter through kidneys and passes through breast milk to child who could have serious side effects on both kidney damage patients and child who is breastfed. Also in older people, this drug is proceed slowly.

Entecavir is also nucleoside reverse transcriptase inhibitors (NRTIs) drug and works in similar way as Tenofovir do. It is highly recommended for chronic HBV patients in adults and patients with HIV-HBV co-infection. It is also used to treat pediatric children of 2 years and older with HBeAg positive and patients having liver disease with high liver enzymes levels (Ohba et al., 1995). It also prevents the reinfection of HBV after liver transplantation and HIV patients infected with HBV.

1.14) Research Hypothesis:

The present vision of research is to better understand the virology and clinical features of hepatitis B virus and to eliminate it as public health problem.

1.14.1) Null hypothesis:

The epidemiological, serological, immunological and molecular profiles of HBV might not help in diagnosing and monitoring viral hepatitis B as public health problems.

1.14.2) Alternative hypothesis:

The epidemiological, serological, immunological and molecular profiles of HBV help in diagnosing and monitoring viral hepatitis B as public health problems.

1.15) Research Objectives:

1.15.1) General objectives:

Study of epidemiological, serological, immunological and molecular profiles of hepatitis B virus in Nepal.

1.15.2) Specifics objectives:

a) Study of geographical and demographical distribution of HBV among Nepalese population.

b) Estimation of sero- prevalence of the serological markers like anti-HBc, HBsAg and HBeAg in Nepalese population by RDT and ELISA.

c) Collection of details of biochemical tests of HBV positive patients.

d) DNA Extraction of HBV

e) Quantification of HBV DNA.

f) Genotyping of circulating Hepatitis B virus.

g) PCR of the extracted sample.

h) Sequencing of the sample.

1.16) Rationale of the study:

Hepatitis B virus (HBV) is a serious public health problem worldwide and major cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC). Because of the high HBV related morbidity and mortality the global disease burden of hepatitis is substantial. There are many problems associated with the current methods of screening that may account the lack of public awareness leading to increased risk of transmission of hepatitis B infection. Nepal still lacks a facility for the estimation of HBV viral load and genotyping and is entirely dependent on foreign commercial laboratories. The need for exporting the samples to foreign countries for molecular testing often leads to sample degradation and inaccurate results. The challenges in the area of HBV-associated disease are the lack of knowledge in predicting outcome and progression of HBV infection and an unmet need to understand the molecular, cellular, immunological, and genetic basis of various disease manifestations associated with HBV infection. Therefore, this research will help to study and analysis of the patterns, causes and effects of health conditions in defined population of Nepal. The detection of hepatitis B virus DNA will play a significant role in diagnosis and monitoring infection related to HBV. It will also help to assessing therapeutic responses. Genotyping of HBV helps in understanding of geographical distribution of HBV infection.

CHAPTER TWO

LITERATURE REVIEW

2.1) Epidemiology:

Globally, HBV has become one of the life-threatening diseases in this century. According to World Health Organization (2018) an estimated 257 million people all around the world have been infected and around 887,000 deaths resulted due to hepatitis B virus in 2015. Approximately 30 % of world's population has serological evidence of acute and chronic HBV infection. It is the most common chronic viral infection in the world. 650, 000 from cirrhosis and liver cancer due to chronic hepatitis B infection and another 130 000 from acute hepatitis B. In the 2010 Global Burden of Disease study, HBV infection ranked in the top health priorities in the world, and was the tenth leading cause of death (786, 000 deaths per year). These data have led WHO to include viral hepatitis in its major public health priorities.

2.2) HBV Scenario of World:

Globally the prevalence of HBV infection is divided into three different geographical categories in terms of positivity of HBsAg in chronically infected people: highly endemic area with more than 8% HBsAg positive chronic carrier, moderate endemic area with 2-8 % and low endemic area with less than 2 % HBsAg positive chronic carrier. These categories help in understanding the incidence, patterns of transmission, possible control of diseases and other factors relating to consequences of HBV infection (Maclachlan & Cowie, 2015).

2.2.1) Highly endemic areas:

Those areas that have the positivity of HBsAg in more than 8 % of total populations are categories as highly endemic areas. According to WHO (2018), the prevalence of HBV is high in Western Pacific Region, Asia Pacific and sub- Saharan African Region. It has been estimated that globally 45 % of the world's population live in these areas. HBV infection has become third major causes of death in Pacific Region, Asia and Africa (McGlynn, Tsao, Hsing, Devesa, & Fraumeni, 2001) leading to 60-80 % of liver cancer in adults

(Chang et al., 2009). The lifetime risk of HBV infection is more than 60% in these areas and most of individuals are infected during childhood or infancy and have high rate of HCC (Lavanchy, 2009). This is because most early childhood HBV infections are asymptomatic and there is little recognition of acute disease. HBV infection is associated with 80-90 % cases in Southeast Asian countries such as India, Vietnam and Singapore as well as in China and Korea. The mechanisms of early childhood transmission in areas of high endemicity are variable because infections cluster in households of persons with chronic infection. Perinatal or vertical mode of transmission is common in Southeast Asian countries where women are highly affected than men. However, horizontal transmission occurs more frequently in African countries. This is because HBeAg positivity rate among pregnant woman is high in Southeast Asia as compared in Africa. In East and Southeast Asian countries as well as the Pacific, 35 to 50 % of HBsAg-positive women are HBeAg positive (Giacchino & Cappelli, 2010). It is estimated that 3 to 5% of all infants in these countries may develop chronic HBV infection at birth and that up-to 30 to 50% of all chronic infections among children may result from perinatal transmission. The prevalence rate of perinatal transmission is low in African countries with 8.3 % in Tanzania, 8.0 % in Ghana and 7.0 % in Senegal whereas more than 100 % transmission occurs in Southeast Asian countries (Anna Kramvis & Clements, 2010). Vaccination program against HBV infection isn't introduced into national immunization programs in many countries of highly endemic areas. Also, in these areas there is lack of awareness regarding the routine childhood immunization against HBV (Goldstein et al., 2005). Therefore, HBV is common among newborn infants and young children. If a mother is both HBsAg and HBeAg positive, 70 to 90% of infants will become infected if not given immunoprophylaxis. Approximately 5 to 20 % of children are infected at birth, if the mother is HBsAg-positive but HBeAg negative. Infants of HBsAg-positive women who aren't infected at birth are at increased risk of HBV infection during early childhood because of household contact with infected persons. However, those countries having high impact of immunization against HBV, there is markedly reduction in prevalence of HBsAg and chronic liver cancer. In China, the positivity of HBsAg was 6.64 % between the age group of 1-29 years (Yue & Mingshou, 2017). China has both low and high endemicity of HBV infection. In Beijing and Shanghai of China, the HBsAg positivity rate is as low as 3.03%,

while in western and southern provinces of China, it is greater than 8 % (Lu, Li, Liu, & Zhuang, 2010).

2.2.2) Moderate endemic areas:

Those areas that have a positivity of HBsAg in 2-7 % of total populations are categories as moderate endemic areas. It includes North Africa, Middle East, parts of Eastern and Southern Europe, the Eastern Mediterranean area and the Indian subcontinent. Approximately 43 % of world's populations live in these areas. The lifetime risk of HBV infection is 20 to 60 % and infections occur in all age groups. This prevalence is similar in proportion to the global population of high endemic areas (Trépo, Chan, & Lok, 2014). Both perinatal and horizontal mode of transmission is predominant in these areas. However, the perinatal transmission is low that is 10 to 20 % as compared to high endemic areas. The prevalence of HBsAg positivity is 2 to 7 % among pregnant women and 20 % of women are both HBsAg and HBeAg positive (Mahoney, 1999). HBV transmission among the children in early age may be variable in different regions or among different ethnic groups within a country. It has been recorded that most European countries have low endemicity of HBV infection after the impact of vaccination programs in their national immunization programs (Salleras et al., 2005) .

2.2.3) Low endemic areas:

Areas having 2 % HBsAg positivity of total populations are categories in low endemic areas and include North America, Western Europe, Australia, some countries in South America, and Japan. Only 12 % of world's total populations live in these areas. The lifetime risks of HBV infection is less than 20 % and most infections occur in adolescents and adult. Horizontal mode of transmission is predominant in these areas among intravenous drug user, unsafe homosexual and heterosexual contact with multiple partners, household contacts of persons with chronic HBV infection, hemophiliacs, hemodialysis patients and health workers (Gust, 1996). Other sources of infection include contaminated surgical instruments and donor organs. Health care workers, dentists, and others who have frequent contact with infected blood or blood products are at highest risk. One- third of chronic infection in United States occurs through the perinatal route during childhood while acute infection occurs among adults. The prevalence of chronic HBV infection is about 0.1 - 2.0 % in the United States and Western Europe. Immigration has

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pronounced effect on prevalence of chronic HBV infection in these areas. It was estimated that immigration accounts 90 % of newly diagnosed cases of chronic HBV infection in U.S. Most of the imported cases were from highly endemic countries such as Philippines, China, and Vietnam which contributed 13.4%, 12.5%, and 11.0%, respectively (Mitchell, Armstrong, Hu, Wasley, & Painter, 2011).

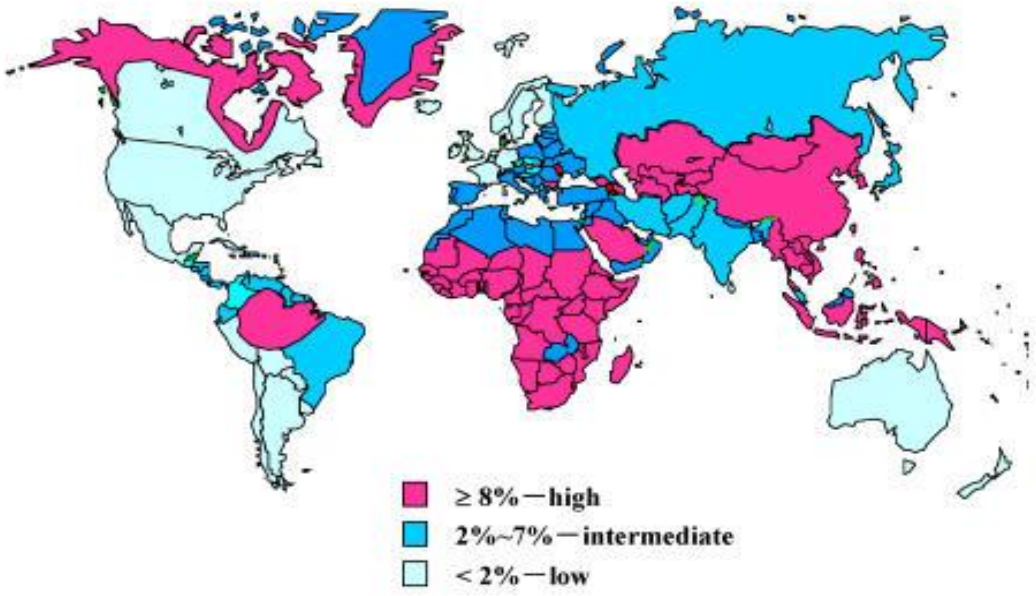


Figure 2.1: Geographical distribution of HBV infection based on positivity of HBsAg.

2.3) HBV Scenario in Nepal:

Nepal is a small Himalayan country sandwiched between China in the north and India in the south. China has the highest prevalence of HBV infection with the disease burden of nearly 90 million (Yue & Mingshou, 2017) while India has the intermediate prevalence with the disease burden of nearly 50 million (Ray, 2017). Nepal has the low prevalence rate of HBV infection (Santosh Man Shrestha & Shrestha, 2012). As there is no any national representative data to confirm the prevalence rate of HBV infection among the Nepalese people. However, it was recorded that rural areas have highest prevalence of HBsAg and anti-HBc than in urban (Bidya, 2002).

The prevalence of HBsAg among Nepalese samples was first done at Yale University in 1973. HBsAg was found positive in these samples and reported HBV was epidemic in Nepal. A 1990 community seroprevalence study was the first recorded survey done on

the prevalence of HBV infection in Nepal. It showed an overall prevalence of HBsAg positivity of 0.9 % on an average 1.6 % was among males and 0.5 % among females (A. Shrestha, 2016). However, various studies conducted from 1990 to 2003 showed variable seroprevalence of HBsAg in Nepal ranging from 0.3 % to 4.0 % (S. Khan, Siddiqui, & Ansari, 2012). Similarly, the prevalence of HBV among blood donors in whole Nepal and Kathmandu was found to be 0.82 % and 0.92 % respectively (Kandel Sr, P Ghimire, Tiwari Br, 2013).

The prevalence of HBV infection is heterogeneous in Nepal due to diversity within regions as well as in ethnic groups. It was reported that HBV infection is highly prevalence among some ethnic group of Nepal as compared to general population. Sherpas residing at Mt. Everest Base camp and Gurung from Manang district have intermediate prevalence with HBsAg positivity of 7.35 % and 3.5 % respectively. The prevalence of Anti-HBs and Anti-HBc among these Sherpas are 22.3 % and 24.3 % respectively. Similarly, high prevalence of HBsAg positivity ranging from 21 to 38 % was reported among subpopulation in mountain region of Nepal by various studies (Shedain, Devkota, Banjara, Ling, & Dhital, 2017). Similarly, the Tharus residing at Surkhet have intermediate prevalence of HBsAg. A study conducted among healthy Nepalese males showed that Eastern development region of Nepal had highest rate of HBsAg positivity and this prevalence decreased steadily to Far western development regions. Kailali district of Far western development region was highly infected followed by Rukum and Kaski. Similarly others districts having high HBsAg positivity were Jhapa, Sankhuwasabha, Ramechhap, Sarlahi, Dhanusha, Baglung, Gulmi, Palpa and Dang (Manandhar K & Shrestha B, 2000).

Horizontal mode of transmission of HBV infection is predominant among adults in Nepal. It is noted during early childhood among infected sibling, relatives, family, intravenous drug user, blood donors and sex workers. The positivity of HBsAg among siblings, offspring and spouses was 31 %, 20.5 % and 17.7 % respectively. Commercial sex workers, drug addicts, hemodialysis patients, soldiers and health workers were also included in high-risk groups for HBV infection in Nepal as in other countries with HBsAg positivity of 10.9 % , 3-6%, 2%, 3% and 2% respectively. Perinatal or vertical mode of transmission is low because the rate of HBsAg positivity among pregnant women is 0.5 % with HBeAg negative. However, other studies reported that prevalence of HBV is high among HBsAg

positive mothers and their children in the indigenous population of the most remote mountain community of Nepal. It is also reported that HBsAg carrier rate is lower in Nepal as compared to other Asian countries. Adults between age group of 21-30 years showed the highest HBV infection (A. C. Shrestha, Ghimre, Tiwari, & Rajkarnikar, 2009).

HBV accounts for 4 % of acute hepatitis infection. People with chronic HBV infection have high risk of developing liver cirrhosis and hepatocellular carcinoma. The most important etiological agents of chronic liver cancer in world are HBV, HCV and alcohol. HBV infection accounts for 40% of the liver cirrhosis in Nepal. A study conducted between 1990 to 1997 showed that two common causes of liver diseases in Nepal were alcohol and Hepatic IVC Disease (HVD) (S. M. Shrestha, 2005). HBV is found to be responsible cause of Jaundice in Nepal.

The prevalence of co-infections associated with HBV infection is variable in the different geographical area around the world. However, very few studies have been conducted regarding with occurrence of co-infections with HBV virus in most countries of the world including Nepal. A study conducted among volunteer blood donors in Nepal showed that the prevalence of HBV/HIV co-infection was 0.033% (Thapa & Ghimire, 2008). Very few studies regarding the incidence of co-infection of HBV and HCV in HIV patients has been reported in Nepal. However, no any study concerning to incidence of co-infections with these viruses has been done in western Nepal till date. The transmission of HBV is higher among unsafe sexual partner and IDUs as compared to HCV. Therefore, the incidence of HBV among drug addicts becomes the main concern in Nepal. Co-infection of HBV/HCV/HIV has the highest risk of acute and chronic liver cancer, cirrhosis and hepatic failure in comparison to monoinfection. It was reported that male were significantly at a higher risk to develop HBV co-infection than female (Supram, Gokhale, Sathian, & Bhatta, 2015).

Three major genotypes of HBV in Nepal were found to be A, C and D. Despite being a low prevalence area, Nepal has a diversity of hepatitis B genotypes (Paudel et al., 2015). 'ayw', 'adw' are the common HBV sub-genotype in Nepal. 47 % of HBsAg were 'ayw', 34.3 % were 'adw' and 4 % were 'adr'. The remaining 15 % 'ad', 'ay', and 'a' were rarely occurring subtypes in Nepal (Hadiwandowo et al., 1994) .

2.4) Genotypes of HBV:

HBV is classified into ten (A-J) genotypes and forty sub-genotypes based on nucleotides sequences divergences of more than 8% and at least 4% respectively in whole genome. The emergence of HBV genotypes and sub-genotypes is due to the lack of proof reading activity of DNA and RNA dependent DNA polymerase and mis-incorporation of nucleotides during viral replication. Each genotype and sub-genotype of HBV has distinct geographical and ethnic distribution (Jung & Pape, 2002). Genotypes and sub genotypes also give us the information about disease progression, clinical progression, antiviral treatment and outcomes of disease. Genotype A is distributed globally and frequently occurs in Central Africa, Europe, and India. Genotypes B and C occur frequently in highly endemic areas like Asian countries and Australia where perinatal or vertical transmission are the main vehicles for the transmission of virus. Genotype E is predominant in West Africa, and F is common among American native, Polynesia and Central and South America. Genotype G has been reported in France, Germany, and the United States. Genotype H was described in Central and South America (Panduro, Maldonado-Gonzalez, Fierro, & Roman, 2013). Genotype I and J are predominant in Vietnam and Laos (Raimondo et al., 2008). Moreover, the final genotype H has been reported from the Ryukyu Islands in Japan (Sunbul, 2014). Genotypes A, D, E, F, and G are frequently found in areas where horizontal transmission is the main mode of transmission (Kao, Chen, & Chen, 2009). Genotype H is prevalent in an area of low endemicity of HBV infection.

