Complete Genome Sequence Analysis of Hepatitis B Virus Isolated from Pakistani Patients with Chronic HBV Infection

BY

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(2008-NUST-BS-V&I-05)

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2012.

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

of Bachelors of Applied Biosciences

With

Majors in Biotechnology

Atta-ur-Rahman School of Applied Biosciences
National University of Sciences & Technology
Islamabad, Pakistan
2012

National University of Sciences and Technology

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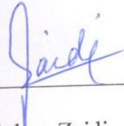
Supervisor: 

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Dedication

I Dedicate this Thesis to
My Grand Parents
And
My Family

ACKNOWLEDGEMENTS

First and foremost I thank ALLAH (SWT) for making it all possible. Secondly I would offer my sincerest gratitude to our Principle Dr Muhammad Ashraf and my supervisor, Dr. Najam us Sahar Sadaf Zaidi, who supported me throughout my thesis with her patience and knowledge whilst allowing me the room to work in my own way. One simply could not wish for a better or friendlier supervisor.

I would like to acknowledge Dr. Hajra Sadia for their kind guidance and support during thee entire period of my work. My sincere thanks to Kashif Mehmood, Abbas Raza, Fakhira Begum, Abida Siddiqa, Muhammad Suleman Riaz and Dr. Mustafeez Mujtaba for helping me a lot during my research work. I would like to acknowledge my fellow lab mates; Rubina, Habiba, Humaira, Zaira and Ammad faheem for all the fun we had during the last one year, my class fellows and my hostel friends for their help and sincerity. Last but not the least; I would like to thank my family for all their love, for supporting me both financially and spiritually throughout my university days, and being with me on every step of my career and life.

Lord Umer Javed

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LIST OF ABBREVIATIONS

HBV	Hepatitis B Virus
HCC	Hepatocellular carcinoma
DNA	Deoxyribonucleic acid
C gene	Core gene
S gene	Surface gene
CDC	Centre of disease control
Prec/c	Precore and core
HBcAg	Hepatitis B core antigen
HBeAg	Hepatitis B e antigen
NCBI	National Center for Biotechnology Information
Nt	Nucleotide
ER	Endoplasmic Reticulum
Pg	RNA Pregenomic RNA
mRNA	Messenger RNA
S	Surface
Pol (P)	Polymerase
C	Core
En1	Enhancer element 1
En2	Enhancer element 2
3D	Three dimensional
P	Proline
ORF	Open reading frame

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ABSTRACT

Hepatitis B Virus (HBV)has been etiologically linked to several liver related ailments of both chronic and acute nature, including fatal fulminant hepatitis, cirrhosis and hepatocellular carcinoma, which is a highly prevalent human cancer and is responsible for significant morbidity and mortality internationally. A large number of genomic discrepancies have been reported in HBV over the past two decades and these induce a specific change in virus biology. The variants involved in host immune system evasion radically modify the virus-host interactions when present and are a serious challenge to healthcare personnel.To investigate this, a cross sectional study was conducted in HBV infected patients identified randomly in different tertiary care hospitals of Islamabad and Rawalpindi, which will lead to the characterization of sequence variations in HBV genome after amplification and cloning.The purpose of this study was to demonstrate full length genome mutations in chronic HBV infected patients in the Pakistani population and to individually map the mutations in the viral gene products of the HBV to extensively describe their molecular characteristics in viral life cycle and course of infection. New treatment alternatives have to be employed to treat accumulated viral mutations as seen in the case of Hepatitis B virus mutants in Pakistan. There is a dire need that pathologists, pharmaceutical and the healthcare industry augment their awareness of HBV mutants and how these mutants may modify existing diagnostic and treatment options in the perspective of Pakistan.

Chapter 1

INTRODUCTION

Hepatitis B Virus (HBV)has been etiologically linked to several liver related ailments of both chronic and acute nature, including fatal fulminant hepatitis, cirrhosis and hepatocellular carcinoma, which is a highly prevalent human cancer and is responsible for significant morbidity and mortality internationally(Lee, 1997). HBVis a small enveloped DNA virus and is prototypic member of the *Hepadnaviridae* that replicates via reverse transcription.

Estimated 400 million people get chronically infected with HBV every year (Tong *et al.,* 2010). HBV infection has been reported to cause approximately 1.5 million fatalities annually, most of which are in Asian and African region(Fan *et al.,* 2011). Chronic HBV infection is a key public health issue in Pakistan and has known association with a wide range of clinical states ranging from an asymptomatic carrier state with no observable alteration in liver function to critical liver damage conditions including liver cirrhosis and hepatocellular carcinoma (Ali *et al.,* 2011).

Although the estimated increase in liver cancer is mainly attributed to hepatitis C, hepatitis B is a also marked as potential major contributor to burden of liver cancer in near future (Liang and Ghany, 2002). HBV is classified into genotypes A-F, each having different clinical and etiological importance (Mizokami *et al.,* 1999) and recent data backed up by studies in animals demonstrates that genotype of virus may influence the spectrum of liver disease,

including nature and incidence of hepatocellular carcinoma, (Li *et al.,* 1993; Ahmed *et al.,* 2009; Khawaja and Khawaja, 2009). HBV genotypes display a distinct geographical distribution which in turn correlates with extent of liver disease (Kidd-Ljunggren *et al*., 2002). No explanation is currently available for these differences.

HBV reverse transcriptase (RT) bears resemblance with human immunodeficiency virus (HIV) RT lacking 3'–5'proofreading capacity and consequently being an error-prone enzyme (Gunther *et al*., 1999; Cane *et al*., 1999) this corresponds to a large number of nucleotide substitutions during replication process. Locating specific mutations with higher prevalence in viral genome is of significant importance, the reason being that such information provides valuable insight regarding their influence on pathological and clinical aspects of the virus (Kim *et al*., 2007).

Recent studies show that nucleic acid substitutions in post-HCC isolates impart them with both structural and functional changes as well as influence their interaction with host cells (e.g., enhanced replication efficiency, escape from host immune responses, highly cytopathic or increased transactivating effect, etc). This could reveal important clues regarding variables implicated in progression of HBV chronic infection to malignancy (Lin *et al*., 2001).

Each of the six sub genotypes and genotypes of hepatitis B virus (HBV) is associated with a specific combination of geographic distribution, ethnicity, and anthropological history. Following Phylogenetic analysis of HBV whole genome six genotypes were designated ranging from A-G with a minimum of 8% intergenotypic diversity among them (Stuyver *et al*., 2000; Norder *et al*., 1994

; Okamoto *et al*., 1988). A unique pattern is shown by each of the seven genotypes in terms of geographical distribution. Higher prevalence has been reported for genotype A in North America, Africa and North-western Europe (Norder *et al*., 1993). Genotypes B and C are typically more frequent in Asia (Okamoto *et al*., 1988), while genotype D has a worldwide distribution although it is majorly in Mediterranean region. Genotype E is present in Africans and genotype F has been reported to dominate in native South American populations. (Norder *et al*., 1993; Arauz-Ruiz *et al*., 1997). Currently, the newly isolated genotype G seems to be restricted to HBV carriers in USA, Georgia and France (Stuyver *et al*., 2000).

This study would help in better understanding of HBV genotypes prevailing in Pakistan, mutations persisting in them and the evolutionary lineage of Pakistani prevalent HBV. Such knowledge would be of high value for research experts and health care professionals.

The aims of this study are:

- To amplify full length HBV genome from Pakistani HBV infected patients.
- To clone full length HBV genome in Pakistani population
- Analysis of complete genome sequence of HBV isolated in Pakistan and comparing it with published sequences by molecular evolutionary analysis.