The presence of different genotypes within a same geographical area results in recombination between genotypes in a host following simultaneous co-infection of several genotypes or from superinfection with different genotypes (Kidd-Ljunggren, Miyakawa, & Kidd, 2002). Recombination is the key factor for the evolution and genesis of HBV genotypes and sub-genotypes. It provides a mechanism by which virus can improve their fitness by fighting with the host immune response which further results in the evolution of drug resistant virus strain or strain with enhanced transmissibility and pathogenicity. The recombinant hybrid genotype B/C has been reported from the East Asia; Japan (Sakamoto et al., 2007), C/D from Tibet (Muhammad, Rashid, & Salih, 2014), A/D from Italy and Northern India and D/E from the Arica, D/C from the Eastern India (Ghosh et al., 2013).

Each genotype of HBV has a distinct genomic length. Genotype A is 3221 nucleotide long with the insertion of six nucleotides in the terminus of polymerase gene and core gene. Genotypes B, C, F and H have genomic length of 3215 nucleotides. Genotype D is 3182 nucleotides long while genotype E has genomic length of 3212 nucleotides. Moreover, genotype G is 3248 nucleotides long. These three genotype D, E and G have the nucleotide deletion of thirty-three and three nucleotides deletion in preS1 region of polymerase gene respectively (Bartholomeusz & Schaefer, 2004).

HBV was categorized into nine subtypes, i.e., ayw1 to ayw4, ayr, adw2, adw4, $adrq^-$, and $adrq^+$, according to the antigenic determinants (Matuszewski, 2008). The full nucleotide sequence of 18 HBV strains was first compared by Okomato and his colleagues and found that these clustered into four groups, genotype A, B, C and D with more than 8 % divergence between the groups (Okamoto et al., 1988). This degree of divergence has since become the definition criterion for HBV genotypes.

All the genotypes of HBV have sub-genotypes expect genotypes E, G and H. Genotypes B and C have at least 9 and 16 sub-genotypes respectively. Genotypes I and J are newly reported genotypes. In genotypes A, B, C, D, F, respective sub-genotypes Aa/A1, Ae/A2, Bj/B1, Ba/B2, B3, B4, B5, Ce/C1, Cs/C2, C3, C4, D1-D9, and F1-F4 differ widely in virologic aspects.

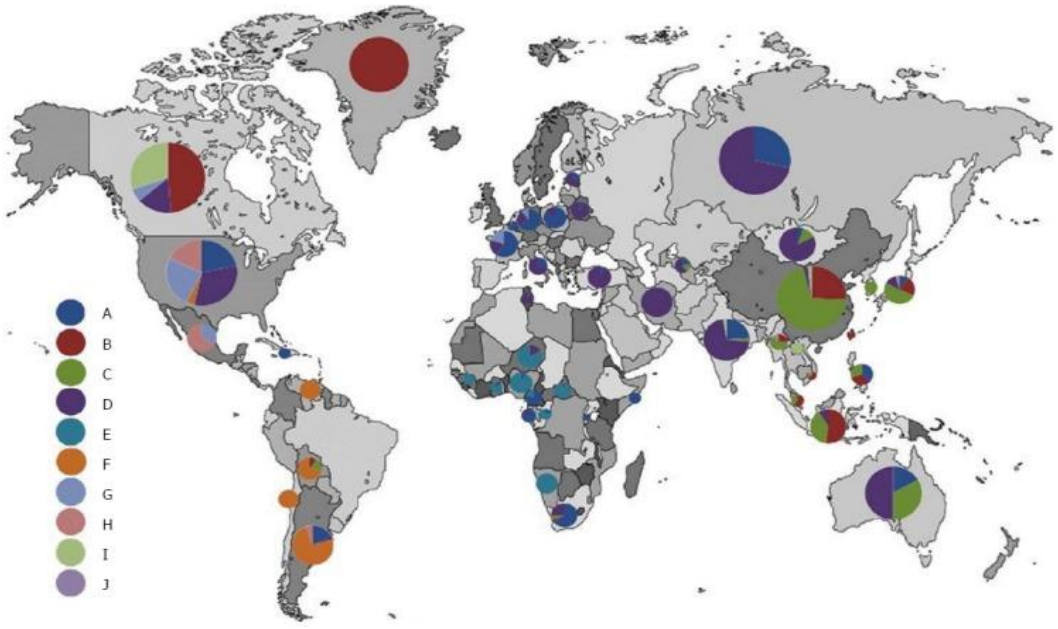


Figure 2.2: Worldwide Geographical distribution of HBV genotypes

Genotype A:

Genotype A consists of six (A1-A6) sub-genotypes. Sub-genotype A1 is predominant in South Africa, Malawi, Somalia, Brazil (Anna Kramvis & Paraskevis, 2013), Nepal, Yemen, India (Gopalakrishnan et al., 2013). Majority of sub- genotype A1 belongs to 'adw2' serotype (Kimbi, Kramvis, & Kew, 2004) but fewer belongs to 'ayw' serotypes isolated from Philippians (Sugauchi et al., 2004). Sub-genotype A2 is common in Northern Europe, the U.S.A, Alaska and Greenland (Schaefer, Magnius, & Norder, 2009) while sub-genotype A3 in West Africa (Fuat Kurbanov et al., 2005). Sub-genotypes A4 and A5 was recorded from Mali and Nigeria (Andernach, Nolte, Pape, & Muller, 2009) and sub-genotype A6 has been found to be circulating in African-Belgian patients. Genotype A lacks the precore mutation, which consists of G to A substitution at 1896, that creates a stop codon. However, it consists of C at position 1858 which stabilize the precore loop. Carboxyl terminal of HBV core gene contains a 6 nucleotide insert in genotype A which is absent in other genotypes (A Kramvis, Weitzmann, Owiredu, & Kew, 2002).

Genotype B:

Genotype B is 3215 nucleotide long and is divided into B1-B9 sub-genotypes (Thedja et al., 2011). The sub-genotype B1 is predominant in Japan, B2 in China and is widely distributed in Asian countries, B3 in Indonesia, B4 in Vietnam and sub-genotype B5 in Philippines. Similarly, a newly discovered sub-genotype B6 is found in Alaska, Canada and Greenland. Sub-genotype B7, B8 and B9 has been isolated from Indonesia (Thedja et al., 2011). Sub-genotype B1 is considered as a pure strain as there is no recombination in this sub-genotype. The precore/core ORF of the sub-genotypes B2-B5 have a genotype C recombinant region (Sugauchi et al., 2003). The difference between sub-genotype B1 and B2 is the presence of G at nucleotide 1838 in sub-genotype B1 while A at nucleotide 1838 in sub-genotype B2. Sub-genotypes B1, B2 and B3 have adw serotype while 'ayw' serotype was found among the sub-genotypes B4 and B5 (W. C. Liu et al., 2007).

Genotype C

Genotype C is 3215 base pair long and has 16 sub-genotypes. It is predominant in Eastern and Southern Asia especially in Indian subcontinent (Miyakawa & Mizokami, 2003), in pacific islands and in people who migrate from these areas in the U.S.A, Australia,

New Zealand and other European countries. Sub-genotype C1 is common in Vietnam, Myanmar and Thailand, C2 in Japan, Korea and China and sub-genotype C3 dominates the Micronesia, Melanesia and Polynesia (Schaefer, 2005). Sub-genotype C4 is dominant in Australia, C5 in Vietnam and Philippines. Sub-genotype C6 was discovered in Papua of Indonesia (Lusida et al., 2008). Sub-genotype C7 to C16 was also recorded from Indonesia (W. Shi et al., 2012). It has been observed that genotype C with C mutation at 1858 instead of usual mutation of T at 1858 has been found in 10-25 % of East Asia patients infected with genotype C (U.S., S., & A.S.F., 1994).

Genotype D

Genotype D is widely distributed throughout the world while its sub-genotypes show distinct geographical distribution. It is predominant in Northern Asia, Eastern Europe, Russia, Middle East, Asia, Mediterranean region and North Africa (Anna Kramvis & Kew, 2007). Genotype D has 6 sub-genotypes D1-D6. Sub-genotype D1 is predominant in Europe, Middle East, Egypt, India, and Pakistan, D2 in Europe, Japan and India, D3 in Europe, South Africa, United States, India and Pakistan, D4 in Australia, Japan and Papua (W. Shi et al., 2012). Sub-genotype D5 has been reported from India (Datta, 2008), D6 from Kenya, Russia and Indonesia, D7 is common in Morocco and Tunisia. Sub- genotype D8 and D9 was reported from India and Nigeria. Sub-genotype D9 was a recombinant of genotype D & C (Ghosh et al., 2013). Sub-genotype has ayw2 serotype and D2 have ayw3 serotype (Anna Kramvis & Kew, 2007).

Genotype E

Genotype E is predominant genotype of West and central Africa (Fujiwara et al., 2005). It was also found in India (Singh et al., 2009). Genotype E is known to have a very low genetic diversity (Brian J. McMahon, 2009) and no any sub-genotype has been reported yet. It has ayw4 serotype. This genotype has distinguished features like deletion of 3 nucleotides at the N-terminal region of preS region and presence of a nucleotide motif 'CCAGCTTCC' at 18 nucleotides upstream from the stop codon of core gene. It was recorded that patients with anti-HBe positive infected with genotype E have 1856 mutation (Anna Kramvis & Kew, 2005). It is hypothesize that genotype E has been strain of chim-

panzee and other non-human primates and may have been entered in human population or vice versa (Takahashi, Brotman, Usuda, Mishiro, & Prince, 2000).

Genotype F

Genotype F is known as "new world" genotypes and has highest divergence of 14 % from other genotypes. It is predominant among indigenous population of North & South America. It consists of four sub-genotypes F1-F4 (Santos et al., 2010) and has adw4 serotype (Anna Kramvis & Kew, 2007). Sub-genotype F1 is common in Alaska, Argentina and Bolivia, F2 in Venezuela and Brazil, F3 is found in Venezuela, Columbia and Panama whereas sub-genotype F4 is predominant in Argentina and Bolivia (A. Khan et al., 2008). Sub-genotype F2 has C at 1858 so it lacks PC mutation whereas F1 has T at 1858 so PC mutation occurs in F1 (Schaefer, 2005). A study also suggested that F1 and F2 have specific amino acids residues, Leu45 and Tr45 respectively in sub-genotype F2 in the S gene product (Mello et al., 2007).

Genotype G

Genotype G has 3248 base pairs and exclusively found in France, United States and Vietnam (Stuyver et al., 2000). It is characterized by 36 bp insertion at 3' position of 1905 and 3 bp deletion in the N-terminal of preS1 region. and it is found along with genotype A and genotype C (Sugauchi et al., 2004). Genotype G genome has been found to be least divergent from the genotype E (11%) and most divergent (15%) from the genotype F (Sugauchi et al., 2004). The Precore/core region of this genotype has 2 stop codons at position 2 and 28 and therefore expression of HBeAg protein does not takes place but if HBeAg is present in serum it may be due to the co-infection with other genotypes, mainly genotype A (Kato et al., 2002). This genotype is not divided into further sub-genotypes. More isolates having genotype G are needed for their sub classification into sub-genotypes.

Genotype H

HBV genotype H is predominant in Mexico, Central America and Nicaragua. It is similar to genotype F but divergence of 7.5-9.6 % on the basis of whole genome from genotype F makes it new genotype (Arauz-Ruiz, Norder, Robertson, & Magnius, 2002). No any sub-genotypes of this genotype is yet reported.

Genotype I

Genotype I is 3215 nucleotide long and possesses adr serotype. It was isolated from Vietnam (Thien, Huy, Ngoc, & Abe, 2008) and also reported from a primitive tribe Idu-Mishmi of northeast. However, this genome has divergence of only 7.0 % $\pm$ 0.4 % in whole genome from genotype C. It has been proposed as a complex recombinant of genotype C, A and G along with seven unique conserved amino acid residue not present in any of the known genotypes (F. Kurbanov, Tanaka, Kramvis, Simmonds, & Mizokami, 2008). Genotype I from the Indian tribe are found to be the recombinants of genotypes G, C and A. adw2 and ayw2 serotype of genotype I have been reported from Taiwanese population. More studies are needed to establish it as a new genotype (Yu et al., 2010).

Genotype J

Genotype J is 3182 nucleotides long and belongs to 'ayw' serotype. The 10th genotype J was isolated from HCC patients in Japan. It was known that this genotype is neither a recombinant of nine (A-I) genotypes nor a recombinant from Gorilla, Chimpanzee, Gibbon and Orangutan. This genotype is differ from genotype I by 9.9 -16.5 % on the basis of whole genome (Anna Kramvis, 2014).

Table 2.1: Worldwide distribution of sub-genotypes of HBV

Genotype	Sub-genotype	Distribution
A	Aa/A1	Nepal, Philippines Japan, South Africa
	Ae/A2	Germany , Poland France, United States and United Kingdom
	A3	Cameroon and Gabon
	A4	Mali
	A5	Nigeria
	A6	African-Belgium people
	A7	Cameroonians
B	Bj/B1	Japan
	Ba/B2	China, Taiwan, Vietnam
	B3	Indonesia
	B4	Vietnam

	B5	Philippines
	B6	Arctic indigenous population,
	B7-B9	Eastern Nusa Tenggara islands of Indonesia
C	Cs/C1	China, Bangladesh, Malaysia, Hong Kong, Thailand, Vietnam
	Ce/C2	China, Korea, Japan, Taiwan
	C3	Polynesia
	C4	Northeast Australia
	C5	Philippines and Vietnam
	C7	Thailand
	C8	Indonesia
	C9	Thailand and Tibet, China
	C10-16	Indonesia
D	D1	Pakistan, India, Iran
	D2	Russia, India, and the Baltic region
	D3	Pakistan, India
	D4	Solomon Islands
	D5-D8	India, Indonesia Tunisia and Nigeria
	D9	India
E	ND	ND
F	F1	Peru, Central America, Venezuela
	F2	Venezuela
	F3	Panama, Venezuela and Colombia
	F4	Bolivia, Brazil, Argentina
G	ND	ND
H	ND	ND
I	ND	ND
J	ND	ND

*ND: Data not available

2.5) Clinical and virological characteristics associated with HBV genotypes:

The clinical and virological course of infection with HBV depends upon the age, immune response and viral strains infecting an individual**.** Acute infections lead to fulminant hepatitis in less than 1 % of an individual. While chronic infections leads to development of liver cirrhosis (LC) and HCC in approximately 0-1 % of an individual. Various viral factors such as host factors, specific viral mutations, viral load, HBV genotype, HBV DNA level help in the prediction of clinical and viral outcomes of chronic HBV infection as well as treatment of interferon (IFN)- α treatment response (C. J. Liu & Kao, 2013). It was known that patients with genotype C have higher level of ALT and hepatic histopathological activity as compared to genotype B. The reason behind this is still unknown. It was suggested that patients with genotype C have lower level of HBV specific cytotoxic T lymphocyte (CTL) than that of patients with genotype B. In addition, genotype C have higher level of programmed death receptor-1 (PD 1) expression on HBV specific CTL against HBcAg and non-specific CTL (Xibing et al., 2013). Patients with genotype C have longer immune clearance periods because of higher viral loads, high levels of ALT, and low response to interferon treatment. Patients with acute infection due to genotype A have higher tendency of developing chronicity (Ogawa, Hasegawa, Naritomi, Torii, & Hayashi, 2003) while patients with Genotype D have higher risk of acute liver damage as compared to other genotypes (Wai et al., 2005). Similarly, a study carried out in China also suggested that that sub-genotype C2 is associated with chronicity development. CHB patients with sub- genotype B2 have higher viral loads and higher HBeAg positivity. It is more predominant among males showing that horizontal transmission is main route of spreading HBV sub-genotype B2 (Wai et al., 2005). Among genotype A, B, C and D, the rate of developing chronicity in patients is higher with genotype A and D as compared to genotype B and C (Mariko Kobayashi et al., 2004). A study conducted in Taiwan showed that patients with genotype A and B have higher rates of HBeAg seroconversion as compared to genotype C and D. This occurrence of HBeAg seroconversion is earlier in genotype A and B. Earlier seroconversion indicates positive outcome while delayed seroconversion indicates the progression of chronic hepatitis to LC. Patients with genotype C have higher DNA replication, higher frequency of PC A1896 mutation, basal core promoter (BCP) A1762T/G1764 A mutation,

pre-S deletion as compared to genotype B (Lin & Kao, 2011). Similarly, genotype A has a lesser occurrence of BCP A1762T/G1764A mutation than genotype D. Various factors like age, gender, level of HBV DNA and ALT, HBeAg and genotype influence response to anti-viral treatment. Various studies showed that with regard to reduction in the expression of HBsAg, HBeAg and the HBV DNA level, genotype C and D and other recombinant hybrid genotype has lesser response to interferon treatment as compared to genotype A and B , with the worst response to IFN being observed with genotype D(Y.-H. Shi, 2012). Another study suggested that genotype A and B show strong potent to IFN/ Peg-IFN than other genotypes C, D and I (Zhang et al., 2013).

A current meta-analysis revealed that there was no significant difference in response between HBV genotypes and nucleos(t)-ide analogs like lamivudine, adefovir, entecavir and telbivudine (Wiegand, Hasenclever, & Tillmann, 2008). The comparison of clinical and virological features among HBV genotypes is summarized in the below table:

Table 2.2: Comparison of clinical and virological features among hepatitis B virus genotypes

Genotype	**B**	**C**	**A**	**D**	**E-J**
Clinical characteristics					
Modes of transmission	Perinatal/vertical	Perinatal/vertical	Horizontal	Horizontal	Horizontal
Tendency of chronicity	Lower	Higher	Higher	Lower	ND
Positivity of HBeAg	Lower	Higher	Higher	Lower	ND
HBeAg seroconversion	Earlier	Later	Earlier	Later	ND
HBsAg seroconversion	More	Less	More	Less	ND
Histological activity	Lower	Higher	Lower	Higher	ND
Clinical outcomes (LC, HCC)	Better	Worse	Better	Worse	Worse in genotype F

Response to INFs	Higher	Lower	Higher	Lower	Lower in genotype G
Response to nucleoside analog	No significant differences among genotypes A to D				ND
Virological characteristics					
Serum HBV DNA level	Lower	Higher	ND	ND	ND
Frequency of PC A1896 mutation	Higher	Lower	Lower	Higher	ND
Frequency of basal core promoter T1762/A1764 mutation	Lower	Higher	Higher	Lower	ND
Frequency of preS deletion mutation	Lower	Higher	ND	ND	ND

Work plan:

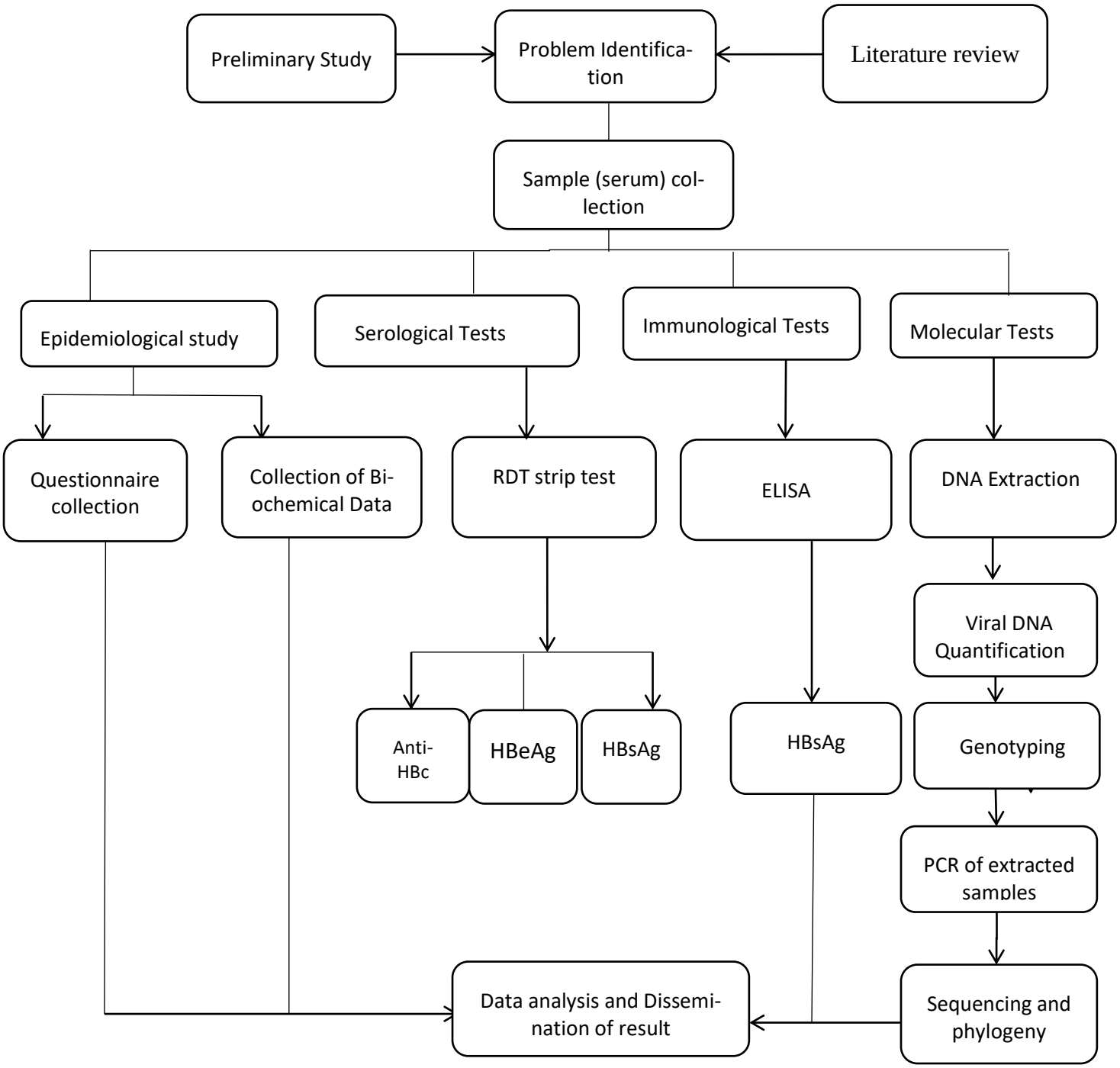


Figure 2.3: Work plan

CHAPTER: THREE

METHODS AND METHODOLOGIES

3.0) Study Area:

This study was conducted in Central Department of Biotechnology, TU, Kirtipur and De-code Genomic research Center, Sinamangal, Kathmandu.

3.1) Ethical Approvals:

For the collection and processing of human blood specimens, approvals was taken from the ethical review board of Nepal Health Research Council (NHRC) and Department has coordinated with the hospitals and research labs from where samples were taken. Before sample collections, all the suspected patients of HBV infection were informed that their blood samples were being used for research purpose and they didn't have to pay for this.

3.2) Inclusion criteria and Exclusion criteria:

Samples confirmed by ELISA after the rapid diagnostic test of HBsAg were selected and proceed for further test in this study. All the information about the hepatitis B positive patients including name, location and ethic were kept de-identified.

3.3) Sample Collection:

Sera from 500 Nepalese populations, inhabiting various district of five-development region of Nepal were collected from June 2017 to January 2018. The sera samples were collected from Sukaraj Tropical and Infectious Disease Hospital (STIDH), Teku, Kathmandu, National lab research(nrl) and Decode Genomic lab and research centre (DGRC), Sinamangal, Kathmandu. Thus, collected sera were stored at -20˚C and taken to research laboratory of Central Department of Biotechnology, Tribhuvan University, Kirtipur. During transportation, all the sera samples kept at deep-freeze were directly transferred into an ice box labeled as "High Risk Samples" and transported safely. After reaching to the research laboratory, processing of samples for different tests was done.

3.4) Collection of Questionnaire from HBV positive patients:

Demographic parameters like ethnicity, location, age, sex, family history of hepatitis B and other opportunistic infections (OIs), clinical symptoms, duration of disease; associated risk factors for HBV transmission and sero-positivity against its surface antigen were recorded by direct interview from the patients on a pre-designed questionnaire for epidemiological studies of hepatitis B. The questionnaire was prepared as:

I. Ethnicity:
II. Place of birth
III. Any vaccination against hepatitis
    a) No b) Yes (how long)
IV. Vaccination source:
    a) Hospital b) Community vaccination
V. Any medication taken during jaundice.
    a) Yes b) No
VI. If yes, name of the medicine used and how long it has been taken
VII. How long did patient was diagnose
    a) 1 month b) 6 months
    c) More than 6 months d) repeated infections in few years
VIII. Family history of hepatitis B/ Liver cirrhosis
    a) Yes b) No
IX. IV drug use
X. Ever treated for Sexually Transmitted Disease (STD):
    a) Yes b) No
XI. How many sex partner had a patient had in a lifetime.

3.5) Serological test

3.5.1.) One Step Strip Style HBsAg Test:

The test strip with arrow side pointing down was dipped into the vessel of serum for about 10 mins. The strip was left for 10 mins and the result was observed. Only one colored band on the control region indicated negative test while in addition to a pink colored control band, a distinct pink colored band in the test region indicated positive test.

3.5.2) One Step Strip Style HBeAg Serum Test:

The test strip with arrow side pointing down was dipped into the vessel of serum for about 10 minutes. The strip was left for 10 minutes and the result was observed. Only one colored band on the control region indicated negative test while in addition to a pink colored control band, a distinct pink colored band in the test region indicated positive test.

3.5.3) One Step Strip Style Anti-HBcAb Test:

The test strip with arrow side pointing down was dipped into the vessel of serum for about 10 minutes. The strip was left for 10 minutes and the result was observed. Only one colored band on the control region indicated negative test while in addition to a pink colored control band, a distinct pink colored band in the test region indicated positive test.

3.6) Immunological test:

3.6.1) Enzymes Linked Immunosorbent Assay (ELISA):

Those samples, which showed positive tests for RDT, were confirmed by using ELISA. The protocol was followed according to the manufacturer's instruction**.**

Well A1 was labeled as blank well, B1, C1 and D1 as negative control and E1 and F1 were labeled as positive controls. While remaining others wells were labeled for samples. 50 µl of Negative control, positive control and sample was added to their respective wells. 50 µl of enzymes conjugated was added to each well expect the blank well. The plate was shaken on the plate shaker for 30 seconds to completely mix the liquid within the wells. The plate was covered with a lid and incubated at 37˚C for 30 minutes. 350 µl of wash solution was added and aspirated. This process was repeated for 6 additional times for total of 6 washes. At the end of washing, the plate was inverted and tapped out to remove any residual wash solution onto absorbent paper.50 µl of substrate A, 50 µl of substrate B was added to each well including the blank well. The reagents were gently mixed for 15 seconds and the plate was incubated at 37˚C in the dark for 10 minutes without shaking. 50 µl of stop solution was added to each well, including the blank well and mixed gently. Then the absorbance was taken within 20 minutes at 450 nm using a reference wavelength of 630 nm to minimize well imperfection in a micro-

plate reader. Alternatively, the actual absorbance was obtained by subtracting the absorbance of each well at 450 nm with the absorbance of the blank well at 450 nm.

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

3.7) Collection of Biochemistry data and platelets of Hepatitis B positive patients:

Clinical details of the patients included in the study were obtained from their medical records. The parameters considered were gender, whether the patient was an inpatient/outpatient, the district from where the patient came, their occupation and age. Liver function tests like ALT/ SGOT, AST/SGPT, ALP, total bilirubin, and direct bilirubin and platelets count of all the HBV positive patients were collected from the hospital's record. They were analyzed based on their lower, normal and upper range. The fluctuation in their normal ranges indicates the damage in their hepatocytes.

3.8) Molecular tests:

3.8.1) HBV DNA Extraction from Human Serum:

50 µl Proteinase K was pipetted into the bottom of 1.5 ml microcentrifuge tube. 200 µl sample was added to the microcentrifuge tube and mixed. 200 µl lysis buffer SSVL, 15 µl carrier RNA and 5 µl internal controls was mixed to the sample and vortex for 15 seconds. The microcentrifuge tube was incubated at 65˚C for 10 minutes. The 1.5 ml microcentrifuge tube was briefly centrifuged. 280 µl absolute ethanol was added to the sample and vortex for 15 seconds. All mixture from step IV (approx. 745 µl) was transferred to a Spin Star TM column without wetting the rim. The cap was closed and centrifuged at 6,200 xg (8,000 rpm) for 1 minute. The Spin star TM column was placed in a collection in a clean collection tube, and the tube containing the filtrate was discarded. The

Spin star TM column was carefully opened and 500 µl Wash buffer 1, SSW1 was added without wetting the rim. The cap was closed and centrifuged at 6,200 xg (8,000 rpm) for 1 minute. The Spin star TM column was carefully opened and 500 µl Wash buffer 2, SSW2 was added without wetting the rim. Close the cap and centrifuge at 6,200 xg (8,000 rpm) for 1 minute. The filtrate was discarded and collection tube was reused. The tube was centrifuged at 7,000 xg (13,000 rpm) for 10 minutes. The Spin star TM column was transferred to a clean 1.5 ml microcentrifuge and the collection tube containing trace Wash Buffer 2 was discarded. The Spin star TM column was carefully opened and 30- 60 µl Elution buffer SSE was added on the center of the membrane. The tube was incubated at room temperature (15-30˚C) for 5 minutes and then centrifuged at 6,200 xg (8,000 rpm) for 1 minute. The process was proceed to downstream (eg. Real time PCR) reaction set up or eluate was stored at - 20˚C if not used immediately.

3.8.2) HBV DNA Quantification:

In those HBsAg that tested positive by the ELISA method, we randomly selected forty-eight samples and DNA was extracted from each sample using Spin star TM column for HBV DNA quantifications. Viral load was quantified by Applied Biosystem; Step one Real-Time PCR using TaqMan probe.

All the reagents and samples were thawed completely, mixed by pipetting and gently vortexing and centrifuged briefly before used. Here, the internal control was added during the sample preparation. Then 25 µl of the master Mix was added into each required well of an appropriate optical reaction tube. 25 µl of the sample and 25 µl of the control i.e. Quantification Standard, Positive control, and Negative Control were added into their respective tube. The sample and controls were thoroughly mixed with the Master Mix by up and down pipetting. The 48- wells reaction plate was closed with an appropriate optical adhesive film and the reaction tubes with appropriate lids. The 48-well reaction plate was centrifuged in a centrifuge with a microtiter plate for 30 seconds at appropriately 1,000 xg (3,000 rpm).

The PCR test channel was selected as FAM channel (Reporter: FAM, Quencher: None) to test HBV specific DNA. JOE channel (Reporter: JOE, quencher: None) to test Internal control.

Table 3.1: Preparation of master mix for viral load using RT-PCR

No.of reaction	1	12
Master A	5µl	60 µl
Master B	20µl	240µl
Vol. of Master mix	25µl	300µl

Table 3.2: The PCR temperature profile and Dye Acquisition for viral load using RT- PCR

	Stage	Cycle repeat	Acquisition	Tempera-ture	Time
Denaturation	Hold	1	-	95˚C	2 mins
Amplification	Cycling	45	-	95˚C	0.15 min
			√	58˚C	0.20 min
			-	72˚C	0.30 min

3.8.3) Genotyping of HBV

Each component from the diagnostic kit was taken out and placed them at room temperature. The reagents were allowed to equilibrium at room temperature, and each of them was mixed well respectively for future use. According to quantity of test specimens, negative control and positive control the approximate volumes of PCR mix, enzymes mix, and internal control was pipetted. They are fully mixed into PCR master mix and centrifuged instantaneously for future use. Then 5 µl of lysis buffer was added into each PCR tube and mixed with 5 µl of negative control, positive control and test specimens respectively. The tubes were pipetted gently for 3-5 times, avoiding air bubbles. Every specimen was repeated to two PCR tubes for the further genotyping reaction. After 10 minutes, 40 µl of two PCR-Master mixes were added into two PCR tubes of each specimen respectively and then the tubes lid was covered. PCR reaction tubes were placed into the specimen wells of the amplification device. The negative control, positive control and unknown samples were set up in the corresponding sequence and input sample information.

The PCR test channel was selected as: FAM channel (Reporter: FAM, Quencher: None) to test HBV genotype and C – DNA. HEX or VIC channel (Reporter: VIC, Quencher: None) to test HBV genotype D and IC. The passive Reference was set as ROX.

Table 3.3: The preparation of master mix for genotype using RT-PCR

PCR master mix	PCR mix	Enzyme mix	Internal control
HBV-B/D PCR master mix	38 µl/ test	3 µl/ test	-
HBV-C/IC PCR master mix	38 µl/test	3 µl/ test	0.4 µl/ test

Table 3.4: The PCR cycle parameters for genotypes using RT-PCR

S.N	Step	Temperature	Time	Cycle number
1	UNG enzymes activation	50˚C	2 mins	1
2	Taq enzymes activation	94˚C	5 mins	1
3	Denaturation	94˚C	15 sec	45
4	Annealing, extension, fluorescence collection	57˚C	30 sec	
5	Device cooling	25˚C	10 sec	1

3.8.4) PCR amplification of HBV polymerase region using specific primer:

The DNA extracted of the samples collected from STIDH were subjected to conventional PCR. The primers were designed from the conserved region of all the genotypes (A-H) to target P gene with partial S gene due to overlapping ORF. The PCR reaction mixture and condition was shown in table 3.5 and 3.6. The PCR products were gel purified and sequenced bi-directionally. The sequence data obtained was used for genotype.

The primer sequences used for PCR are as follow:

HBV-all-GEN-FP: 5’ – CTCAATGTTAGTATCCCTTGGAC – 3’

HBV-Gen-BCD-RP: 5’ – AGGAATATGATAAAACGCCGCAGACA – 3’

Table 3.5: The reagent for PCR condition

S.N	Reagents	For single reaction
1	Forward primer (100 ng/µl)	2.0 µl
2	Reverse primer (100 ng/ µl)	2.0 µl
3	10 X Assay buffer	5 µl
4	dNTPs (10mM)	2.0 µl
5	TaqPol (3U/ µl)	0.5 µl
6	Water	38.5 µl
Total volume		50.0 µl

Table 3.6: Parameters for PCR cycle

Step	Temperature	Time	Cycle
Initial denaturation	94˚C	5 min	
Denaturation	94˚C	30 sec	35
Annealing	52˚C	30 sec	
Extension	72˚C	1.30min	
Final extension	72˚C	7 min	

3.8.5) Agarose gel electrophoresis:

Agarose gel electrophoresis was used to resolve the PCR products for the confirmation of amplification. For this 1 % agarose gel was prepared in 1X TAE (Tris base, acetic acid and EDTA) buffer, stained with ethidium bromide and casted in gel electrophoresis tank. 5 µl of PCR product was mixed with 2 µl of loading buffer and loaded in each lane. From lane 1 to lane 15, samples were loaded in their respective lane. In lane 16, ladder of 500 bp (Bayou Biolab) was added. Then the gel was run at 100 volts for 35 min. The PCR products on agarose gel were visualized under UV Trans Illuminator and gel doc to confirm the amplified fragment.

3.8.6) Chromatogram:

The sequences provided in AB1 file format were opened FinchTv, bad sequences were trimmed manually and was processed in Bioedit Sequence Alignment Editor for contig preparation.

3.8.7) Sequence analysis and phylogeny:

The PCR amplicons were sent to Chromous Biotech Pvt. Ltd, Bangalore, India for bidirectional sequencing using the primers sets of HBV-all-GEN-FP: 5'– CTCAATGTTAG-TATCCCTTGGAC – 3'and HBV-Gen-BCD-RP: 5' – AGGAATATGATAAAACGCCGCAGACA – 3'. Sequencing was performed using Big Dye Terminator version 3.1 (Applied-Biosystems) on the ABI 3500 Genetic Analyzer (Applied Biosystems). A database containing previously isolated sequences of HBV (A-H genotype) was downloaded from Genbank and was aligned by using geneious R11 (geneious aligner). Phylogenetic trees were created by distance-matrix and neighbor-joining analyses after bootstrapping to 1000 replicates, using the MEGA V.7.0 software. The tree was visualized and edited in Geneious R11. Besides, using previously isolated HBV sequences from Nepal; India Tree was built using exact same parameter as above using Geneious for S-gene segment.

CHAPTER FOUR

RESULTS

4.1) Questionnaire Collection:

In this research work, all the patients was found to be infected and/or asymptomatic carrier with HBV. The study was carried out in Sukaraj Tropical and Infectious Disease Hospital (STIDH), Kathmandu district. The subjects in this study were analyzed based on epidemiological and demographical distribution, sex, age, risk factor for HBV infection, immunization status and opportunistic infections.