Chapter 2

LITERATURE REVIEW

Liver inflammation or hepatitis can be caused by different infectious or non infectious agents, but there are at least five types of viruses which primarily causes hepatitis as their clinical symptom. These viruses are, thus, named hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D (or delta) virus (HDV) and hepatitis E virus (HEV).

The human hepatitis B virus (HBV) is a small enveloped DNA virus implicated in both chronic and acute hepatitis. Despite the fact that an efficient vaccine is already available, an estimated 360 million individuals are chronically infected all over the world and mortality rate has reached more than 1 million per year due to HBV-associated liver pathologies (Block *et al.,* 2007). Although HBV replication is not considered directly cytopathic, HBV infection causes a wide range of liver disease from acute to chronic viral hepatitis, which may lead to liver cirrhosis and afterwards to hepatocellular carcinoma (HCC) development. Even though large amount of epidemiological data implicates chronic HBV infection as the main risk factor for liver cancer development,(Chemin and Zoulim, 2009; Lok, 2004; Pollicino *et al.,* 2011) the molecular mechanisms underlying HBV persistence and pathogenesis are still unclear. The genomic organization of HBV and its replication strategies are unique and allow it to remain in an infected hepatocyte. A remarkabletrait of HBV infection is the formation in hepatocyte nuclei of a stable HBV-DNA minichromosome, the so-called covalently closed circular DNA (cccDNA), serving as template to generate all RNAs necessary for

protein production and viral replication. Although the reversion of viral activity and the failure of viral clearance after withholding of antiviral therapy is mostly due to the perseverance of cccDNA in chronically infected individuals, the virological and immunological mechanisms that prevent virus abolition leading to the development of chronic infection are still poorly understood. The development of novel experimental infection models and quantitative serological and intrahepatic biomarkers would provide new insight into the strategies adopted by HBV for both persistence and pathogenesis.

2.1 Epidemiology

An estimated 400 million individuals have been reported infected with HBV throughout the world (Tong *et al.,* 2010).The numbers of fatalities reported are approximately 1.5 million individuals per year as a consequence of infection, make it highest no form Asia and Arica (Fan *et al.,*2011). Chronic infection by HBV is a key public health issue in Pakistan and is linked to different clinical illnesses, from less dangerous state with a normal liver activity to more deadly liver diseases (Ali *et al.,* 2011).

2.2 HBV Structure

HBV being the member of*Hepadnaviridae*family are the smallest DNA-containing, enveloped animal viruses known so far. Characteristic of HBV is its high specificity of tissue and species, and a unique genomic organization and replication mechanism. Even thougha lot of research has been done, vital steps of the viral life cycle, such as viral entry and organization of the cccDNA minichromosome, are still poorly understood (Glebe and Urban, 2007). Only recently, new infection models and molecular techniques have opened new

promises to investigate specific steps of the lifecycle and virus-host interactions influencing viral activity in infected hepatocytes (Levrero*et al.,* 2009).

HBV demonstrate three major structural phenotypes, the dane particles which are double-shelled and have a size ranging from 42- to 47-nm (Dane *et al.,* 1970), second phenotype is sphere form with size of approximate 22 nm, while third is filamental form with diameter of 22nm and variable length (Dane *et al.,* 1970; Robinson and Lutwick, 1976). Furthermore, HBV virions with tadpole shape have been reported as well, characterized by a head sized 42 nm and a tail 120nm in length and 22 nm in diameter (Dane *et al.,* 1970).

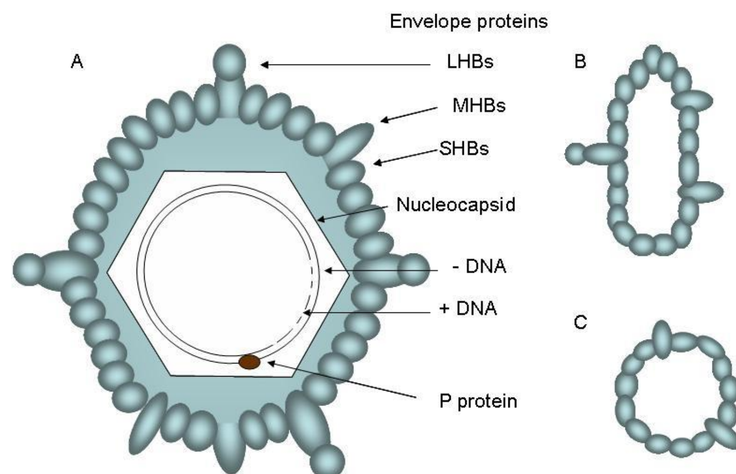

Figure 2.1: A. Diagrammatic representation of a Dane particle or complete HBV virion. The envelope is made up of LHBs, MHBs and SHBs. **B**. Filamentous sub-viral particle, which is composed of SHBs and MHBs. **C**. Spherical sub-viral particle, is composed mainly of SHBs and MHBs. (Gerber and Thung, 1985; Ganem and Varmus, 1987; Seeger and Mason, 2000).

Infectious Dane particles are 42nm in size; their double-shelled consists of an outer envelope and an inner nucleocapsid of icosahedral morphology (figure

1.2A). The envelope consists of three proteins which make up the surface, namely cocarboxyterminal large (LHBs), middle (MHBs) and small (SHBs) surface proteins (figure 2.1A). Nucleocapsid consists of a core protein (HBcAg) which covers viral DNA (Seeger *et al.,* 1991; Gerber and Thung, 1985; Seeger and Mason, 2000; Ganem and Varmus, 1987).At least two proteins are associated withviral DNA: a protein kinase and an endogenous DNA polymerase (PK) (Gerber and Thung, 1985; Robinson and Greenman, 1974;Ganem and Varmus, 1987). Diameter of spherical particles is typically 22 nm while filamentous particles have variable length but identical diameter. The two aforementioned particles are noninfectious in nature and relatively deficient in LHBs as well (figure 2.1 B & C)

2.3 Genome Organization

HBV genome consists of partially double stranded DNA virus with a size of approximately 3.2 kb. A protein is covalently attached to 5' end of minus strand which has unit length, whereas plus strand has variable length but is less than unit length, and its 5' end has an RNA oligonucleotide attached to it. Therefore cohesive ends only maintain circularity of DNA strand rather than imparting a closed configuration (Gao and Hu, 2007). HBV genome consists of four overlapping open reading frames (ORFs); coding seven different proteins by multiple in-frame start codons (Figure 2.1). Parts are present on HBV genome which regulate the determination of site for polyadenylation as well as control encapsidation of specific transcript into nucleocapsid. Genomic arrangement of hepatitis B virus family differentiates it from other viruses with non-conserved DNA replication pattern (Knaus and Nassal, 1993).

There are four ORFs which are displayed by HBV genome (Block *et al.,* 2007): as shown in the figure 2.2, the preS/S encoding the three viral surface proteins; the precore (PreC)/core encoding the core protein and the non structural PreC protein, also known as secreted e-antigen (HBeAg); the pol ORF encoding the viral polymerase, which possesses DNA polymerase, RNase H and reverse transcriptase activities, and also acts as the terminal protein for priming; and the X ORF, encoding the small regulatory X protein, which is crucial in vivo for viral replication (Zoulim *et al.,* 1994; Lucifora*et al.,* 2011). All four ORFs use a single mutualpolyadenylation signal motif; hence, the HBV-RNA transcripts are polyadenylated and capped (Nassal, 2008).