4.2) Epidemiology and demography of hepatitis B in Nepal

4.2.1) Geographical distribution of hepatitis B:

The patients enrolled for this study were from all the districts of seven provinces of Nepal. Most of the patients were from the Siraha, Saptari, and Sarlahi districts of province 2 with the prevalence rate of 38.35 % (n=51), 24.06 % (n=32) and 15.04 % (n=20) respectively. Province 3 had second highest prevalence rate of HBV infection with Kathmandu and Lalitpur districts of prevalent rate of 18.45 % (n=19) and 7.77 % (n=08) respectively.. Out of seventy-seven districts of seven provinces of Nepal, the prevalence of HBV infection was not found in Khotang district of province no: 1.

Figure 4.1: Incidence of HBV cases with highest prevalence in the districts and province of Nepal

Table 4.1: Distribution of patients according to Districts and Provinces:

S. N	Prov No:	Districts	No.of sub-jects	S.N	Prov-No:	Districts	No.of sub-jects
1		Taplejung	5	39		Kaski	3
2		Jhapa	7	40		Baglung	1
3		Morang	6	41		Lamjung	4
4		Sunsari	5	42	4	Nawalpur	2
5		Okaldhunga	4	43		Prabhat	2
6		Solukhumbu	2	44		Mustang	4
7	1	Panchthar	2	45		Manang	1
8		Bhojpur	6	46		Kapilvastu	2
9		Dhankuta	6	47		Arghakhanchi	1
10		Sankhuwasaba	2	48		Bardiya	2
11		Udayapur	2	49	5	Banke	1
12		Terathum	4	50		Dang	4
13		Illam	2	51		Gulmi	3
14		Saptari	32	52		Nawalparasi	2
15		Mahottari	1	53		Palpa	2
16		Dhanusha	5	54	5	Pyuthan	2
17	2	Sarlahi	20	55		Rolpa	3
18		Rautahat	3	56		Rukum	2
19		Siraha	51	57		Rupandehi	2
20		Bara	6	58		Dailekh	4
21		Parsa	4	59		Dolpa	1
22		Chitwan	5	60		Humla	4
23		Dhading	6	61		Jajarkot	1
24		Kavrepalanchok	3	62		Jumla	1
25		Kathmandu	19	63	6	Kalikot	1
26		Rasuwa	1	64		Mugu	4
27		Dhanusha	2	65		Salyan	1
28	3	Nuwakot	4	66		Surkhet	3

29		Sindhuli	3	67		Achham	2
30		Bhaktapur	7	68		Baitadi	1
31		Lalitpur	8	69		Bajhang	1
32		Ramechhap	3	70		Bajura	2
33		Sindhupalan-chowk	3	71	7	Dadeldhura	1
34		Dolakha	2	72		Doti	2
35		Makwanpur	5	73		Darchula	1
36		Gorkha	5	74		Kailali	4
37	4	Tanahu	4	75		Kanchanpur	4
38		Myagdi	1	76			
Total							320

4.2.1.1) Prevalence of HBsAg in suspected HBV patients:

The prevalence of HBV infection was found highest in province number 2 followed by province number 3. In overall, the prevalence of HBsAg positivity was 68 %(n=53) in province number: 1, 77 % (n=103) in province number: 2, 69 % (n=71) in province number: 3. Similarly, the prevalence of HBsAg was 59 % (n=29), 51 % (n=26), 44 % (n=20), 40 %(n=17) in province number: 4, 5, 6 and 7 respectively. The prevalence rate was found to be decreased on moving from east to west region of Nepal with lowest prevalence being on province number 7.

Table 4.2: Distribution of suspected HBV patients in seven provinces

Prov. No	No. of sera examined	No. of positive count	Percent positive
1	78	53	68%
2	133	103	77%
3	103	71	69%
4	49	29	59%
5	51	26	51%
6	45	20	44%
7	42	17	40%
Total	500	320	100%

4.2.2) Distribution of patients according to Gender:

The study cohort consisted of 66.56 % (n=213) males and 33.12 % (n=106) females. The male population was found to be higher than female population. Male: Female ratio was found to be 2.01:1. One cases of other gender infected with HIV co- infection was also found in this study.

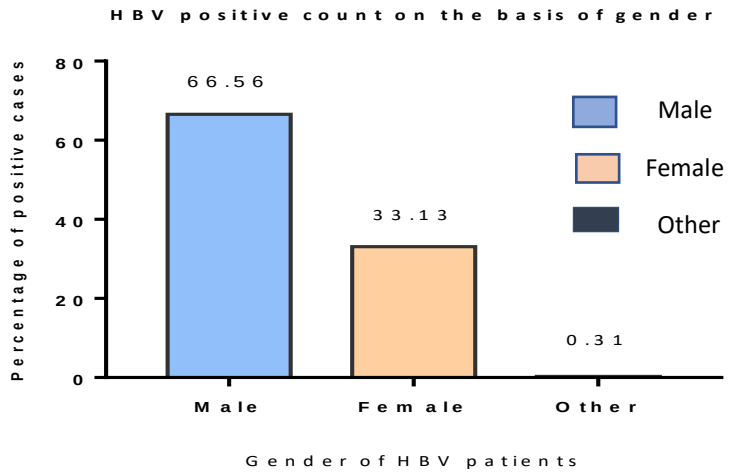


Figure 4.2: Gender wise distribution of HBV positive patients

4.2.3) Distribution of patients according to age group:

A total of 320 HBV positive patients ranging from 13 to 76 years with mean age 32.28± 13.07 years were involved in this study. Most of the patients infected were with-in the age group of 20-40 years (n=190) followed by age group of 40-60 years. The prevalence of HBV in pediatric age group below 10 years was not found in this study.

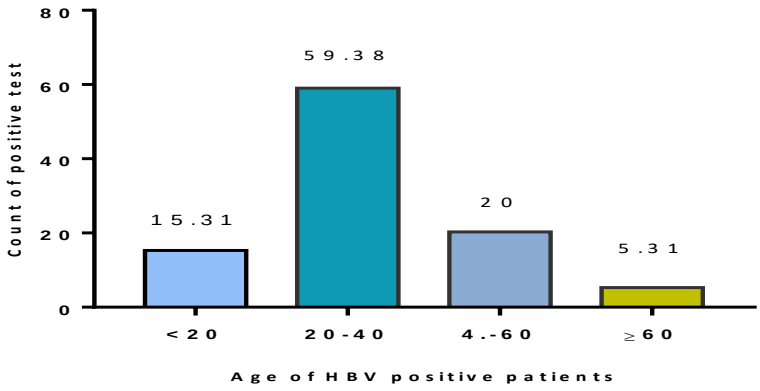


Figure 4.3 : Age-specific prevalence of HBsAg, HBeAg and Anti-HBc in HBV postive patients.

4.2.4) Risk factors of HBV infection:

Risk factors for chronic HBV infection include sex, age, cigarette smoking, alcohol consumption, chemical carcinogens, hormonal factors, and genetic susceptibility. In this study, it was found that HBV was common among intravenous drug user and alcoholic, health workers, patients having tattoos and patients having family history of HBV infection with HBsAg positivity of 16.0 % (n=51/320), 2 % (n=06/320), 2.0 % (n=08/320) and 7.0 % (n=24/320) respectively. The prevalence rate of HBV/HIV co-infection was 17.0 % (n=53/320), HBV/HCV was 3.0 % (n=10/320) and HBV/HCV/HIV was 2.0 % (n=5/320).

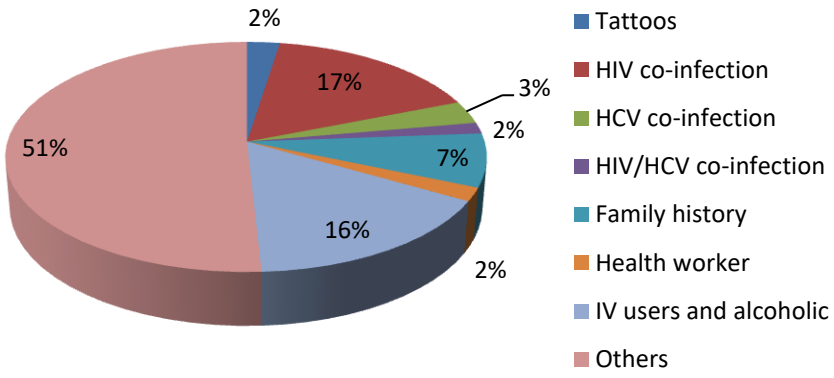


Figure 4.4: Risk factor associated with HBV infection

4.2.5) Vaccination status:

Among three hundred and twenty HBV positive patients, 4.68 % (n=15) were found to be vaccinated against HBV while remaining 95.32 % (n=305) were non vaccinated. Inaddition comparing whole patients, HBV infection was found to be negative in 36% (n=180) patients.

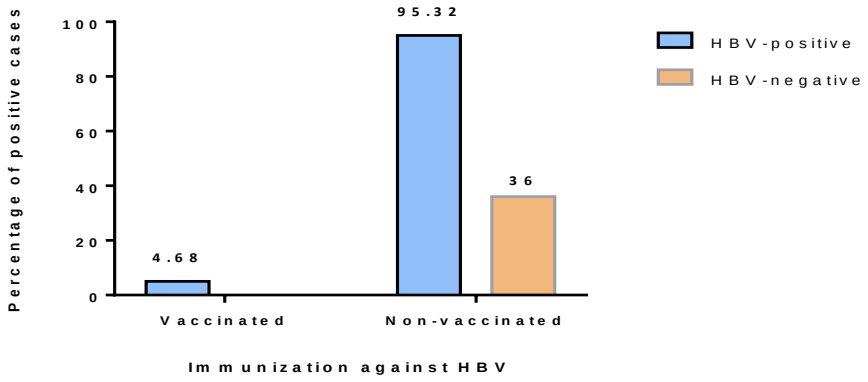


Figure 4.5: Vaccination status among HBV infected patients

4.3) Serological results

4.3.1) Rapid Diagnostics test (RDT):

Out of 500 suspected HBV patients, 64 % (n=320) patients showed positive HBV infection and 36 % (n=180) patients were HBV negative. The positivity of HBV infection was carried out on the basis of positivity of HBsAg. Among 320 positive patients, 21.56 % (n=69) were HBeAg positive and 84.06 % (n=269) patients were anti-HBc positive.

Figure 4.6: Sample showing positive result by RDT

4.3.2) Enzymes Linked Immunosorbent Assay (ELISA):

Out of 320 positve patients identified from RDT, 97.17 % (n=311) patients showed positive tests toward ELISA and 3.43 % (n=11) were found to be of occult hepatitis B infection (OBI). The development of intensive yellow color after the addition of stop solution indicates the positive result test for HBV. The evaluation was done taking the cutoff value according to manufacturer's instruction for positive test

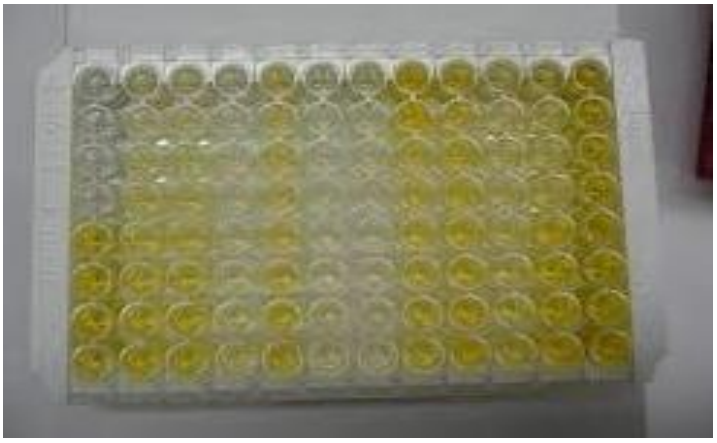


Figure 4.7: Samples with positive result test for ELISA

Table 4.3: Calculation of absorbance, absorbance/cutoff value of HBsAg by ELISA

Sample	Absorbance	Absorbance/Cutoff-value	Remarks
PC	2.694±0.107	25.179±1.826	Positive
NC	0.0115±0.005	0.106±0.002	Negative
Sample			
PC	2.458 ± 0.614	23.449 ±5.861	Positive
NC	0.026±0.045	0.276 ±0.433	Negative

4.3.3) Prevalence of HBsAg, HBeAg and Anti-HBc with Gender:

In male, 64.06 % (n=205) patients were positive to HBsAg, 12.5 % (n=40) were positive to HBeAg and 55 % (n=176) patients were positive to Anti-HBc whereas in female 32.81 % (n=105) were positive to HBsAg, 8.75 % (n=28) were HBsAg positive and 28.75 % (n=92) were Anti-HBc positive. Similarly, in other gender, 0.31 % was HBsAg, HBeAg and Anti-HBc positive respectively.

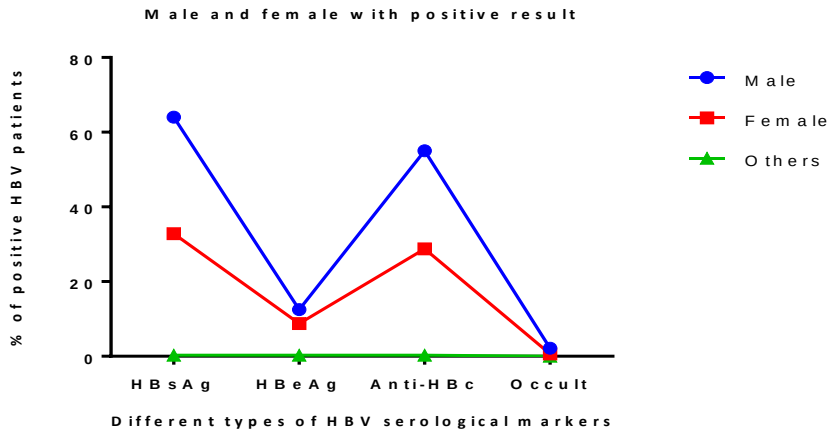


Figure 4.8: The relation between gender of patients and occurrence of HBsAg, HBeAg and Anti-HBc due to an infection by HBV.

4.3.4) Prevalence of HBsAg, HBeAg and Anti-HBc due to infection by HBV.

Out of 320 HBV patients, 18.57 % (n=60) patients showed both HBsAg and HBeAg positivity, 81.87 % (n=262) showed HBsAg and Anti-HBc positivity, 18.57 % (n=60) showed

HBsAg and Anti-HBc positivity while only 10.31 % (n=33) showed all HBsAg, HBeAg and Anti-HBc positivity.

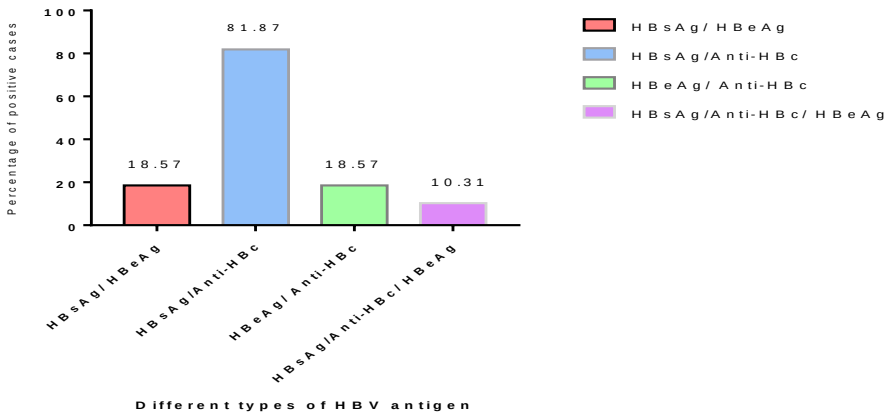


Figure 4.9: The relation between the occurrence of HBsAg, HBeAg and Anti-HBc due to infection by HBV.

4.4) Acute and Chronic infection:

In this study, out of 320 HBV patients 67.50 % (n=216) of patients have acute infection and 32.50 % (n=104) of patients have chronic infection.

Table 4.4: Table showing acute and chronic cases of HBV infection

Infection	No. of subjects	Percentage (%)
Acute	214	67.50 %
Chronic	106	32.50 %
Total	**320**	**100 %**

4.4) Biochemical Results:

4.4.1) Total bilirubin:

A total of 320 HBV patients, 25.13 % (n=81) of patients have high, 69.69 % (n=223) have normal and 5.00 % (n=16) of patients have low levels of total bilirubin.

Table 4.5: Table showing total bilirubin level in HBV positive patients

		No. of subject	Percentage (%)
Bili T-categorized	**High (≥1.4)**	81	25.31 %
	Normal (0.30-1.4)	223	69.69 %
	Low (>0.3)	16	5.00 %
	Total	**320**	**100.0%**

4.4.2) Direct bilirubin:

In this study, 87.1 % (n=155) of patients have normal and 12.9 % (n=165) of patients have high level of direct bilirubin.

Table 4.6: Table showing direct bilirubin level in HBV positive patients

		Count	Column N %
Bili D_Categorized	**Normal (0.0-0.4)**	155	87.1%
	High (≥0.4)	165	12.9%
	Total	**320**	**100.0%**

4.4.3) Alanine Aminotransferase (ALT) :

Out of 320 HBV patients, 45.31 % (n=145) of patients have normal ALT levels while 54.69 % (n=175) of patients have high ALT levels.

Table 4.7: Table showing ALT level in HBV positive patients

		Count	Column %
ALT- categories	Normal (up to 42)	123	45.31 %
	High (≥ 42)	197	54.69 %
	Total	**320**	100 %

4.4.4) Aspartate Aminotransferase (AST):

The normal value of AST is up to 37 U/L for both male and female. In this study, 47.19 % have normal and 52.81 % have high levels of AST.

Table 4.8: Table showing AST levels in HBV positive patients

		Count	Column %
AST-categories	**Normal (up to 37)**	151	47.19 %
	High (≥ 37)	169	52.81 %
	Total	**320**	**100 %**

4.4.5) Alkaline phosphatase (ALP):

Out of 320 HBV patients, 29.97 % (n= 96) of patients have lower level, 16.25 % (n=52) of patients have normal levels and 53.75 % (n= 172)of patients have higher levels of ALP.

Table 4.9: Table showing ALP level in HBV positive patients

			Count	Column %
ALP_Categorized	Male	Low (<42)	74	23.13%
		Normal (53-128)	13	4.06%
		High (≥128)	113	35.31%
	Female	Low (<42)	22	6.87%
		Normal (42-98)	3	0.94%
		High(>98)	59	18.44%
	Children	Normal (54-369)	36	11.25%
		Total	320	100%

4.4.6) Platelets :

Out of 320 HBV patients, 60.62 % (n=194) had lower platelets counts, 38.43 % (n=123) have normal platelets count and 0.94 % (n=3) have thromboctyopenia.