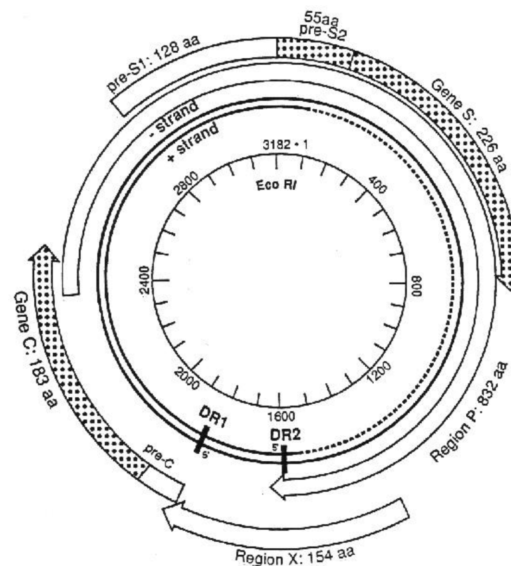

Figure 2.2: Genome of hepatitis B virus (HBV) as present in virion, the 3.2-kb genome of HBV exists in a partially double-stranded state. Negative sense has the Long/negative sense strand has constant length and contains a nick at unique site. The short/positive sense has a variable 3' end but a unique 5' end that is which differs among individual

virions. All viral proteins are coded by negative strand, which is in from of four overlapping open reading frames (ORFs). These four ORF encode surface proteins, precore/Core protein, polymerase having reverse transcriptase activity and most important X protein which is involved in development of hepatocellular carcinoma (Feitelson and Larkin, 2001).

2.4 Genotypes

Each of the six sub genotypes and genotypes of hepatitis B virus (HBV) is associated with a specific combination of geographic distribution, ethnicity, and anthropological history. Following Phylogenetic analysis of HBV whole genome six genotypes were designated ranging from A-G with a minimum of 8% intergenotypic diversity among them(Stuyver *et al.,* 2000; Norder *et al.,* 1994;Okamoto *et al.,* 1988). A unique pattern is shown by each of the seven genotypes in terms of geographical distribution. Higher prevalence has been reported for genotype A in North America, Africa and North-western Europe (Norder *et al.,* 1993). Genotypes B and C are typically more frequent in Asia (Okamoto *et al.,* 1988), while genotype D has a global distribution although it is mainly in Mediterranean region. Genotype E is present in Africans and genotype F has been reported to dominate in native South American populations.(Norder *et al.,* 1993; Arauz-Ruiz *et al.,* 1997). Currently, the newly isolatedgenotype G seems to be restricted to HBV carriers in USA, Georgia and France (Stuyver *et al.,* 2000).

Studies from Pakistani origin report variable HBV genotypes and their occurrence. A study by Idress, et al, reported a prevalence of genotype C, while Alam, *et al*, found genotype D in 85%, Baig, *et al*, reported genotype D in 64% of patients (Baig*et al.,* 2008; Idrees*et al.,* 2004;Alam*et al.,* 2007) and Abbas, et al,

reported genotype D as the prime genotype; this study however, mostly included hemodialysis patients with HBV infection (Abbas *et al.,* 2006).

2.5 Relation of Mutations to Genotypes

Like HBV genotypes, HBV mutant species also show particular distribution pattern. For example the PreC mutants are found more recurrent in areas such as the Mediterranean basin and Asia, where genotypes B, C and D are predominant and are uncommon in North America and Europe, where genotype A predominates (Hunt *et al.,* 2000; Lindh *et al.,* 1997). The differences between HBV genotypes in the selection of BCP and/or PC mutations and disease progression have been verified in studies from Europe (Sanchez-Tapias*et al.,* 2002). Studies from Asian origin supply strong evidence that HBV genotype B is related with insidious progressive liver disease compared with genotype C, but the basis for such dissimilarity in pathogenicity could not be proved. Additional studies are required to outline the relation of different HBV genotypes and course and outcomes of HBV infection (Sakugawa*et al.,* 2002).

2.6 Transmission of HBV

Hepatitis B virus is transmitted parentally via apparent or unapparent, percutaneous or per mucosal contact with septic blood or other human body liquids. Major aspects for infection comprise unscreened blood transfer, sexual contact, re-using or sharing of syringes amidst injection drug users, tattooing, working or stayingin a health-care setting, visiting/residing in a correctional facility, long-term household or intimate non-sexual contact with an HBsAg-positive individual and renal dialysis (Lavanchy, 2004; Wasley*et al.,* 2006). In areas where the prevalence is low, hepatitis B is acquired through unsafe

activities—such as insecure sexual contact or syringe sharing with HBsAg-positive individuals—and by contact with contaminated apparatus used for therapeutic procedures. In high-frequency regions, majority of infections occur during early childhood. About 90% of HBeAg-seropositive mothers with high viral load transmit hepatitis B virus to their off spring, compared with 10–20% of HBeAg-seronegative carrier mothers (Okada *et al.,* 1976). The prevalence of HBeAg is greater in Asian than in African HBsAg carrier mothers (40 vs. 15%), so perinatal transmission is greater in Asians, but mainly horizontal in Africans (Botha *et al.,* 1984; Lin *et al.,* 2003).

2.7 Factors Affecting the Course of HBV Infections

The progression of HBV infections can be related to a variety of factors, mainly categorized as viral factors, host-related factors and external factors. A number of host-associated causes are interrelated with an augmented hazard of progressing chronic HBV infectivity (Hyams, 1995; Polish *et al.,* 1992), such as host defense response, time of disease and host genetic factors (Lindh *et al.,* 1999). External factors include alcohol, smoking, and dietary carcinogens. Study demonstrates that HBV chronic hepatitis patients with heavy alcohol history have as much as 6 folds higher risk of progression to cirrhosis (Ikeda *et al.,* 1998). Similarly other study have shown that the progression of HBV infection to cancer can be highly effected due to smoking and consumption of dietary carcinogen, like aflatoxins, which contaminate food stored in moist and humid environment (Yang *et al.,* 2002; Ming *et al.,* 2002). Thirdly, viral factors play a huge role in progression of HBV infections. HBV sequence mutations have also been gradually acknowledged as a feature that changes the progress and result of HBVliver

infection (Wang *et al.,* 2005). Variations in the genes and genome of HBV can manipulate the host response, as well as it can create stronger mutants with greater surviving abilities. Mutations in HBV regulatory regions and promoter sequences can cause over production of viral proteins. The role of Major HistocompatibilityComplex II (MHC II) polymorphism in the aftermath of HBV pathogenicity has also been inspected in different studies (Chakravarty, 2005; Thursz, 1997), yet the accurate antigen presentation with the support of MHC II to the helper T cells is imperative for HBV removal (Thursz, 1997).

2.8 Mutant HBV Viral Gene Products

HBV uses reverse transcriptase (RT) like human immunodeficiency virus (HIV) RT, which is an error-prone enzyme lacking 3'–5' proofreading capacity (Cane *et al.,* 1999; Gunther *et al.,* 1999), and hencecauses a huge amount of nucleotide replacements during virus life cycle. Finding of commonness of various specific mutations in the viral genome proves to be of a lot ofsignificance, because they present information regarding their effect on the virological and clinical aspects of the virus (Kim*et al.,* 2007).

Although HBV have a high replication efficiency(with a daily production of 10^{11} circulating virusparticles as compared to 10^{9} particles per day of humanimmunodeficiency virus; HIV), the rate of mutant generation of HBV is interestingly lower than $2x10^{4}$ base substitutions/site/year(Girones and Miller, 1989). This proves that HBV is one to two orders of magnitude lower thanother viruses that lack polymerase-associated proof-readingfunctions. The decrease in production rate and number of viable mutants is also due to theorganization of theHBV genome having multiple overlapping open readingframes. However, the

driving force behind the choice and take-over of amutant strain appear to be due tocellular and humoral host immune response and anti viral therapy. In vitro studies suggested that defectivemutants could play a significant role by interfering at replicative and transcriptional levels of HBV(Gunther *et al.,* 1997).The occurrence of mutations along HBV genomeis random,and the selection of one over the others authorize a biologicalgain to the prevalent mutation during the replicationcycle of the virus or the mutant gets a selective advantage over wild-type virus in host-virus interactions (Oldstone, 1991). Yet these interactions are not considered in most of the molecular epidemiology ofHBV mutational studies. The importance of the presence or absence of a givenmutation is analysed by simple means ofassociative statistics with the clinicopathological or diseasepatterns of the infected hosts.

The replication of HBV population causes many changes in its genome. Thus considering the replication rate ofany HBV population, within a time frame of weeks and months in a single patient, its evolution can be compared to the evolution of human populations during centuries or millennia. Thus it would be much more important to study the dynamic changesof diverse viral mutations within the overall viral populationin relation with the series of events taking place inthe same host or changes of the host-virus interactions. Therefore there is a need to modify the methodologies so that these biological or pathogenetic traits of viral mutations can be better understood.

Studies have shown that structural and functional changes of the genome in post-HCC isolates and their interactions with host cells (e.g., increased replication efficiency, evading immune responses of host, exceedingly cytopathic or increased

transactivating effect, etc.), however, could expose significant clues related to progression from HBV chronic infection to malignancy (Lin *et al.,* 2001).

Table 2.1: HBV variants and their potential impact for pathogenesis of HBV infection (Baumert and Blum, 2000; Baumert *et al.,* 2005; Zoulim, 2004; Pawlotsky, 2005).

HBV region	Mutation	Molecular phenotype	Clinical relevance
Pre-S/S	Pre-S1/ pre-S2/ S-promotor	Misassembly	Fibrosing cholestatic hepatitis
	S	Alteration of B- and T-cell epitopes	Vaccine escape
	S splicing		Immune escape Diagnostic escape
Pre-C	Pre-C-stop	Loss of HBeAg	Severe hepatitis HBeAg-deficiency
Core	Core	Alteration of T-cell epitopes	Viral persistence Severe hepatitis
RT/Pol	Pol	Replication deficiency	Viral latency Viral persistence
	Pol	Resistance to antivirals	Therapy escape
Regulatory Elements	Core promotor	Enhanced replication and core expression	Severe hepatitis Modulation of drug resistance
		Decreased HBeAg synthesis	HBeAg seronegativity
	Enhancer I	Decreased replication	Chronic hepatitis

Data collected shows that(Table 2.1) HBV mutants can affect clinical outcomes by modifying the natural course of infection and giving rise to antiviral resistance (Baumert and Blum, 2000; Baumert *et al.,* 2005; Zoulim, 2004; Pawlotsky, 2005). In the perspective of various genotypes, natural occurring mutations have been explored in the structural and non-structural genes as well as regulatory features of the virus. Mutations produced in BCP, are interlinked with anti-HBe, HBsAg and

positive serological shape, most importantly includes G1764A and A1762T.BCP and PreC mutations are observed at one place and are correlated to HBeAg-negative ailments after liver transplantation, introduction of HCC and FHF (Hunt *et al.,* 2000; Takahashi *et al.,* 1995). The PreC stop codon mutations result inloss of hepatitis B e antigen (Liang *et al.,* 1991), explained groups of mutations in the core promoter result in improved viral infection (Baumert*et al.,* 2005), and alterations in the reverse transcriptase/polymerase genes give resistance to antivirals (Zoulim, 2004;Schildgen*et al.,* 2006). In addition, several modifications in the surface region of HBV genome have been documented which may change the immune modulation of the viral surface proteins (HBsAg) and morphology of viral envelope proteins (Baumert *et al.,* 2005;Kannand Gerlich, 2005).

By complete HBV genomic analysis, it is possible to discover whether there are major nucleic acid sequence variations between Pakistani HBV infected isolates from chronically infected patients (without treatment), chronically infected patients (with treatment) and from HBV induced HCC patients, and by further cell lines studies, functional differences between proteins and the host-virus interactions for such HBV isolates might also be exposed.

Chapter *3*

MATERIAL AND METHODS

3.1 SAMPLE COLLECTION

3.1.1 Study Population

HBV positive patients Sample were collected from different hospitals including polyclinic hospital, Military hospital, and Holy family hospital. For extraction of serum, samples were centrifuged at 8000 rpm for 3 min. The sera was then stored at -80°C to prevent degradation of nucleicacids and protein. The study was authorized by the Institutional review board of National University of Sciences and Technology.

3.2. VIRAL DNA EXTRACTION AND AMPLIFICATION

3.2.1 Viral DNA extraction

The Nuleospin® blood kit (Macherey-Nagel Germany) was used for nucleicacid purification (Whole blood DNA extraction) according to the manufacturer’s instructions. A 200µl aliquot of serum was used for theextraction. The DNA was eluted from the Nuleospin column and the extracted DNA was stored at -20°C.

3.2.2 Primer Designing for Complete Genome of Hepatitis B Virus

Universal primers reported by(Zhang*et al*., 2007) were used for the amplification of Hepatitis B virus genome amplification. Sequences of the primers are given in the table3.1.

Table 3.1: Primers used for PCR amplification of HBV genome

Name	**Sequence**	**Length**	**Annealing Temp**	**Reference**
WAL	ACTGTTCAAGCCTCCAAGCTGTGC	24	66	Zhang*et al.,* 2007
WAF	AGCAAAAAGTTGCATGGTGCTGGT	24	62	Zhang*et al.,* 2007

3.2.3 Full Length Genome Amplification by Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was carried out to amplify HBV genome using the Sample Serum DNA isolated in section 3.2.1. The reaction mixture contained 200ng (5µL) Sample DNA template along with 10µM genome specific forward and reverse primer (1µL each), 2mM dNTPS (3µL)**,** 2mM $MgCl_2$ (2µL), 0.5 units of Dream Taq polymerase (Fermentas, USA) (0.5µL) along with 1X Dream Taq Polymerase Buffer (2µL) and nuclease free water (6.5µL) making a total reaction volume of 20µl. PCR mixture was then placed in Thermal Cycler

(Swift™ MaxPro Thermal Cycler, Esco, Singapore), under the following PCR conditions as shown in figure 3.1;

1. Initial denaturation (95 °C) 5 min
2. Denaturation (95 °C) 45 min
3. Annealing (67 °C) 50 min
4. Extension (72 °C) 23 min
5. Final extension (72 °C) 15 min
6. Held at 4 °C

Steps 2, 3 and 4 were repeated for 35 times between initial denaturation and final extension.

PCR Program for HBV Genome

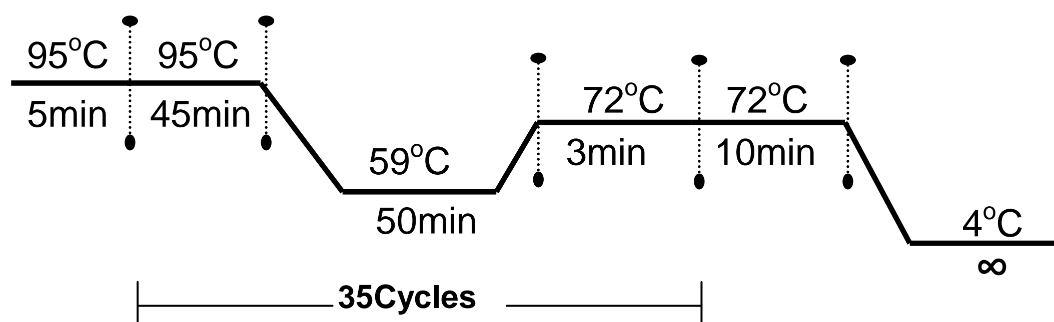

Figure 3.1: Polymerase chain reaction (PCR) program for amplification of Hepatitis B virus Complete Genome (HBV).

The PCR product was visualized afterwards on 0.8% agarose gel, added with ethidium bromide on Wealtec Dolphin Doc (S/N470883) gel documentation system. Patient's history data for positive samples are shown in Table 3.2.