Table 4.10: Table showing platelets level in HBV positive patients

		Count	Column N %
Platelets_	Low (<150,000)	194	60.62%
	Normal(150,00-400,000)	123	38.43%

	High (≥400,0000)	3	0.94%
	Total	320	100.00%

4.4.7) Comparision of biochemical tests between HBV patients and healthy subjects:

The mean of biochemical tests like Bili T, Bili D, ALT, AST and ALP in HBV patients was found as 1.188$\pm$ 1.605, 0.607$\pm$0.568, 46.359 $\pm$ 20.519, 45.960 $\pm$ 25.338 and 196.744 $\pm$ 137.516 respectively. Similarly, the mean of Bili T, Bili D, ALT, AST and ALP in healthy subjects was found as 0.982 $\pm$ 0.522, 0.426 $\pm$0.400, 31.979 $\pm$ 13.201, 27.787 $\pm$ 11.524, 142.495 $\pm$ 120.455 respectively. This showed that there was no statistically significant difference between the mean biochemical tests count in the patients with HBV and healthy subjects (p>0.99). The mean platelets count in HBV patients was 173.781 $\pm$100.466 and in healthy subjects was 258.372$\pm$84.647. This showed that there was statistically significant difference between the platelets count of HBV patients and healthy subjects (p<0.0001). Thrombocytopenia was reported in 0.94 % (n=3) of HBV patients.

4.5) Molecular test

4.5.1) HBV DNA Quantification:

Out of 48 samples, six samples contained more than 10 million IU/ml virus while forty-two samples contained less than 10 million IU/ml virus and shown in the table 4.17 . The lower and upper limit for the quantification of viral load is 10 IU/ml and more than 10 million IU/ml respectively. Sample with high Ct value indicates lesser amount of target nucleic acid present in the sample while the sample having less Ct value have greater amount of target nucleic acid.

Table 4.11: Table showing the viral load of HBV patients.

Viral load	Count	Column N %
<10 millions	42	85.5%
>10 millions	6	14.5%
Total	**48**	**100%**

Table 4.12: Table showing Ct. Value of samples quantified by using real time PCR

Sample ID	Ct value	Viral load (IU/ml)	Sample ID	Ct value	Viral load (IU/ml)
01	23.08	2,500,000	25	34.07	2,280
02	19.96	>10 million	26	24.14	1,270,000
03	11.32	>10 million	27	33.70	2,890
04	24.62	935,000	28	33.84	2,630
05	11.32	>10 million	29	11.32	>10 million
06	26.35	2,110,000	30	23.87	1,505,305
07	23.36	2,090,000	31	33.70	2,890
08	23.90	1,480,000	32	31.90	7,956
09	28.38	84,600	33	19.96	>10 million
10	28.47	80,900	34	21.92	5,647,577
11	11.32	>10 million	35	19.96	>10 million
12	29.78	34,600	36	23.33	2,233,771
13	32.63	5,720	37	30.18	24,664
14	34.18	2,130	38	32.55	5,188
15	32.34	6,870	39	26.56	219,199
16	33.96	2,440	40	31.75	8,781
17	34.68	1,540	41	3.96	16,688
18	29.13	53,100	42	27.94	146,158
19	34.23	2,060	43	27.93	81,816
20	30.19	27,000	44	33.15	1,915
21	31.17	14,400	45	23.93	2,622,120

22	34.96	1,280	46	31.28	6,492
23	33.90	2,540	47	22.96	78,479
24	31.81	9,590	48	32.67	2,405

4.5.2) Genotype:

Genotype of forty-eight positive patients with both acute and chronic cases was done. Out of 48 patients, 68.75 % (n=33) have chronic hepatitis B (CHB) infection and 37.5 % (n=18) patients have acute hepatitis B (AHB) infection. Genotype D was found in 60.42 % (n=29) patients, genotypes B in 6.25 % (n=3) patients, genotype C in 6.25 % (n=3) patients, recombinant hybrid of C/D in 2.08 % (n=1) and unknown genotypes in 25.0% (n=12) patients. Samples with unknown genotypes were subjected to convectional PCR.

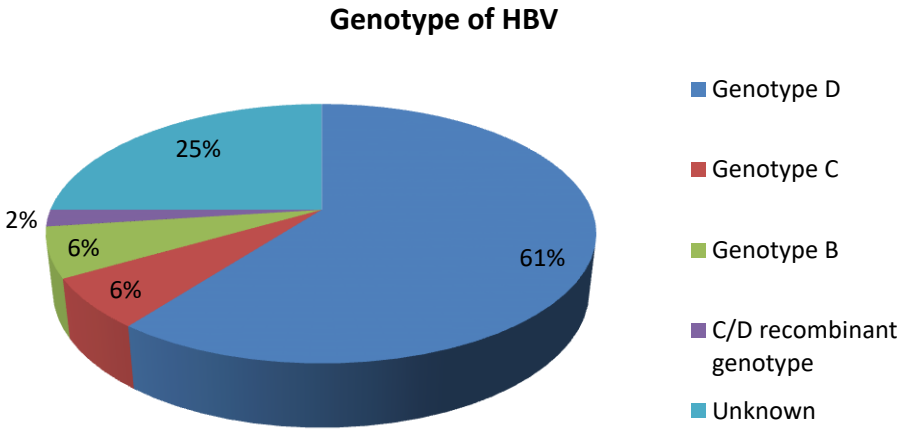


Figure 4.10: Prevalence rate of HBV genotype

Figure 4.11: Graph of HBV genotypes

Table 4.13: The results of under-test specimens are determined as below:

HBV-B/D PCR mix		HBV-C/Internal control PCR mix	Result Determination
FAM	HEX	FAM	
Ct≤39	No Ct	No Ct	HBV B genotype
No Ct	No Ct	Ct≤39	HBV C genotype
No Ct	Ct≤39	No Ct	HBV D genotype
Ct≤39	No Ct	Ct≤39	HBV B/C recombinant
Ct≤39	Ct≤39	No Ct	HBV B/D recombinant
No Ct	Ct≤39	Ct≤39	HBV C/D recombinant
Ct≤39	Ct≤39	Ct≤39	HBV B/C/D recombinant

Table 4.14: Table showing genotype with Ct. Value

Sample ID	Ct value	Genotype	Sample ID	Ct value	Genotype
01	-	Unknown	25	-	D
02	37.13	D	26	24.84	D
03	37.3	D	27	34.71	D
04	35.98	D	28	31.86	D
05	-	Unknown	29	35.71	D
06	26.87	D	30	31.97	D
07	20.25	D	31	32.98	D
08	35.14	D	32	19.94	C
09	24.18	D	33	36.50	B
10	36.01	D	34	36.66	C
11	36.66	C	35	-	Unknown
12	36.05	D	36	34.98	D
13	-	Unknown	37	35.02	D
14	-	D	38	31.12	D
15	-	D	39	-	Unknown
16	-	D	40	-	Unknown

17	38	D	41	-	Unknown
18	37.83	D	42	30.98	D
19	33.95	D	43	41.45	D
20	36.85	B	44	34.34	B
21	36.28 19.97	C/D recom-binant	45	-	Unknown
22	41.89	D	46	-	Unknown
23	37.89	Unknown	47	-	Unknown
24	-	Unknown	48	-	Unknown

4.5.3) PCR amplification of HBV polymerase region:

All the fifteen samples (1012, 8436, 8998, 36572, 36662, 32970, 33290, 5461, 8815, 440, 33666, 36318, 36372, 429 and 7426) from Lane 1 to Lane 15 having PCR amplicon of size just above 1.5 kb indicated positive result. The PCR products were gel purified and sent to Chromous Biotech Pvt. Ltd, Bangalore, India for further sequencing.

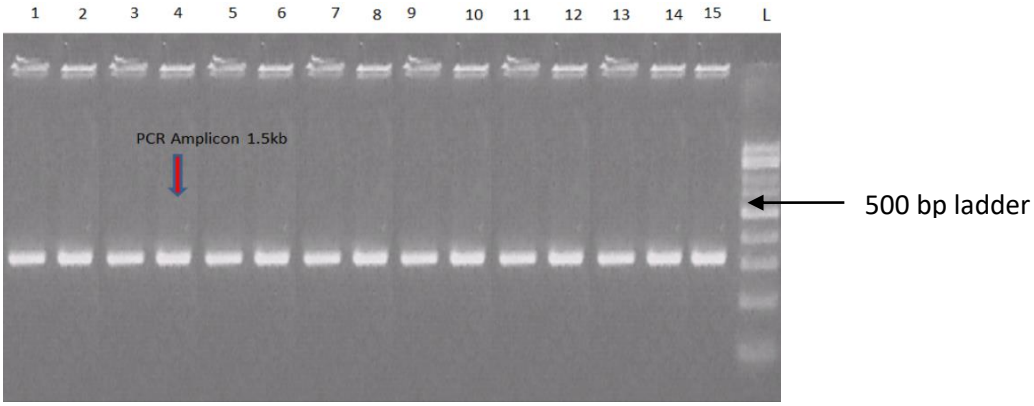


Figure 4.12: PCR amplification of HBV fragments from given sample. The size of PCR amplified product is ~1.5kb.

4.5.4) Sequencing analysis:

Fifteen samples were analyzed for sequence. Out of fifteen samples, four sub-genotypes namely A1, C1, D1 and D4 were detected. Four samples (36572, 36662, 8436, and 8998) were of sub-genotype A1, two samples (33290, 440) were of sub-genotypes C1, two samples (1012, 429) were of sub-genotypes D1 and four samples (32970, 33666, 5461, and 7426) were of sub-genotypes D4. However, three (8815, 36318, 36372) of them

turned out to be CD recombinant genotype. The best nucleotide identity match with the database ranged from 89.7% to 100 %.

4.5.5) Phylogenetic analysis:

Phylogenetic tree was constructed using sequences obtained in this study along with sequences obtained from Genbank. P-gene based tree was obtained after multiple sequence alignment of sequences of all samples and different genotype (A-H) showed agreement of genotyping result from web based online tools. S-gene based tree showed disparity as 'CD' recombinant strain is closely related to D genotype whereas in P-gene based tree its more closely related to C-genotype.

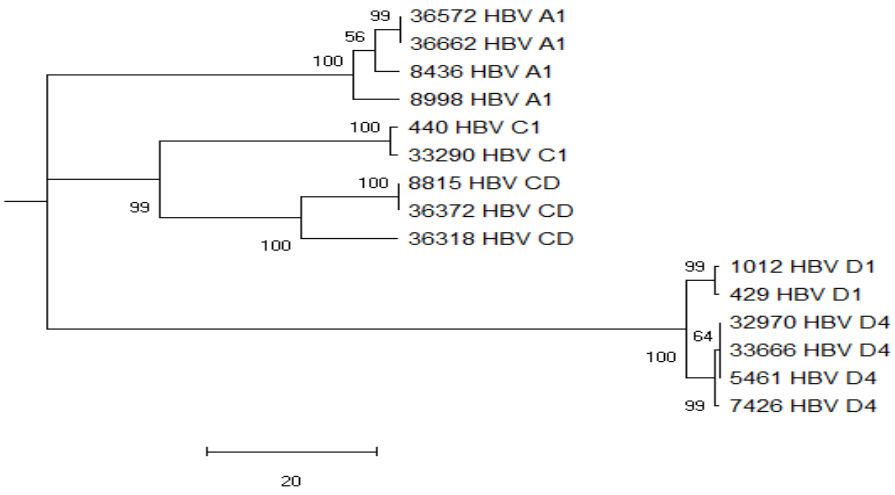
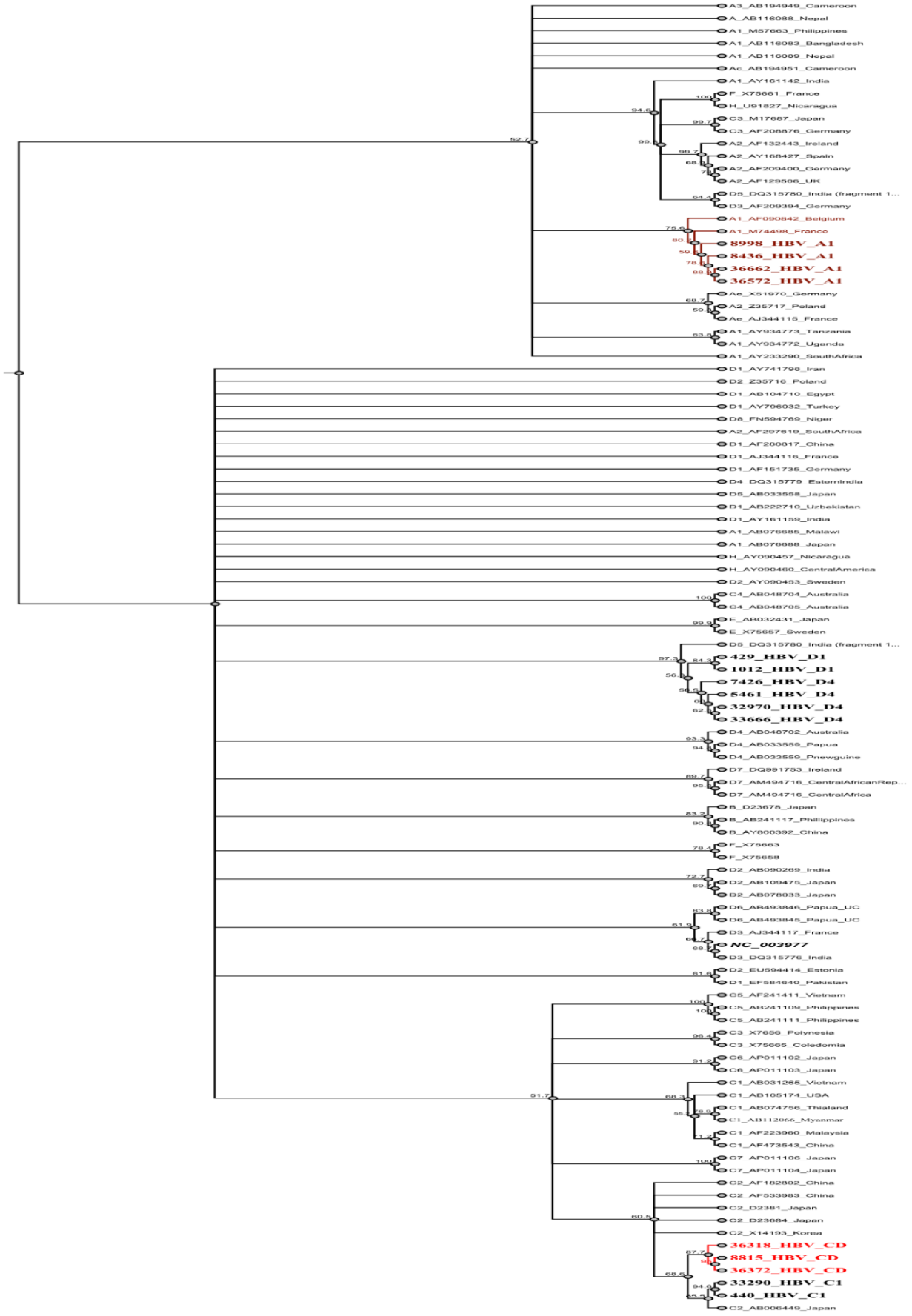


Figure 4.13: Phylogenetic tree of all the samples based on the partial sequence of P-gene and S-gene.


A3_AB194949_Cameroon
A_AB116088_Nepal
A1_M57663_Philippines
A1_AB116083_Bangladesh
A1_AB116089_Nepal
Ac_AB194951_Cameroon
A1_AY161142_India
F_X75661_France
H_U91827_Nicaragua
C3_M17687_Japan
C3_AF208876_Germany
A2_AF132443_Ireland
A2_AY168427_Spain
A2_AF209400_Germany
A2_AF129506_UK
D5_DQ315780_India (fragment 1...
D3_AF209394_Germany
A1_AF090842_Belgium
A1_M74498_France
8998_HBV_A1
8436_HBV_A1
36662_HBV_A1
36572_HBV_A1
Ae_X51970_Germany
A2_Z35717_Poland
Ae_AJ344115_France
A1_AY934773_Tanzania
A1_AY934772_Uganda
A1_AY233290_SouthAfrica
D1_AY741798_Iran
D2_Z35716_Poland
D1_AB104710_Egypt
D1_AY796032_Turkey
D8_FN594769_Niger
A2_AF297619_SouthAfrica
D1_AF280817_China
D1_AJ344116_France
D1_AF151735_Germany
D4_DQ315779_Estomindia
D5_AB033558_Japan
D1_AB222710_Uzbekistan
D1_AY161159_India
A1_AB076685_Malawi
A1_AB076688_Japan
H_AY090457_Nicaragua
H_AY090460_CentralAmerica
D2_AY090453_Sweden
C4_AB048704_Australia
C4_AB048705_Australia
E_AB032431_Japan
E_X75657_Sweden
D5_DQ315780_India (fragment 1...
429_HBV_D1
1012_HBV_D1
7426_HBV_D4
5461_HBV_D4
32970_HBV_D4
33666_HBV_D4
D4_AB048702_Australia
D4_AB033559_Papua
D4_AB033559_Pnewguine
D7_DQ991753_Ireland
D7_AM494716_CentralAfricanRep...
D7_AM494716_CentralAfrica
B_D23678_Japan
B_AB241117_Phillippines
B_AY800392_China
F_X75663
F_X75658
D2_AB090269_India
D2_AB109475_Japan
D2_AB078033_Japan
D6_AB493846_Papua_UC
D6_AB493845_Papua_UC
D3_AJ344117_France
NC_003977
D3_DQ315776_India
D2_EU594414_Estonia
D1_EF584640_Pakistan
C5_AF241411_Vietnam
C5_AB241109_Philippines
C5_AB241111_Philippines
C3_X7656_Polynesia
C3_X75665_Coledomia
C6_AP011102_Japan
C6_AP011103_Japan
C1_AB031265_Vietnam
C1_AB105174_USA
C1_AB074756_Thialand
C1_AB112066_Myanmar
C1_AF223960_Malaysia
C1_AF473543_China
C7_AP011106_Japan
C7_AP011104_Japan
C2_AF182802_China
C2_AF533983_China
C2_D2381_Japan
C2_D23684_Japan
C2_X14193_Korea
36318_HBV_CD
8815_HBV_CD
36372_HBV_CD
33290_HBV_C1
440_HBV_C1
C2_AB006449_Japan

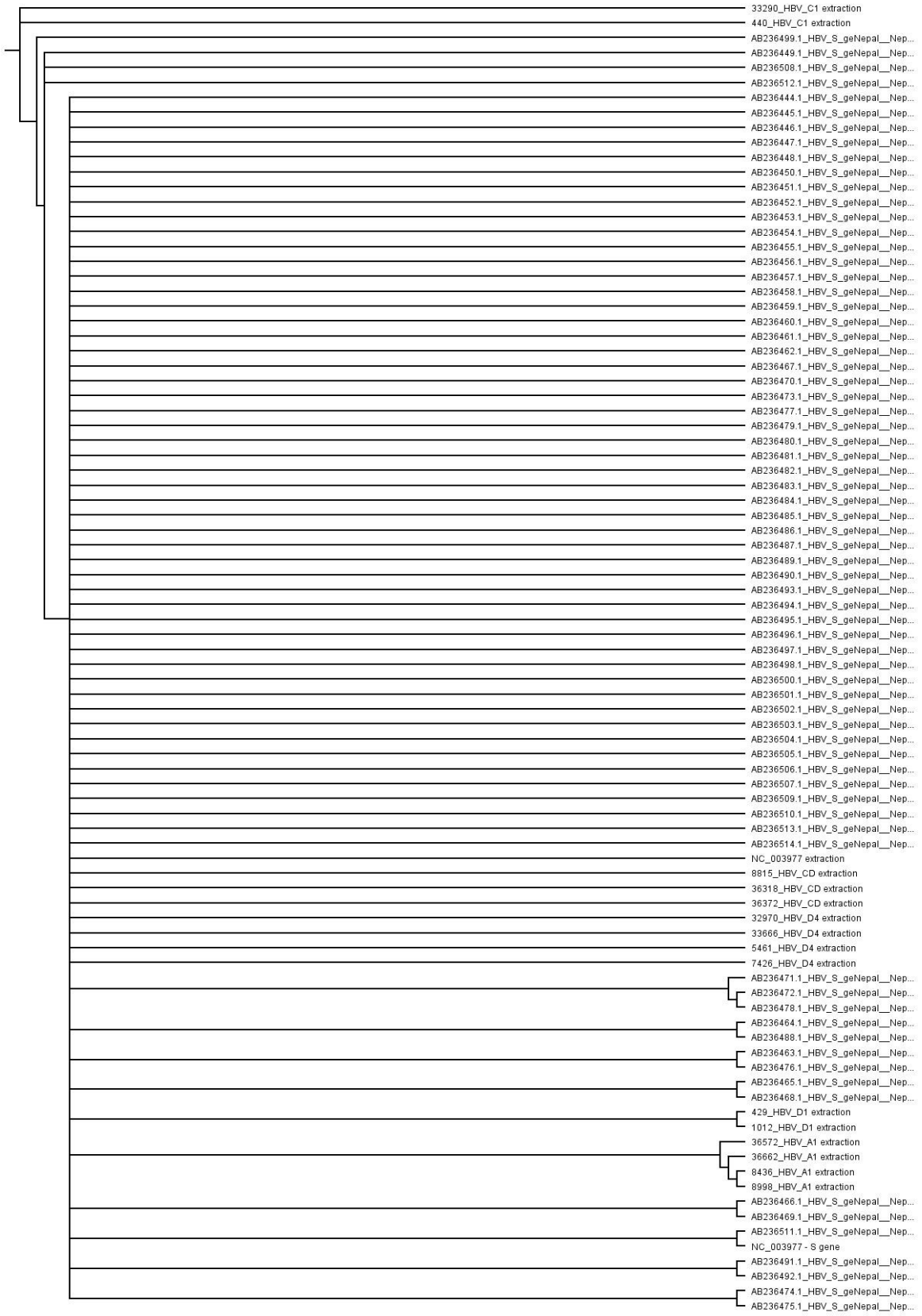


Figure: Phylogenetic analysis of HBV P and S-gene region and reference sequences from Genbank.

CHAPTER FIVE

DISCUSSION

Currently the studies of epidemiology, sero-prevalence rate and prophylaxis of HBV infection have been focused in most parts of the world, as it is tenth most common cause of death world widely. Nepalese populations, especially those in rural area, are not aware of existence and sequel of infection. Nepal has have lowest prevalence of HBV infection with average prevalence rate of HBsAg carrier 0.9 % (Santosh Man Shrestha & Shrestha, 2012). This study reflects the sero-prevalence of hepatitis B using the RDT and ELISA and Real-Time PCR techniques for the quantification of HBV DNA and genotyping of HBV. However, in this study the sero-prevalence rate was found in variable degree according to geographical areas of the country. In overall, the prevalence of HBsAg positivity was 67.95 % (n=53) in province number: 1, 77. 44 % (n=103) in province number: 2, 68.93 % (n=71) in province number: 3. Similarly, the prevalence of HBsAg was 59.2 % (n=29), 50.98 % (n=26), 44.44 % (n=20), 40.48 % (n=17) in province number: 4, 5, 6 and 7 respectively. From the results, it is clear that, province no: 2 and province no: 7 are the most and the least prevalent areas of the country. Such variation in prevalence rate of HBsAg from one part of country to another part was also observed in other countries of Asia (Gust, 1996). In the neighbor country India, variation in prevalence were significantly observed with lowest prevalence of 0.97 % in Chandigarh of northern part and highest prevalence of 5.5 % in Madras of southern part (Tandon, Acharya, & Tandon, 1996).

Serological study by ELISA showed that 18.57 % (n=60) patients had both HBsAg and HBeAg positivity. This means that 18.57 % of CHB patients were in immune tolerance phase. 81.87 % (n=262) of patients had HBsAg and Anti-HBc positivity indicating either acute or chronic infection. 18.57 % (n=60) have HBeAg and anti-HBc positivity indicating chronic infection. Meanwhile 10.31 % (n=33) had HBsAg, HBeAg and Anti-HBc positivity. 3.44 % (n=11) showed HBsAg negativity and Anti-HBc positivity indicating occult hepatitis B infection.

In this study, highest number of HBV patients was found common among the productive age that is 20-40 age group with mean 28.083 ± 5.742 followed by 40-60 age group (47.031 ± 5.623). This result is in accordance with the similar study conducted among

blood donors in Dharan districts of Nepal where the highest prevalence was detected in the same age group (Rai, Dongol, & Khanal, 2016). Many studies suggested that pediatric age group below 10 years was observed as the most susceptible age group for HBV infection. However, in this study none of pediatric age group below 10 years were found to be infected with HBV infection. The prevalence of HBV infection was found to be decreased from the age group above 60 years. Nevertheless, this pattern of increasing prevalence in HBsAg positivity from 20-25 years onward is also in agreement with the result obtained in Singapore (Guan, 1996). The factors which govern such increased prevalence rate in accordance to the age is not clearly understood.

Regarding the gender distribution, males were found to be at higher risk of HBV infection as compared to females. Similar result was observed in our study including one positive case of other gender with HIV co-infection. The ratio of male to female was found 2.01:1, with 66.56 % (n=213) male and 33.12 % (n=1.0) female. The male population was higher because they are involved in different kinds of outdoor activities and exposed to various kinds of risk factors of HBV infection.

The prevalence of co-infections associated with HBV infection is variable in the different geographical area around the world. However, very few studies have been conducted regarding with occurrence of co-infections with HBV virus in most countries of the world including Nepal. In countries with low endemicity of HBsAg positivity like US and Europe, HBV/HIV co-infection was reported to be 6-14 % (Ahuja, Malhotra, Chauhan, & Hans, 2013). In India the overall rate of HBV/HCV and HBV/HIV co-infection were 4.7 % and 1.68 % respectively (Kosaraju, Padukone, & Bairy, 2011). A study conducted among volunteer blood donors in Nepal showed that the prevalence of HBV/HIV co-infection was 0.033% (Thapa & Ghimire, 2008). The rate of HBV and HCV co-infection in HIV patients has been reported in few studies in Nepal. In our study, the co-prevalence of HBV/HIV was 16.56 % (53/320), HBV/HCV was 3.13 (10/320) and HBV/HCV/HIV was 1.56 (5/320).

It is not plausible to distinguish the acute and chronic forms of Hepatitis B only on the basis of serological analysis. This is because HBsAg can be detected in both acute and chronic state of the infections. It is also true that, in case of healthy individuals, no sign and symptoms will appear and occurrence of climax condition of the infection is not possible. Therefore, the details of biochemical tests as well as liver function test (LFT)

are necessary. The mean of biochemical tests like Bili T, Bili D, ALT, AST and ALP in HBV patients was found as 1.188± 1.605, 0.607±0.568, 46.359 ± 20.519, 45.960 ± 25.338 and 196.744 ± 137.516 respectively. Similarly, the mean of Bili T, Bili D, ALT, AST and ALP in healthy subjects was found as 0.982 ± 0.522, 0.426 ±0.400, 31.979 ± 13.201, 27.787 ± 11.524, 142.495 ± 120.455 respectively. This study showed that there was no statistically significant difference between the biochemical tests or LFT like Bili T, Bili D, ALT, AST and ALP between healthy subjects and HBV infected patients. However the result obtained from the estimation of ALT in HBsAg positive cases showed that 54.69 % (n=197) have elevated level of ALT indicating that these samples seem to have carrier state of the infection. The remaining 45.31 % (n=123) of the total HBsAg positive cases represent either for the infection at incubation period or at the prodromal period of the infection or as the persistent carrier. The mean platelets count in HBV patients was significantly lower than the mean platelets count in healthy subjects. Thrombocytopenia is a common feature of chronic liver disease and was reported in 0.94 % (n=3) of total HBV infected patients.

The quantification of HBV DNA by real- time PCR showed that 12.5 % (n=6) had viral load of more than 10 million IU/ml while 87.5 % (n=42) had less than 10 million IU/ml of viral load. It was seen that all the samples with viral load greater than 10 million IU/ml are HBeAg positive. This indicates that they were chronic carrier of HBV infection. In this study it was found that Genotype D was found to be predominantly circulating genotype in serum of HBV patients. This also showed that horizontal mode of transmission of HBV infection is common among Nepalese population. The C/D recombinant genotype was supposed to be transmitted from Tibetans people. Similar result was also found among Nepalese population (Paudel et al., 2015).

Phylogeny was performed using S and P-gene sequences. Phylogeny using P-gene showed agreement of genotyping result as all sequences clustered with their respective genotypes. Many isolates from around world were used to construct the tree. Samples included in this study showed close relation with other isolated from the around the world according to genotype. Genotype-A1 clustered in its respective branch and was very closely related to isolates from France and Belgium than previous A1 genotype reported from Nepal, showing possible origin from Europe. Genotype-C1 also was found to

be more closely related to isolates from Japan, but Genotype-D1 & D4 was related to isolate from India giving hint to their respective probable origin. CD-recombinant genotype however, was found to be related to C1 genotype from this study indicating probable divergence of Genotype C to Genotype D or recombinant event might have occurred in S-gene as S-gene determines Genotype.

CHAPTER SIX

SUMMARY

Hepatitis B infection is a self-limiting disease caused by HBV virus. It is transmitted through blood and other body fluids, including saliva, tears, semen and vaginal secretions. Depending on the epidemiological pattern within a geographic area, the main ways of transmission are sexual intercourse, parenteral contact or infection of the baby at birth from an infected mother. Globally, HBV has become one of the life-threatening diseases in this century. According to World Health Organization (2018), an estimated 257 million people all around the world have been infected and around 887,000 deaths resulted due to hepatitis B virus in 2015. Approximately 30 % of world's population has serological evidence of acute and chronic HBV infection. It is the most common chronic viral infection in the world. . On the basis of the HBV carrier rate, the world can be divided in to high, medium and low endemicity regions. The major concern is about high endemicity countries, especially in Asia and Africa, where the most common routes of infection remain vertical transmission from mother to child and horizontal transmission between family members. Nepal has low prevalence of HBV infection with HBsAg positivity of 0.9 %. The infection is common in rural areas as compared to Urban areas. Horizontal mode of transmission of HBV infection is common among the Nepalese population.

During the periods of 7 months, June 2017 to January 2018, five hundred suspected cases of HBV was collected from one regional hospital; STIDH, two-research centre; nrl and DGRC of Kathmandu districts. They were studied epidemiologically, serologically, immunologically and molecularly. RDT was used for serological, ELISA for immunological and Real- time PCR for molecular study. Along with demographic and clinical details like geographical location, duration of infection, vaccinated status, risk factor was also recorded. Conventional PCR and sequencing of the amplified product of the HBV was performed to study the genotype and sub-genotype of HBV. Statistical analysis was done by using Graph pad prism version 7.

From this study, it was found that province 2 have the highest prevalence rate of HBV infection followed by province no: 3. The prevalence rate was found to be decreased on moving from east to west region of Nepal with lowest prevalence being on province number 7. Jaundice was the main factor for HBV infection in Nepal. Beside Jaundice, HBV infection was common among Drug addicts, alcoholic, HBV/HIV co-infected patients, and family members.

A total of 320 positive HBV patients with age ranging 13 years to 76 years were included in this study. Mean age was 32.281 ± 13.07 years. Most patients were within the most productive and active age group of 20-40 years followed by 40-60 age group. The infection was found high in males as compared to female with male: female ratio of 2.01:1. One cases was of other gender with HIV co-infection. It was found that acute hepatitis infection was high among the Nepalese population. Quantification of HBV DNA from real time PCR showed patients with chronic infection had more than 10 million IU/ml of virus. All the patients with chronic infection are HBeAg positive. Genotype D was found to be predominantly circulating genotype in the serum of HBV infected patients of Nepal.

Sequencing of the fifteen HBV amplified PCR products showed that sub-genotype A1 was very closely related to isolates from France and Belgium than previous A1 genotype reported from Nepal, showing possible origin from Europe. Genotype-C1 also was found to be more closely related to isolates from Japan, but Genotype-D1 & D4 was related to isolate from India giving hint to their respective probable origin. CD-recombinant genotype however, was found to be related to C1 genotype from this study indicating probable divergence of Genotype C to Genotype D or recombinant event might have occurred in S-gene as S-gene determines Genotype.

CHAPTER SEVEN

CONCLUSION

Nepal has low to intermediate prevalence of hepatitis B infection. As there is no any national representative data to confirm the prevalence rate of HBV infection among the Nepalese population. In our study, we had collected five hundred suspected case of HBV from one regional hospital and two-research lab from Kathmandu districts in six months of period. It was found that out of five hundred cases, 64 % (n=320) were HBsAg positive. Most of the patients were from terai region and they were unaware of transmission of this infection. This indicates that HBV infection is spreading among the people of this region. Beside jaundice, it was found that HIV co-infection and drug addicts and alcohol was the main risk factor of HBV in Nepal.

It was found that HBV infection was common among the most productive age group of 20-40 years followed by 40-60 years. It is prodigious burden to our country as most of the active and economically viable age group is being affected by HBV infection. The disease was common among the males who were found to be drug addicts and alcoholic and co-infected with HIV as compared to females**.** As many people have no symptoms during the initial infection. Some develop a rapid onset of sickness with vomiting, yellowish skin, dark urine and abdominal pain. Often these symptoms last a few weeks and rarely does the initial infection result in death. It may take 30 to 180 days for symptoms to begin**.** Lack of proper awareness, vaccination strategy and facility for the estimation of HBV viral load and genotyping are the main problems faced by HBV infected patients of our country. Most of the patients have to entirely dependent on foreign commercial laboratories for the diagnosis of this disease. Therefore, special awareness programs and efforts should be necessary for the prevention of HBV infection in Nepal where HBV infection is less endemic.

This research will help to study and analysis of the patterns, causes and effects of health conditions in defined population of Nepal. The detection of hepatitis B virus DNA will play a significant role in diagnosis and monitoring infection related to HBV. It will also help to assessing therapeutic responses. Genotyping of HBV helps in understanding of

geographical distribution of HBV infection. Sequencing of clinical defined samples helps to reveal the extent of viral genetic variation, response to antiviral therapies and enhance the understanding of the course of infection

RECOMMENDATION

- Each HBsAg cases must be monitored with the liver function test and the possibility of hepatitis B infection must be looked in each jaundice cases.
- Further research in this topic needs to be focused on the Hepatitis B prevention in the high-risk Nepalese population, including vaccination strategy.
- The results of this research may be reflected in providing awareness among the Nepalese people about Hepatitis B risk factors, particularly HIV co-infection, HCV co-infection, tattoo and piercing procedures and safe-sex practices.
- We also recommend the update for public health policies regarding vaccination against Hepatitis B in high-risk Nepalese communities and ethnic and prioritization of antiviral treatment.