Table 3.2: Patient history data of samples used for the amplification and cloning of HBV genome.

Patient Data for HBV Genome				
Patient no	**Patients name**	**Patient Age/year**	**Patient Gender**	**Genotype**
1	Pc 02	10	M	D
2	CTL 1	22	M	D
3	CTL 2	25	F	D
4	Pims 2	32	M	D
5	Khi 10	38	M	D
6	MH8	35	F	D

3.3 Gel Elusion of PCR Product

Silica bead DNA gel extraction kit, (Fermentas, USA) was used to elute the PCR product in order to continue with the cloning. Standard procedure was followed to extract the product from the gel.

3.4 CLONING

3.4.1 Cloning in to TA vector

Using Invitrogen's Dual Promoter TA cloning kit, eluted product of HBV genome was cloned into TA vector (pCR®II-TOPO®) after amplification. Refer to figure 3.2 for map of the aforementioned plasmid. A 50ng mixture of linearized pCR®II and 150ng of eluted HBV genome product were co-incubated with 4 units

of T4 DNA ligase at 14°C overnight along with appropriate buffer and nuclease free water.

3.4.2 Preparation of Competent Cells

For competent cells preparation, anisolated colony was picked and inoculated in 10 ml LB media. It was then incubated overnight at 37°C in Refrigerated Shaking incubator TSS-40-250 (Technico Scientific Supply, Pakistan). Subculturing was performed in 50 ml LB media using 2 ml of inoculum from previous culture and incubated for two hours at 37°C in Refrigerated Shaking incubator TSS-40-250 (Technico Scientific Supply, Pakistan). This culture was then centrifuged at 4000 rpm in centrifuge 5810R (Eppendorf, Germany) at 4oC. Supernatant was then discarded and pellet was resuspended in 20 ml of chilled 50mMcalcium chloride ($CaCl_2$). It was then incubated for 10 minutes on ice and then again centrifuged at 4000 rpm in centrifuge 5810R (Eppendorf, Germany) at 40^{0}C. Afterwards pellet was resuspended in 2 ml 50 mM $CaCl_2$.Competent cells were henceforth kept on ice prior to use.

3.4.3Transformation of Ligated Product

Transformation was performed via heat shock method. 150 µl of competent cells (DH5 alpha strain of *E coli*) were co-incubated with 10 ul of ligated product on ice, for duration of 40 minutes. For heat shock temperature was elevated to 42°C for 90 seconds, subsequently the product was transferred to ice and incubated for 5 minutes. After adding Luria Bertani (LB) media this product was incubated at 37 ^{o}C for 2 hours. Pelleting of cells was performed at 14000 rpm for 2 minutes using table top centrifuge (Sigma Germany). After discarding supernatant ~ 200 µl media was left behind in which the pellet was resuspended. After that it was spread

on agar plate supplemented with 100 μg/ml ampicillin, 20mg/ ml Isopropyl β-D-1-thiogalactopyranoside (IPTG), and 20 mg/ml of 5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside (Xgal) to obtain blue and white colonies for selection. The plates were then incubated overnight at 37^oC.

3.4.4 Clone Confirmation

Blue/white selection was used for identification of transformants, following colony PCR and restriction digestion, DNA sequencing were performed.

3.4.5 Replica Platting:

For replica plating single white colonies were selected and streaked on agar plate containing 100μg/ml ampicillin. These plates were then incubated overnight at 37^oC. Colonies with positive results were then picked from the plate and grown in 5 ml LB media containing 100μg/ml ampicillin. After was then incubated for 16 hours in Refrigerated Shaking incubator TSS-40-250 (Technico Scientific Supply, Pakistan) at 37 ^oC and 125 rpm.

3.4.6 Colony PCR:

Single colonies were picked from replica plates and mixed in 15 μl of nuclease free water. It was then incubated at 95^oC for 15 minutes followed by 10 minutes at 25^oC. It was then centrifuged in Sigma 1-14 Microfuge (MBI, Canada) at maximum speed and supernatant was used as template for PCR with all conditions aforementioned in amplification section. Each sample was loaded on 0.8% agarose gel to check presence of genome in the vector. The gel was visualized on Wealtec Dolphin Doc (S/N470883) gel documentation system.

3.4.7 Mini Prep of Positive Clones

Gene jet plasmid mini prep kit (Fermatas, USA) was used for the extraction of plasmid. Standard manufacturer protocol was followed for this method.

3.4.8 Restriction Digestion of Plasmid DNA

The extracted plasmid wasconfirmed by subjecting the isolated plasmid to digestion with *EcoR*1 enzyme (Fermentas, USA) as the vector contains restriction sites on both sides of the cloned gene. Isolated plasmid was subjected to digestion with *EcoR*1enzyme to confirm presence of genome. The isolated plasmid (5µL) was treated with 10 units of enzyme (1µL), 10 X Digest reaction buffer (1µL) and nuclease free water (3µL). The reaction mixture was incubated at 37°C for 3 hours and confirmation was made by resolving the digested product on .8% agarose gel, and visualized on gel documentation system (Wealtec Dolphin Doc, Sparks, USA) under UV light.

3.4.9 Sequencing

The plasmid isolated in section 3.4.7 was sequenced using M13 Universal forward and reverse primers at Macrogen (Korea).

Chapter 4

RESULTS

4.1 PCR Amplification of Complete HBV Genome

PCR amplification of Hepatitis B complete genome was carried out using DNA template extracted from patient's serum as mentioned in section 3.2.1 (Viral DNA extraction). Complete genome specific primers were used for amplicon amplification. Figure 4.1 represents the amplified amplicon HBV genome of approximately 3.2kb.

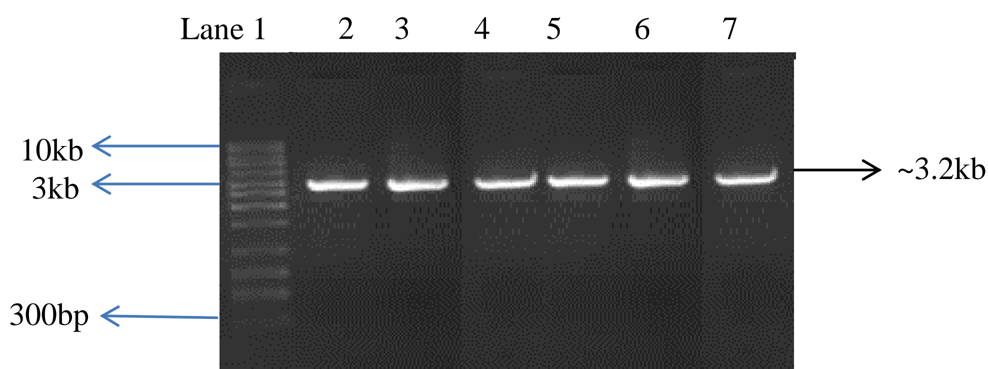

Figure 4.1: Representative gel of amplified complete genome of HBV on 0.8% agarose gel. Lane 1: 1Kb, DNA ladder (300-10,000 bp) (Axygen Scientific, Inc). Lanes, 2-7 show the amplified HBV genome from different chronic patients.

4.2 Gel Elusion of Complete HBV Genome

The amplified fragment was eluted from gel to obtain purified PCR product for further processing. As shown in Figure 4.2 Lane 1 represents the band size of approximately 3.2kb of full length genome of HBV, whereas lane 2 contains 1kb DNA ladder (Axygen Scientific, Inc).