CHAPTER SEVEN

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APPENDICES:

Details of Biochemical report of HBV positive patients:

S.N	Sex	Age	Billi (D)	Billi (T)	SGPT	SGOT	ALP	HBeAg	HBsAg	Anti-HBc
1	M	18	1.4	0.5	58	63	102	Positive	Negative	Positive
2	M	19	1.5	0.2	56	40	91	Positive	Positive	Positive
3	M	19	1.2	0.4	62	75	124	Positive	Positive	Negative
4	M	18	1.2	0.6	25	36	88	Positive	Negative	Positive
5	F	18	1	0.1	32	24	368	Positive	Negative	Positive
6	M	16	1	0.3	27	38	238	Positive	Negative	Positive
7	M	13	1.2	0.2	26	45	134	Positive	Negative	Positive
8	M	19	0.88	0.32	54	28	214	Positive	Positive	Positive
9	M	19	1.3	0.3	60	41	92	Positive	Negative	Positive
10	F	18	1.4	0.3	76	48	188	Positive	Negative	Positive
11	M	14	1.4	0.2	54	41	75	Positive	Negative	Positive
12	F	19	0.7	0.1	38	36	192	Positive	Positive	Positive
13	M	19	0.8	0.1	25	58	79	Positive	Negative	Positive
14	F	14	0.6	0.4	36	54	158	Positive	Negative	Positive
15	M	18	0.8	0.2	37	30	61	Positive	Negative	Positive
16	F	17	1.6	1.2	88	94	196	Positive	Negative	Positive
17	M	16	1.2	0.1	25	32	65	Positive	Negative	Positive
18	M	15	1.4	0.4	48	56	142	Positive	Negative	Negative
19	M	16	0.7	0.3	27	38	138	Positive	Negative	Positive
20	M	16	1	0.1	20	42	125	Positive	Negative	Positive
21	F	15	1.2	0.3	18	41	132	Positive	Negative	Positive
22	M	19	0.9	0.2	49	43	61	Positive	Positive	Positive
23	F	14	0.2	0.3	56	34	134	Positive	Positive	Positive
24	F	19	1	0.2	25	24	120	Positive	Positive	Positive
25	M	17	0.2	1.9	23	61	92	Positive	Positive	Negative
26	M	13	1.2	0.2	38	30	94	Positive	Negative	Negative

27	F	19	1.6	0.5	65	98	214	Positive	Negative	Positive
28	F	15	0.88	0.6	84	96	164	Positive	Negative	Positive
29	M	17	0.6	1.2	58	105	218	Positive	Negative	Positive
30	M	16	0.58	0.65	12	69	98	Positive	Negative	Positive
31	F	17	0.96	0.12	36	85	56	Positive	Negative	Negative
32	M	15	0.58	0.48	39	98	65	Positive	Negative	Positive
33	M	14	0.47	0.85	36	86	101	Positive	Negative	Positive
34	M	18	0.84	0.34	76	76	99	Positive	Negative	Positive
35	M	15	0.74	0.88	36	67	81	Positive	Negative	Positive
36	F	15	0.33	0.85	64	65	54	Positive	Negative	Positive
37	F	19	0.99	0.36	52	66	56	Positive	Negative	Negative
38	M	16	1.8	0.6	52	48	188	Positive	Negative	Positive
39	M	19	0.32	0.56	45	25	145	Positive	Negative	Positive
40	M	18	1.2	0.2	17	19	263	Positive	Negative	Negative
41	F	17	0.8	0.2	10	54	80	Positive	Negative	Positive
42	M	18	0.23	0.3	36	34	78	Positive	Negative	Positive
43	M	15	0.54	0.2	25	41	64	Positive	Negative	Positive
44	M	15	0.4	0.1	94	64	116	Positive	Positive	Positive
45	F	17	1	0.2	67	33	71	Positive	Positive	Positive
46	F	19	0.4	0.1	31	25	211	Positive	Positive	Positive
47	M	17	0.6	0.1	27	24	137	Positive	Negative	Positive
48	M	19	0.5	0.13	37	27	74	Positive	Negative	Positive
49	F	17	1.2	0.4	58	80	183	Positive	Negative	Positive
50	M	23	1.8	0.4	25	27	99	Positive	Negative	Positive
51	M	25	1	0.22	36	34	154	Positive	Positive	Positive
52	M	22	1.8	0.6	25	13	96	Positive	Negative	Positive
53	M	23	0.33	0.21	56	61	154	Positive	Negative	Positive
54	M	29	1.2	0.32	32	23	119	Positive	Negative	Positive
55	M	20	1.3	0.2	25	27	93	Positive	Negative	Negative

56	M	23	1.2	0.6	35	71	164	Positive	Negative	Positive
57	M	21	1.32	0.1	45	71	144	Positive	Negative	Positive
58	M	23	1.2	0.3	28	27	65	Positive	Positive	Positive
59	M	27	0.32	0.2	47	36	67	Positive	Negative	Positive
60	M	22	1.2	0.3	21	28	66	Positive	Positive	Positive
61	M	27	0.7	0.2	44	56	90	Positive	Negative	Positive
62	M	24	0.8	0.2	21	24	102	Positive	Positive	Positive
63	F	25	1.23	0.8	25	51	133	Positive	Negative	Positive
64	M	24	0.8	0.2	55	36	47	Positive	Negative	Positive
65	M	22	1.2	0.2	38	30	94	Positive	Negative	Negative
66	M	26	0.2	0.6	81	34	27	Positive	Negative	Positive
67	M	25	0.21	1.3	56	53	28	Positive	Negative	Positive
68	F	21	0.8	0.1	56	188	107	Positive	Positive	Negative
69	M	26	0.4	0.1	66	85	88	Positive	Negative	Positive
70	M	21	1	0.2	103	109	104	Positive	Positive	Positive
71	F	20	0.7	0.2	19	23	61	Positive	Positive	Positive
72	F	23	1.3	0.3	19	45	195	Positive	Negative	Positive
73	M	23	0.9	0.1	34	24	28	Positive	Positive	Positive
74	M	26	0.32	0.9	65	74	99	Positive	Negative	Positive
75	M	29	1.5	0.4	31	59	238	Positive	Negative	Positive
76	M	26	1.6	0.2	20	25	68	Positive	Negative	Positive
77	M	28	1.32	0.8	54	66	111	Positive	Positive	Positive
78	M	29	1.88	0.6	57	68	165	Positive	Negative	Positive
79	M	21	0.6	0.4	54	68	158	Positive	Negative	Positive
80	M	29	1.2	0.3	19	26	80	Positive	Negative	Negative
81	F	21	1.2	0.6	90	60	102	Positive	Positive	Positive
82	F	23	0.7	0.4	58	39	80	Positive	Negative	Positive
83	M	21	1	0.2	51	35	91	Positive	Negative	Positive
84	M	20	1.36	0.52	84	45	124	Positive	Negative	Positive

85	M	25	0.9	0.7	64	78	268	Positive	Negative	Positive
86	F	22	1.8	0.6	61	70	90	Positive	Positive	Positive
87	F	25	2.1	0.8	58	24	188	Positive	Negative	Positive
88	M	26	1.4	0.2	41	35	74	Positive	Negative	Positive
89	M	20	2	0.8	80	96	254	Positive	Negative	Positive
90	M	22	1.88	1.64	94	88	324	Positive	Negative	Positive
91	F	21	1.6	0.6	66	84	198	Positive	Negative	Negative
92	M	23	1.5	0.6	23	65	420	Positive	Negative	Positive
93	M	24	1.25	0.6	37	43	261	Positive	Negative	Positive
94	F	21	1	0.2	20	19	61	Positive	Negative	Positive
95	M	24	1	0.1	36	28	104	Positive	Negative	Positive
96	F	22	1.8	0.6	61	70	90	Positive	Negative	Positive
97	M	22	1.6	0.5	78	72	358	Positive	Negative	Positive
98	M	21	1	0.4	51	35	91	Positive	Negative	Positive
99	F	20	2	0.7	62	68	259	Positive	Positive	Positive
100	M	27	1.4	0.3	42	32	102	Positive	Negative	Negative
101	M	20	1.3	0.3	30	36	84	Positive	Negative	Positive
102	M	29	2.1	0.8	71	24	176	Positive	Negative	Positive
103	F	28	1	0.3	27	38	138	Positive	Negative	Positive
104	M	22	1.4	0.4	45	38	198	Positive	Negative	Positive
105	F	25	0.4	0.1	42	22	96	Positive	Negative	Positive
106	F	27	1.4	0.3	54	51	75	Positive	Positive	Positive
107	M	25	0.9	0.1	24	37	74	Positive	Negative	Positive
108	F	22	0.3	0.1	56	48	325	Positive	Positive	Positive
109	F	25	1.5	0.4	31	59	352	Positive	Negative	Positive
110	M	20	2.2	0.5	59	68	245	Positive	Positive	Positive
112	M	20	2	0.6	45	39	280	Positive	Positive	Positive
113	M	27	18	0.5	50	38	159	Positive	Negative	Positive
114	M	24	0.1	0.3	32	31	79	Positive	Negative	Positive

115	F	20	0.8	0.2	10	17	43	Positive	Negative	Negative
116	M	22	1.3	0.2	46	18	74	Positive	Negative	Positive
117	F	28	1.1	0.2	21	13	73	Positive	Negative	Positive
118	M	22	0.8	0.2	61	37	73	Positive	Negative	Positive
119	M	20	1.2	0.2	35	26	126	Positive	Positive	Positive
120	F	22	0.9	0.2	71	176	75	Positive	Negative	Negative
121	M	20	0.9	0.2	73	50	78	Positive	Negative	Positive
122	F	25	1.6	0.7	20	26	58	Positive	Positive	Positive
123	F	24	0.23	0.63	41	27	78	Positive	Negative	Negative
124	M	21	0.5	0.1	42	66	86	Positive	Negative	Positive
125	M	23	2.5	0.4	29	25	84	Positive	Negative	Positive
126	M	27	0.8	0.4	16	26	98	Positive	Negative	Positive
127	F	26	2.5	0.5	25	21	74	Positive	Positive	Positive
128	M	21	0.6	0.2	35	24	68	Positive	Negative	Positive
129	M	24	1.6	0.8	48	39	258	Positive	Negative	Negative
130	M	26	0.8	0.2	50	36	83	Positive	Positive	Positive
131	M	22	0.7	0.2	45	39	68	Negative	Positive	Positive
132	M	23	1.1	0.2	26	31	53	Positive	Negative	Positive
133	M	20	0.9	0.2	16	20	114	Positive	Negative	Negative
134	M	27	1.2	0.3	56	58	43	Positive	Negative	Positive
135	F	26	1.65	0.56	31	67	36	Positive	Negative	Positive
136	M	22	0.64	0.36	147	35		Positive	Negative	Negative
137	M	21	1	0.2	106	156	153	Positive	Negative	Positive
138	M	29	0.55	0.12	92	41	51	Negative	Positive	Positive
139	F	27	0.54	0.16	35	20	92	Negative	Positive	Positive
140	M	25	0.86	0.18	48	34	138	Negative	Positive	Positive
141	M	27	0.4	0.09	28	21	88	Positive	Negative	Positive
142	M	24	0.92	0.21	21	15	47	Positive	Negative	Negative
143	F	27	0.54	0.16	35	20	163	Positive	Negative	Positive

144	M	27	0.4	0.09	28	21	47	Positive	Negative	Positive
145	M	29	0.55	0.12	92	41	88	Positive	Negative	Positive
146	F	29	0.86	0.18	48	34	138	Positive	Negative	Negative
147	F	26	0.6	0.1	34	24	28	Positive	Negative	Positive
148	F	28	0.72	0.43	46	38	257	Positive	Negative	Positive
149	M	24	0.68	0.54	54	67	354	Positive	Negative	Positive
150	M	24	0.3	0.2	40	35	256	Positive	Negative	Positive
151	M	26	0.88	0.66	56	67	125	Positive	Negative	Positive
152	M	21	0.91	0.62	3	36	312	Positive	Negative	Positive
153	M	28	0.35	0.1	62	38	251	Positive	Negative	Negative
154	M	26	0.98	0.61	58	45	136	Positive	Negative	Positive
155	M	23	1	0.33	91	81	123	Positive	Negative	Positive
156	F	25	1.4	2.33	27	89	148	Positive	Negative	Positive
157	F	28	0.8	1.5	37	56	164	Positive	Negative	Negative
158	F	29	2.1	1.4	84	70	254	Positive	Negative	Positive
159	M	38	0.6	1.2	82	24	247	Positive	Negative	Positive
160	M	32	0.9	1.4	84	84	236	Positive	Positive	Negative
161	F	35	0.23	0.2	20	21	89	Positive	Negative	Positive
162	F	32	1	0.7	42	64	512	Positive	Negative	Positive
163	F	30	0.8	1.3	61	44	459	Positive	Negative	Positive
164	M	30	1	0.9	26	24	435	Positive	Negative	Positive
165	M	32	1	0.2	44	56	315	Positive	Negative	Negative
166	M	33	1.3	0.4	108	143	215	Negative	Positive	Positive
167	M	30	0.63	0.2	42	101	154	Positive	Negative	Positive
168	M	37	0.8	0.2	34	27	341	Positive	Negative	Positive
169	M	33	1.2	0.3	22	23	391	Positive	Negative	Negative
170	F	39	0.8	0.6	20	37	489	Positive	Negative	Positive
171	M	34	2.1	1.2	41	41	165	Positive	Negative	Positive
172	M	39	1.4	1.3	43	42	254	Positive	Negative	Positive

173	M	30	1	0.2	44	24	93	Positive	Negative	Positive
174	M	35	0.8	1.65	39	56	108	Positive	Negative	Negative
175	F	36	0.36	1.32	24	37	139	Positive	Negative	Positive
176	F	32	0.7	0.6	44	30	200	Positive	Negative	Positive
177	M	37	1	2.2	84	60	103	Positive	Positive	Positive
178	M	38	2.2	0.8	60	22	160	Positive	Positive	Positive
179	F	32	1	2.4	72	88	160	Positive	Negative	Positive
180	F	32	0.7	0.2	26	24	84	Positive	Negative	Positive
181	F	30	1	0.2	20	46	93	Positive	Negative	Positive
182	M	35	0.6	1.32	65	36	155	Positive	Negative	Negative
183	F	32	0.4	0.56	25	47	148	Positive	Negative	Positive
184	F	30	0.65	2.56	56	48	167	Positive	Negative	Positive
189	M	30	1.6	1.32	58	96	156	Positive	Negative	Positive
190	M	35	1.2	0.2	38	30	94	Positive	Negative	Positive
191	F	38	0.9	0.6	50	48	166	Positive	Negative	Positive
192	M	36	0.9	0.4	22	48	102	Positive	Positive	Positive
193	M	39	0.3	0.1	25	27	136	Positive	Negative	Positive
194	M	32	1.4	0.2	48	35	74	Positive	Negative	Positive
195	M	32	1	0.5	16	28	245	Positive	Negative	Positive
196	M	33	1.3	0.2	56	60	96	Positive	Negative	Positive
197	M	36	0.2	0.8	52	50	622	Positive	Negative	Positive
198	F	32	1.2	0.3	35	41	133	Positive	Positive	Positive
199	M	36	1.4	0.4	17	21	150	Positive	Positive	Negative
200	M	36	0.5	0.1	11	25	41	Positive	Positive	Positive
201	M	37	0.85	1.56	42	63	531	Positive	Negative	Positive
202	F	35	1.2	0.2	40	21	89	Positive	Positive	Positive
203	F	37	1	0.3	25	56	80	Positive	Negative	Positive
204	M	33	0.2	1.33	55	81	254	Positive	Negative	Positive
205	M	30	0.55	1.36	23	57	235	Positive	Negative	Positive

206	M	33	1	0.2	32	25	66	Positive	Negative	Negative
207	M	35	2.13	1	36	23	412	Positive	Negative	Positive
208	F	30	1.2	0	14	20	122	Positive	Negative	Positive
209	F	30	1.8	1.4	45	54	211	Positive	Negative	Positive
210	F	35	2.4	0.7	24	45	119	Positive	Positive	Positive
211	M	38	0.8	0.2	49	18	82	Positive	Negative	Negative
212	M	39	2	1.54	62	51	512	Positive	Negative	Positive
213	F	35	1.65	1.11	23	36	412	Positive	Positive	Positive
214	F	30	0.8	0.2	24	25	431	Positive	Negative	Positive
215	M	34	3	1	47	61	521	Positive	Negative	Positive
216	M	38	0.6	2.31	46	54	621	Positive	Negative	Negative
217	F	35	0.8	2.11	43	64	421	Positive	Negative	Positive
218	M	33	22	1.66	42	2219	433	Positive	Negative	Positive
219	F	30	1.96	1.54	41	25	413	Positive	Negative	Positive
220	F	35	0.8	0.2	109	58	600	Positive	Positive	Positive
221	F	38	1.33	0.63	52	636	233	Positive	Negative	Positive
222	M	37	1.6	0.4	54	38	245	Positive	Negative	Negative
223	M	36	0.9	0.2	54	33	278	Positive	Negative	Positive
224	F	30	0.7	0.33	45	35	299	Positive	Negative	Positive
225	M	31	1	1.63	46	39	351	Positive	Negative	Positive
226	M	30	2.1	1.32	42	34	351	Negative	Positive	Positive
227	M	38	0.5	1.58	43	32	441	Positive	Negative	Positive
228	M	39	0.92	0.21	36	30	551	Positive	Negative	Positive
229	M	30	0.5	0.2	35	34	79	Positive	Negative	Negative
230	M	38	0.8	0.2	10	31	52	Positive	Positive	Positive
231	M	32	1.3	0.4	108	143	156	Positive	Negative	Positive
232	M	36	0.9	0.98	65	88	154	Positive	Negative	Positive
233	M	35	0.55	0.47	45	81	165	Positive	Positive	Positive
234	F	31	1.8	1.78	23	27	144	Positive	Negative	Positive

235	F	31	2.33	2.21	51	26	175	Positive	Positive	Positive
236	M	38	2.3	0.36	41	31	168	Positive	Negative	Positive
237	M	30	1.4	0.96	35	60	187	Positive	Negative	Positive
238	F	32	0.33	0.24	62	76	176	Positive	Negative	Positive
239	F	31	0.45	1.35	45	34	354	Positive	Positive	Positive
240	M	35	1.64	0.48	47	20	346	Positive	Negative	Negative
241	F	38	0.55	1.66	49	22	389	Positive	Positive	Positive
242	M	39	0.45	1.58	48	19	485	Positive	Negative	Positive
243	M	34	1.45	0.65	52	14	413	Positive	Negative	Positive
244	M	40	1	0.2	46	98	120	Positive	Positive	Positive
245	F	45	2.33	0.74	34	58	512	Positive	Negative	Positive
246	M	46	2.45	0.69	28	25	365	Positive	Negative	Positive
247	M	46	1.4	0.3	49	21	354	Positive	Negative	Positive
248	M	40	0.8	0.4	75	28	421	Positive	Negative	Negative
249	F	45	2.1	0.9	59	19	551	Positive	Negative	Positive
250	M	42	1.3	1.52	54	17	532	Positive	Negative	Positive
251	M	40	1.8	0.4	68	23	234	Positive	Negative	Positive
252	M	45	1	0.2	61	70	241	Positive	Negative	Positive
253	F	44	1.4	0.6	20	49	176	Positive	Negative	Positive
254	F	42	0.9	0.2	27	24	119	Positive	Negative	Negative
256	M	42	1.6	0.4	30	26	166	Positive	Negative	Positive
257	F	42	1.2	0.2	22	48	102	Positive	Negative	Positive
258	M	40	0.8	0.4	71	28	143	Positive	Negative	Positive
259	M	48	0.2	0.69	36	24	136	Positive	Negative	Positive
260	F	49	1.9	0.3	25	36	84	Positive	Positive	Weak positive
261	M	45	0.8	0.4	32	28	350	Positive	Negative	Negative
262	F	43	0.6	0.2	60	36	342	Positive	Negative	Positive
263	M	41	0.2	0.5	33	22	56	Positive	Negative	Positive
264	M	45	0.8	0.2	22	34	92	Positive	Positive	Negative