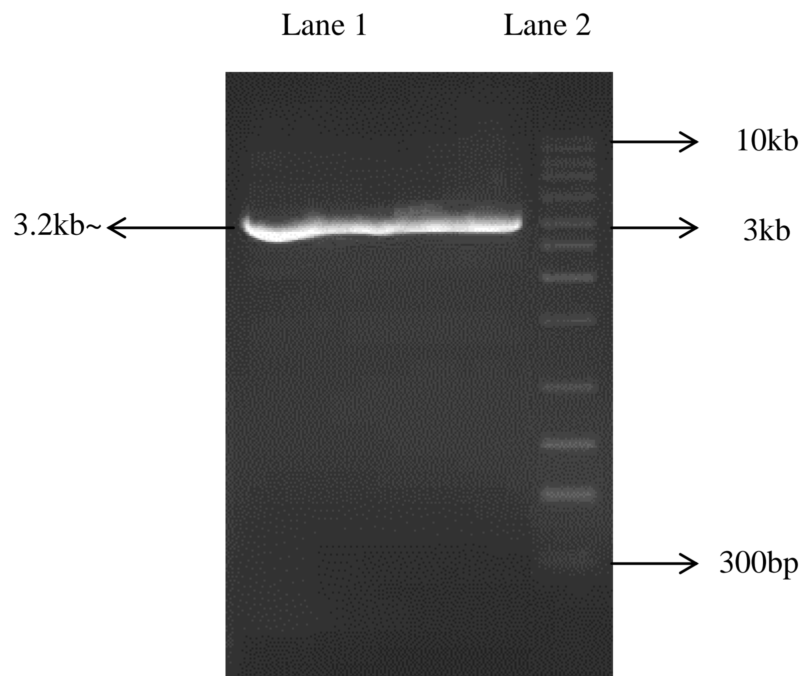

Figure 4.2: Digital image of 0.8% agarose gel of bulk PCR product (HBV genome).Lane1: Bulk PCR product of HBV genome from Pc 02 sample. Lane 2 1kb DNA ladder (Axygen Scientific, Inc).

4.3 Cloning, Transformation & Selection of Full Length HBV Positive Clones

Purified PCR product was then cloned in pCR®II and transformed into E.coli DH5α strain. Transformed colonies were selected on the basis of blue white selection. White colonies were picked and cultured In LB media and colony PCR was performed (results shown in the Figure 4.3.1) representing the band size of 3.2kb approximately.

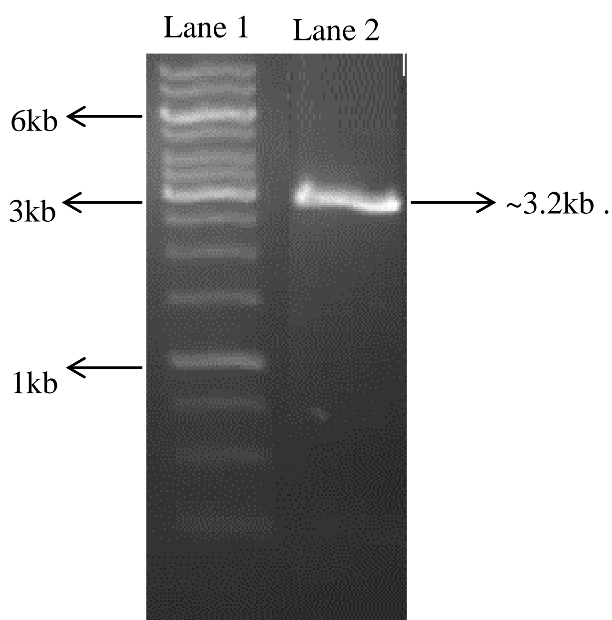

Figure 4.3.1: Digital image of colony PCR product of white colony picked from replica agar plates. Lane 2 shows colony PCR product of inserted HBV genome against DNA size marker of 1Kb DNA ladder (Fermentas. USA) shown in lane 1.

Positive colonies with insert were cultured, plasmid was isolated and digested (figure 4.3.2) for the confirmation of cloning of full length genome of HBV. After digestion of the insert with *EcoR*1 a designating band of approximately 3.2kb was observed on agarose gel.

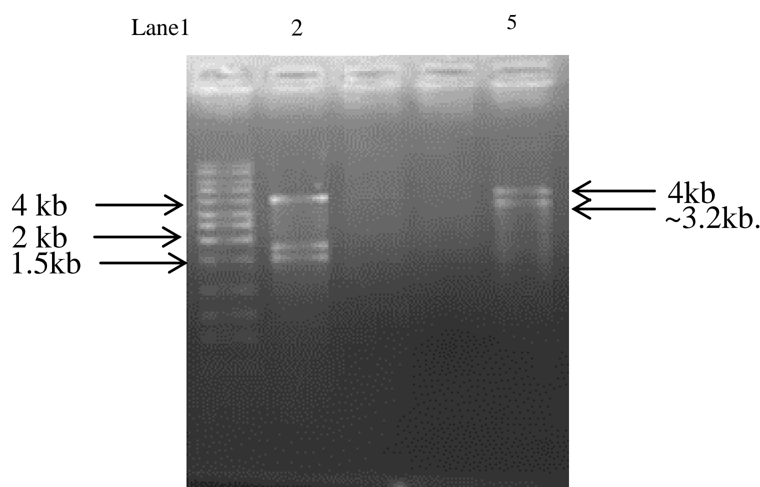

Figure 4.3.2: (A) Digital picture of 0.8% agarose gel showing TA Clones and their restriction digestion. Lane M: 1 kb ladder (Axygen Scientific, Inc). Lanes 5 show the digestion of plasmid with *EcoR*1, where as lane 2 represents double digestion with hindIII and XhoI.

4.4 Sequence Analysis

Cloning of HBV genome was further confirmed by sequencing of the plasmid product. Sequencing was carried out by Macrogen Korea. The sequence obtained after sequencing was then aligned with other complete genome sequences reported from Pakistan and neighboring countries. Sequencing results were aligned using freely available CLC workbench (http://www.clcbio.com). Table 4.1 represents the possible mutations in L2 sample.

Table 4.1 Possible Mutations in Initial Sequence Result (sample L2)

S #	Nucleotide Change	L2 seq position	Consensus Seq position
01	C to T	55	1912
02	A to C	121	1978
03	T to C	250	2107
04	T to G	264	2121
05	G to A	281	2138
06	C to T	282	2139
07	T to C	286	2143
08	N to T	309	2167
09	C to T	313	2170
10	T to C	388	2245
11	A to T	650	2557
12	G to A	731	2588
13	G to T	746	2603
14	T to C	768	2625
15	C to T	887	2744
16	G to A	993	2850
17	Deletion Of A	1093	2950
18	A to C	1108	2965
19	T to C	1151	3008
20	Deletion Of C	1163	3020
21	Deletion Of G	1190	3047
22	C to G	1199	3056
23	C to G	1226	3083
24	C to T	1235	3092
25	T to C	1236	3093
26	C to T	1237	3094
27	C to T	1238	3095

28	T to G	1239	3096
29	Deletion of C	1242	3099
30	Deletion of T	1243	3100
31	C to T	1244	3101
32	T to C	1245	3102
33	A to T	1250	3107
34	Deletion of G	1253	3110
35	A to G	1256	3114
36	A to G	1260	3118
37	Deletion of C	1266	3124
38	C to A	1273	3131
39	C to T	1276	3134
40	A to C	1277	3135
41	Deletion of G	1280	3138
42	C to G	1282	3140
43	C to T	1284	3142
44	Insertions (21)	1192-1212	3157 to 3177
45	Insertions(19)	1217-1235	3181 to 3200

4.5 Nucleotide change in Core Regions (1861-2800)

There were many nucleotide changes observed at various positions 1912, 1978, 2107, 2121, 2138, 2139, 2143, 2167, 2170, 2245, 2557, 2588, 2603, 2625, 2744,2850, in which nucleotide changed form C to T, A to C, T to G, G to A, where as no deletions were observed in this region. This is a partial sequence of core region so at present the role of these changes can not defined properly.