265	F	42	0.8	0.2	42	23	481	Positive	Negative	Positive
266	M	45	2.3	1.36	43	25	225254	Positive	Negative	Negative
267	M	40	0.45	1.65	36	20	366	Positive	Negative	Positive
268	F	49	0.8	0.2	15	45	324	Positive	Positive	Positive
269	F	44	0.7	0.2	20	35	452	Positive	Negative	Positive
270	F	46	0.66	0.36	56	36	421	Positive	Negative	Positive
271	M	40	0.21	0.54	45	71	441	Positive	Negative	Positive
272	M	48	2.4	0.7	49	61	214	Positive	Negative	Negative
273	M	43	2.13	0.69	56	38	321	Positive	Negative	Positive
274	M	40	0.7	0.3	64	43	91	Positive	Positive	Positive
275	M	40	0.65	0.44	28	24	105	Positive	Negative	Positive
276	M	40	0.9	0.2	49	36	167	Positive	Negative	Positive
277	F	47	1.55	0.44	64	20	143	Positive	Negative	Negative
278	M	48	0.56	0.14	90	58	188	Positive	Negative	Positive
279	M	43	0.36	0.33	85	36	79	Positive	Negative	Positive
280	M	48	0.56	0.54	57	59	176	Positive	Negative	Positive
281	M	43	1.47	0.64	67	38	155	Positive	Negative	Positive
282	M	41	1.69	0.55	37	37	134	Positive	Positive	Positive
283	M	45	1.25	1.67	35	56	421	Positive	Negative	Positive
284	M	48	2.13	2.36	36	37	123	Negative	Positive	Negative
285	F	43	2.41	1.98	39	30	255	Positive	Negative	Positive
286	F	43	1.65	0.51	56	34	241	Positive	Negative	Negative
287	F	44	0.85	1.36	54	36	236	Positive	Negative	Positive
288	F	50	0.8	0.2	48	28	48	Positive	Negative	Positive
289	M	51	0.24	2.41	94	29	45	Positive	Positive	Positive
290	M	53	0.65	2.37	65	47	412	Positive	Negative	Positive
291	M	59	1	0.2	35	18	325	Positive	Negative	Positive
292	M	59	1	0.2	54	57	622	Positive	Negative	Positive
293	M	52	1	0.2	48	46	125	Positive	Positive	Positive

294	M	50	1.11	0.64	49	61	452	Positive	Negative	Positive
295	M	55	0.7	0.2	13	38	621	Positive	Negative	Positive
296	M	52	1.33	1.92	46	30	412	Positive	Negative	Positive
297	M	50	0.9	0.3	43	58	8481	Positive	Positive	Positive
298	M	52	1.03	0.26	42	64	285	Negative	Positive	Positive
299	M	52	1.23	0.26	50	72	375	Positive	Negative	Negative
300	F	56	0.95	0.62	56	49	151	Positive	Positive	Positive
301	F	59	0.75	0.56	59	75	259	Positive	Negative	Positive
302	M	58	2.8	0.38	58	75	157	Positive	Negative	Positive
303	F	54	1.55	0.37	54	94	495	Positive	Positive	Negative
304	M	51	1.47	0.78	56	65	381	Positive	Negative	Positive
305	M	58	2.11	0.95	53	17	245	Positive	Positive	Negative
306	M	57	0.56	1.56	77	61	348	Positive	Negative	Positive
307	M	51	0.22	0.11	46	70	347	Positive	Negative	Positive
308	F	53	0.34	0.57	78	32	314	Positive	Negative	Positive
309	M	67	0.59	0.2	20	23	366	Positive	Negative	Negative
310	M	65	0.23	0.88	56	24	351	Positive	Negative	Positive
311	M	63	1	0.2	54	27	165	Positive	Negative	Positive
312	F	60	1.36	0.66	55	28	125	Positive	Negative	Positive
313	M	60	0.8	0.2	52	23	84	Positive	Negative	Positive
314	M	63	1.2	0.3	53	24	136	Positive	Negative	Positive
315	F	67	0.8	0.2	35	48	142	Positive	Negative	Negative
316	M	60	2.31	1.23	55	47	125	Positive	Negative	Positive
317	F	76	1.36	2.22	54	58	145	Positive	Negative	Positive
318	F	62	2.33	1.65	51	54	165	Positive	Negative	Positive
319	F	60	2.14	1.54	61	46	254	Positive	Negative	Positive
320	M	65	0.65	1.98	62	47	255	Positive	Negative	Positive
321	M	62	1.32	2.54	56	52	145	Positive	Negative	Negative
322	F	66	0.65	1.36	48	56	512	Positive	Negative	Positive

323	F	60	1.54	2.31	47	51	236	Negative	Positive	Positive
324	M	67	0.65	0.67	56	48	285	Positive	Negative	Positive
325	F	65	0.33	0.39	64	49	247	Positive	Negative	Positive
326	F/M	45	0.21	0.68	34	67	168	Positive	Positive	Positive

HBsAg ELIZA:

Of the 500 samples tested with conventional ELISA for HBsAg, 311 were HBsAg positive and 9 were occult hepatitis B infection.

S.N	Abs	Abs/ COV	Result	S.N	Abs	Abs/ COV	Result
Blank	0.01	-	-	114	3.002	28.591	Positive
NC	0.011	0.105	Negative	115	2.521	24.01	Positive
NC	0.012	0.108	Negative	116	2.087	19.876	Negative
NC	0.011	0.105	Negative	117	2.065	19.667	Positive
PC	2.761	26.295	Positive	118	2.102	20.019	Positive
PC	2.759	26.096	Positive	119	2.009	19.133	Positive
1	2.721	25.914	Positive	120	2.664	25.372	Positive
2	2.775	25.971	Positive	121	0.015	0.142	Negative
3	2.727	26.505	Positive	122	0.014	0.133	Positive
4	2.783	23.676	Positive	123	2.6	24.762	Positive
5	2.486	26.61	Positive	124	2.264	21.562	Positive
6	0.02	0.19	Negative	125	2.375	22.619	Negative
6	2.739	27.735	Positive	126	2.201	20.962	Positive
7	2.302	16.697	Positive	127	2.282	21.738	Positive
8	1.82	22.619	Positive	128	0.007	0.066	Negative
9	2.375	25.552	Positive	129	2.683	25.552	Positive
10	2.683	25.029	Positive	130	2.87	27.333	Positive
11	2.628	25.372	Positive	131	2.711	25.819	Positive
12	-0.014	0.000	Negative	132	2.653	25.267	Positive
13	2.65	24.915	Positive	134	2.43	23.143	Positive

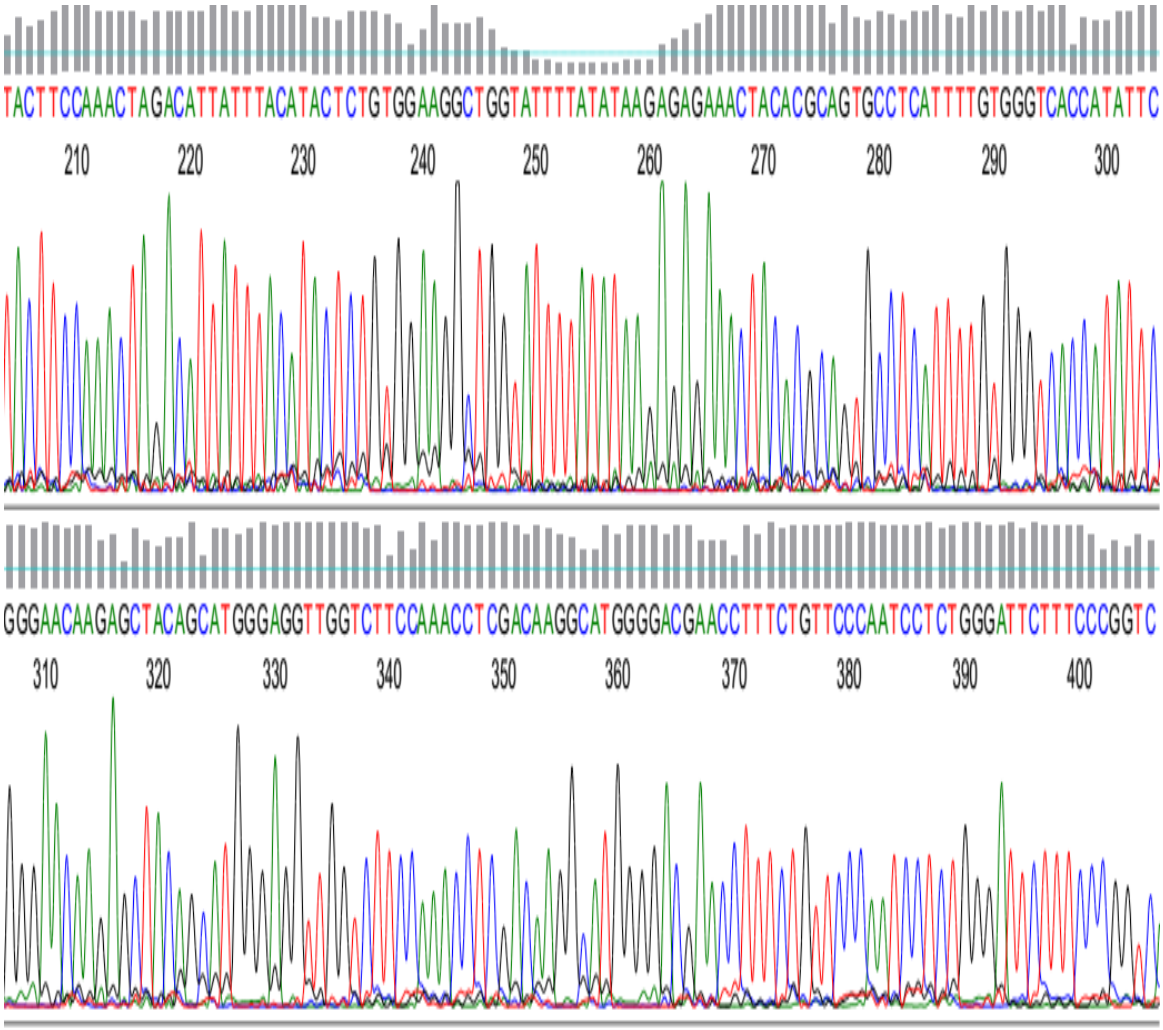
14	2.616	25.448	Positive	135	0.014	0.133	Negative
15	2.672	22.619	Positive	136	2.376	22.629	Positive
16	2.375	24.858	Positive	137	2.008	19.124	Positive
17	2.61	24.858	Positive	138	2.113	20.124	Positive
PC	2.079	19.981	Positive	139	2.053	19.553	Positive
19	2.664	25.239	Positive	140	2.121	20.2	Positive
20	2.794	26.61	Positive	141	2.561	24.39	Positive
21	0.011	0.105	Negative	142	2.651	25.248	Positive
22	0.003	0.029	Positive	143	2.361	22.486	Positive
23	2.392	22.781	Positive	144	2.532	24.114	Positive
24	0.001	0.01	Positive	145	2.423	23.076	Positive
25	0.002	0.019	Negative	146	2.451	23.343	Positive
26	0.015	0.143	Negative	147	2.32	22.095	Positive
27	2.393	22.79	Positive	148	1.998	19.028	Positive
28	0.500	4.762	Positive	149	3.001	28.571	Positive
29	2.794	26.61	Positive	150	0.012	1.114	Negative
30	2.381	22.676	Positive	151	2.118	20.171	Positive
31	2.98	28.381	Positive	152	2.009	19.133	Positive
32	2.468	23.505	Positive	153	2.474	23.561	Positive
33	3.096	29.486	Positive	154	2.269	21.609	Positive
34	2.754	26.229	Positive	155	2.341	22.295	Positive
35	0.008	0.076	Negative	156	2.142	20.4	Positive
36	2.933	27.933	Positive	157	0.01	0.095	Negative
37	2.822	26.876	Positive	158	2.375	22.619	Positive
38	3.001	28.676	Positive	159	2.683	25.552	Positive
39	0.21	2.004	Positive	160	2.628	25.028	Positive
40	3.274	31.181	Positive	161	-0.014	0.133	Negative
41	3.046	29.01	Positive	162	0.005	0.047	Negative
42	0.101	0.962	Negative	163	2.616	24.914	Positive

43	3.103	29.552	Positive	164	2.672	25.447	Positive
44	2.767	26.352	Positive	165	0.01	0.095	Negative
45	0.115	1.095	Negative	166	2.61	24.857	Positive
46	3.316	31.581	Positive	167	2.065	19.666	Positive
47	2.091	19.914	Positive	168	2.557	24.352	Positive
48	0.639	3.086	Negative	169	2.93	27.904	Positive
49	2.982	28.4	Positive	170	0.011	0.104	Negative
50	0.267	2.548	Positive	171	2.051	19.533	Positive
51	0.193	1.842	Negative	172	2.15	20.476	Positive
52	3.209	30.562	Positive	173	0.009	0.085	Negative
53	3.2	30.477	Positive	174	3.107	29.59	Positive
54	3.03	28.858	Positive	175	3.001	28.58	Positive
55	3.221	30.676	Positive	176	2.611	24.866	Positive
56	3.237	30.828	Positive	177	2.103	20.028	Positive
57	3.124	29.753	Positive	178	0.005	0.047	Negative
57	0.22	2.099	Negative	179	2.579	24.562	Positive
58	3.263	31.077	Positive	180	2.18	20.762	Positive
59	0.116	1.107	Negative	181	2.075	19.762	Positive
60	3.203	30.505	Positive	182	2.153	20.505	Positive
61	0.012	0.144	Negative	183	2.008	19.124	Positive
62	2.865	27.201	Positive	184	2.547	23.4	Positive
63	2.532	24.112	Positive	185	2.369	22.562	Positive
64	2.31	21.998	Positive	186	0.006	0.0057	Negative
65	3.044	28.991	Positive	187	0.001	0.000	Negative
66	3.122	29.734	Positive	188	2.647	25.095	Positive
67	3.085	29.382	Positive	189	2.887	27.495	Positive
68	2.71	25.807	Positive	190	2.319	22.086	Positive
69	0.011	0.105	Negative	191	2.11	20.095	Positive
70	3.157	30.067	Positive	192	2.53	24.095	Positive

71	3.214	30.61	Positive	193	2.489	23.704	Positive
72	2.878	27.41	Positive	194	0.002	0.019	Negative
73	3.127	29.781	Positive	195	0.006	0.0571	Negative
74	2.339	22.276	Negative	196	2.656	25.295	Positive
75	2.441	23.247	Positive	197	2.125	20.238	Positive
76	2.61	24.855	Positive	198	2.579	24.562	Positive
77	0.000	0.000	Negative	199	2.343	22.314	Positive
78	3.102	29.543	Positive	200	2.515	23.952	Positive
79	3.04	28.953	Positive	201	0.02	0.19	Negative
80	0.01	0.095	Negative	202	2.739	27.735	Positive
81	3.001	28.578	Positive	203	2.302	16.697	Positive
82	0.102	0.971	Negative	204	1.82	22.619	Positive
83	0.113	1.076	Negative	205	2.375	25.552	Positive
84	0.13	1.238	Negative	206	2.683	25.029	Positive
85	2.53	24.093	Positive	207	2.628	25.372	Positive
86	2.489	23.703	Positive	208	-0.012	0.000	Negative
87	2.325	22.143	Positive	209	2.651	24.916	Positive
88	0.01	0.0952	Negative	210	2.617	25.449	Positive
89	2.656	25.296	Positive	211	2.673	22.619	Positive
90	0.011	0.000	Negative	212	2.365	24.848	Positive
91	0.012	0.114	Negative	213	0.025	0.238	Positive
92	2.343	22.315	Positive	214	2.662	25.353	Positive
93	0	0	Negative	215	3.009	28.657	Positive
94	2.515	23.953	Positive	216	2.557	24.353	Positive
95	2.761	26.298	Positive	217	2.93	27.905	Positive
96	0.042	0.4	Negative	218	2.18	20.762	Positive
97	0.025	0.238	Positive	219	2.051	19.534	Positive
98	2.662	25.353	Positive	220	0.016	0.152	Negative
99	3.009	28.657	Positive	221	0.013	0.124	Negative

100	2.557	24.353	Positive	222	3.107	29.591	Positive
101	2.93	27.905	Positive	223	3.001	28.578	Positive
102	2.18	20.762	Positive	224	2.611	25.343	Positive
103	2.051	19.534	Positive	226	2.103	20.029	Positive
104	0.016	0.152	Negative	227	2.178	20.743	Positive
105	0.013	0.124	Negative	228	2.579	24.562	Positive
106	3.107	29.591	Positive	229	0.013	0.114	Negative
107	3.001	28.578	Positive	230	0.012	0.114	Positive
108	2.611	25.343	Positive	231	3.002	28.591	Positive
109	2.103	20.029	Positive	232	2.521	24.01	Positive
110	2.178	20.743	Positive	233	2.721	25.914	Positive
111	2.579	24.562	Positive	234	2.775	25.971	Positive
112	0.013	0.114	Negative	235	2.727	26.505	Positive
113	0.012	0.114	Positive	236	2.557	24.353	Positive
114	3.002	28.591	Positive	237	2.515	23.953	Positive
115	2.521	24.01	Positive	238	3.002	28.591	Positive
116	2.087	19.876	Negative	239	2.091	19.914	Positive

Chromatogram:



Sequencing Reaction:

The Sequencing mix Composition is as follows:

S.N	Reagents	For single reaction
1	Ready Reaction Mix	4µl
2	Template (100ng/µl)	1µl
3	Primer (10pmol/λ)	2µl
4	Milli Q Water	3µl
	Total volume	10 µl

The sequencing PCR Conditions is as follows: (25 cycles)

Steps	Temperature	Time	Cycles
Initial Denaturation	96°C	5 min	
Denaturation	96°C	30 sec	25 cycles
Hybridization	50 °C	30 sec	
Elongation	60 °C	1.30 min	

Collection of questionnaire from HBV patient

Collection of blood samples from HBV

Collection of questionnaire from HBV patient