4.6 Nucleotide Change in Surface Regions(2800- 3160)

There were nucleotide changes observed at the start of the Surface gene at position 3095, 3096, 3099, 3100, 3101, 3102, 3107, 3110, 3114, 3118, 3124, 3131, 3138, 3140, 3135 and 3134. This region contains deletion at position 3020, 3047, 3099 and 3100. Insertion of 21 and 19 nucleotides was observed from 3157-3177 and 3181-3200. Full genome sequence of HBV would help in providing a better insight in the nucleotide changes in these regions.

```
                                                                        1,860                        1,880                         1,900
Hepatitis B virus DNA, complete genome, isolate: Pkst7912  CATGTCCTAC TGTTCAAGCC TCCAAGCTGT GCCTTGGGTG GCTTTGGGGC 1900
Hepatitis B virus DNA, complete genome, isolate: Pkst8216  CATGTCCTAC TGTTCAAGCC TCCAAGCTGT GCCTTGGGTG GCTTTGGGGC 1900
Hepatitis B virus DNA, complete genome, isolate: Pkst8134  CATGTCCTAC TGTTCAAGCC TCCAAGCTGT GCCTTGGGTG GCTTTGGGGC 1900
Hepatitis B virus strain 3798-91, complete genome          CATGTCCTAC TGTTCAAGCC TCCAAGCTGT GCCTTGGGTG GCTTTGGGGC 1900
Hepatitis B virus isolate T1503, complete genome           CATGTCCTAC TGTTCAAGCC TCCAAGCTGT GCCTTGGGTG GCTTTGGGGC 1900
Hepatitis B virus isolate T1562, complete genome           CATGTCCTAC TGTTCAAGCC TCCAAGCTGT GCCTTGGGTG GCTTTGGGGC 1900
L2 genome                                                  -------TAC TGTTCAAGCC TCCAAGCTGT GCCTTGGGTG GCTTTGGGGC 43
Consensus                                                  CATGTCCTAC TGTTCAAGCC TCCAAGCTGT GCCTTGGGTG GCTTTGGGGC
Conservation
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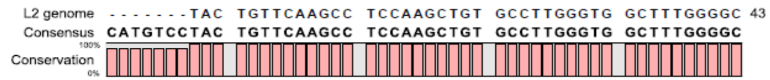

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Hepatitis B virus DNA, complete genome, isolate: Pkst8216  ATGGACATTG ATCCTTATAA AGAATTTGGA GCTACTGAGG AGTTACTCTC 1950
Hepatitis B virus DNA, complete genome, isolate: Pkst8134  ATGGACATTG ACCCTTATAA AGAATTTGGA GCTACTGTGG AGTTACTCTC 1950
Hepatitis B virus strain 3798-91, complete genome          ATGGACATTG ACCCTTATAA AGAATTTGGA GCTACCGTGG AGTTACTCTC 1950
Hepatitis B virus isolate T1503, complete genome           ATGGACATTG ACCCTTATAA AGAATTTGGA GCAACTGTGG AGTTACTCTC 1950
Hepatitis B virus isolate T1562, complete genome           ATGGACATCG ACCCTTATAA AGAATTTGGA GCTACTGTGG AGTTACTCTC 1950
L2 genome                                                  ATGGACATTG ATCCTTATAA AGAATTTGGA GCTACTGTGG AGTTACTCTC 93
Consensus                                                  ATGGACATTG ACCCTTATAA AGAATTTGGA GCTACTGTGG AGTTACTCTC
Conservation
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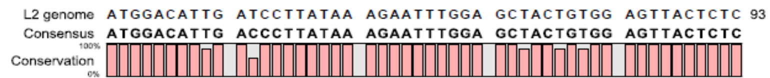

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Hepatitis B virus DNA, complete genome, isolate: Pkst8216  GTTTTTGCCT TCTGACTTCT TTCCTTCAGT ACGAGATCTT CTAGATACCG 2000
Hepatitis B virus DNA, complete genome, isolate: Pkst8134  GTTTTTGCCT TCTGACTTCT TTCCTTCAGT ACGAGATCTT CTAGATACCG 2000
Hepatitis B virus strain 3798-91, complete genome          GTTTTTGCCT TCTGACTTCT TTCCTTCAGT ACGAGATCTT CTAGATACCG 2000
Hepatitis B virus isolate T1503, complete genome           ATTTTTGCCT TCCGATTTCT TTCCGTCTGT CCGAGATCTT CTAGATACCG 2000
Hepatitis B virus isolate T1562, complete genome           GTTTTTGCCT TCTGACTTCT TTCCTTCAGT ACGAGATCTT CTAGATACCG 2000
L2 genome                                                  GTTTTTGCCT TCTGACTTCT TTCCTTCCGT ACGAGATCTT CTAGATACCG 143
Consensus                                                  GTTTTTGCCT TCTGACTTCT TTCCTTCAGT ACGAGATCTT CTAGATACCG
Conservation
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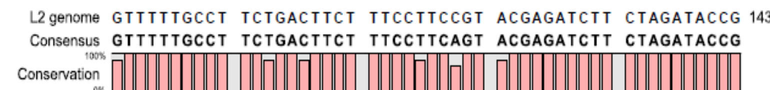

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Hepatitis B virus DNA, complete genome, isolate: Pkst8134  CCTCAGCTCT GTATCGGGAT GCCTTAGAAT CTCCTGAGCA TTGTTCACCT 2050
Hepatitis B virus strain 3798-91, complete genome          CCTCAGCTCT GTATCGGGAT GCCTTAGAGT CTCCTGAACA TTGTTCACCT 2050
Hepatitis B virus isolate T1503, complete genome           CCGCAGCGCT ATATCGGGAT GCGTTAGAGT CTCCTGAGCA TTGTTCACCT 2050
Hepatitis B virus isolate T1562, complete genome           CCTCAGCTCT GTATCGGGAA GCCTTAGAGT CTCCTGAGCA TTGTTCACCT 2050
L2 genome                                                  CCTCAGCTCT ATATCGGGAA GCCTTAGAGT CTCCTGAGCA TTGTTCACCT 193
Consensus                                                  CCTCAGCTCT ATATCGGGAA GCCTTAGAGT CTCCTGAGCA TTGTTCACCT
Conservation
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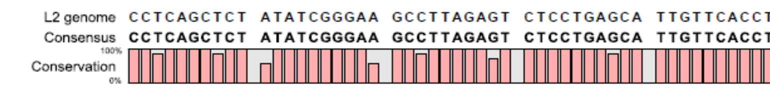

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Hepatitis B virus DNA, complete genome, isolate: Pkst8216  CACCATACTG CACTCAGGCA AGCAATTCTT TGCTGGGGGG AACTAATGAC 2100
Hepatitis B virus DNA, complete genome, isolate: Pkst8134  CACCATACTG CACTCAGGCA AGCAATTCTT TGCTGGGGGG AACTAATGAC 2100
Hepatitis B virus strain 3798-91, complete genome          CACCATACTG CACTCAGGCA AGCAATTCTT TGCTGGGGGG AACTAATGAC 2100
Hepatitis B virus isolate T1503, complete genome           CACCATAATG CACTCAGGCA AGCAATTCTT TGCTGGGGAG AATTAATGAC 2100
Hepatitis B virus isolate T1562, complete genome           CACCATACTG CACTCAGGCA AGCAATTCTT TGCTGGGGGG AACTAATGAC 2100
L2 genome                                                  CACCATACTG CACTCAGGCA AGCAATTCTT TGCTGGGGGG AACTAATGAC 243
Consensus                                                  CACCATACTG CACTCAGGCA AGCAATTCTT TGCTGGGGGG AACTAATGAC
Conservation
```

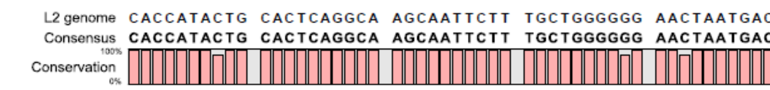

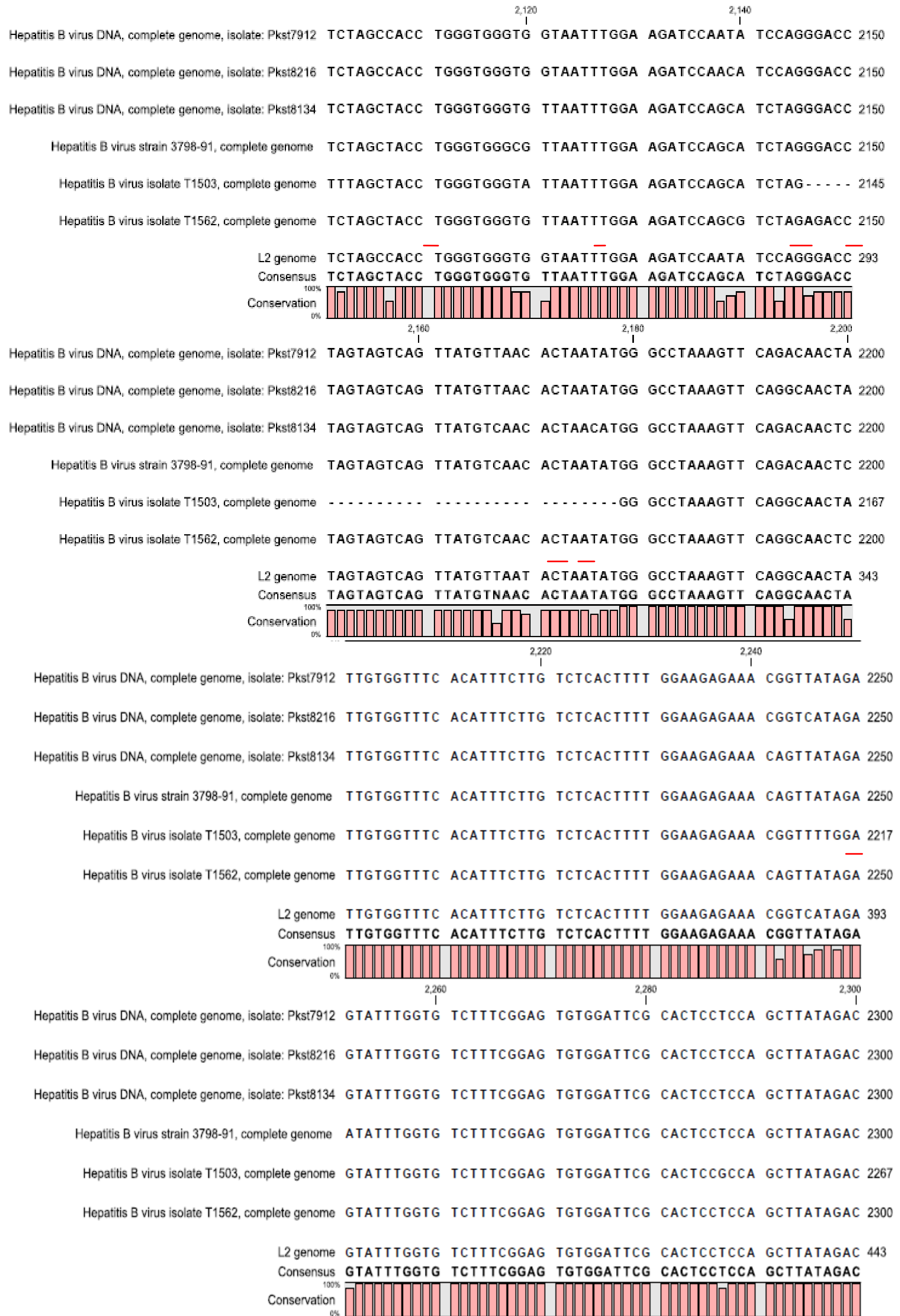
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Hepatitis B virus DNA, complete genome, isolate: Pkst8134 TCTAGCTACC TGGGTGGGTG TTAATTTGGA AGATCCAGCA TCTAGGGACC 2150
Hepatitis B virus strain 3798-91, complete genome TCTAGCTACC TGGGTGGGCG TTAATTTGGA AGATCCAGCA TCTAGGGACC 2150
Hepatitis B virus isolate T1503, complete genome TTTAGCTACC TGGGTGGGTA TTAATTTGGA AGATCCAGCA TCTAG----- 2145
Hepatitis B virus isolate T1562, complete genome TCTAGCTACC TGGGTGGGTG TTAATTTGGA AGATCCAGCG TCTAGAGACC 2150
L2 genome TCTAGCCACC TGGGTGGGTG GTAATTTGGA AGATCCAATA TCCAGGGACC 293
Consensus TCTAGCTACC TGGGTGGGTG TTAATTTGGA AGATCCAGCA TCTAGGGACC
100%
Conservation
0%
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2,180
2,200
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Hepatitis B virus DNA, complete genome, isolate: Pkst8216 TAGTAGTCAG TTATGTTAAC ACTAATATGG GCCTAAAGTT CAGGCAACTA 2200
Hepatitis B virus DNA, complete genome, isolate: Pkst8134 TAGTAGTCAG TTATGTCAAC ACTAACATGG GCCTAAAGTT CAGACAACTC 2200
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L2 genome TAGTAGTCAG TTATGTTAAT ACTAATATGG GCCTAAAGTT CAGGCAACTA 343
Consensus TAGTAGTCAG TTATGTNAAC ACTAATATGG GCCTAAAGTT CAGGCAACTA
100%
Conservation
0%
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2,240
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Hepatitis B virus DNA, complete genome, isolate: Pkst8134 TTGTGGTTTC ACATTTCTTG TCTCACTTTT GGAAGAGAAA CAGTTATAGA 2250
Hepatitis B virus strain 3798-91, complete genome TTGTGGTTTC ACATTTCTTG TCTCACTTTT GGAAGAGAAA CAGTTATAGA 2250
Hepatitis B virus isolate T1503, complete genome TTGTGGTTTC ACATTTCTTG TCTCACTTTT GGAAGAGAAA CGGTTTTGGA 2217
Hepatitis B virus isolate T1562, complete genome TTGTGGTTTC ACATTTCTTG TCTCACTTTT GGAAGAGAAA CAGTTATAGA 2250
L2 genome TTGTGGTTTC ACATTTCTTG TCTCACTTTT GGAAGAGAAA CGGTCATAGA 393
Consensus TTGTGGTTTC ACATTTCTTG TCTCACTTTT GGAAGAGAAA CGGTTATAGA
100%
Conservation
0%
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2,280
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Hepatitis B virus DNA, complete genome, isolate: Pkst8134 GTATTTGGTG TCTTTCGGAG TGTGGATTCG CACTCCTCCA GCTTATAGAC 2300
Hepatitis B virus strain 3798-91, complete genome ATATTTGGTG TCTTTCGGAG TGTGGATTCG CACTCCTCCA GCTTATAGAC 2300
Hepatitis B virus isolate T1503, complete genome GTATTTGGTG TCTTTCGGAG TGTGGATTCG CACTCCGCCA GCTTATAGAC 2267
Hepatitis B virus isolate T1562, complete genome GTATTTGGTG TCTTTCGGAG TGTGGATTCG CACTCCTCCA GCTTATAGAC 2300
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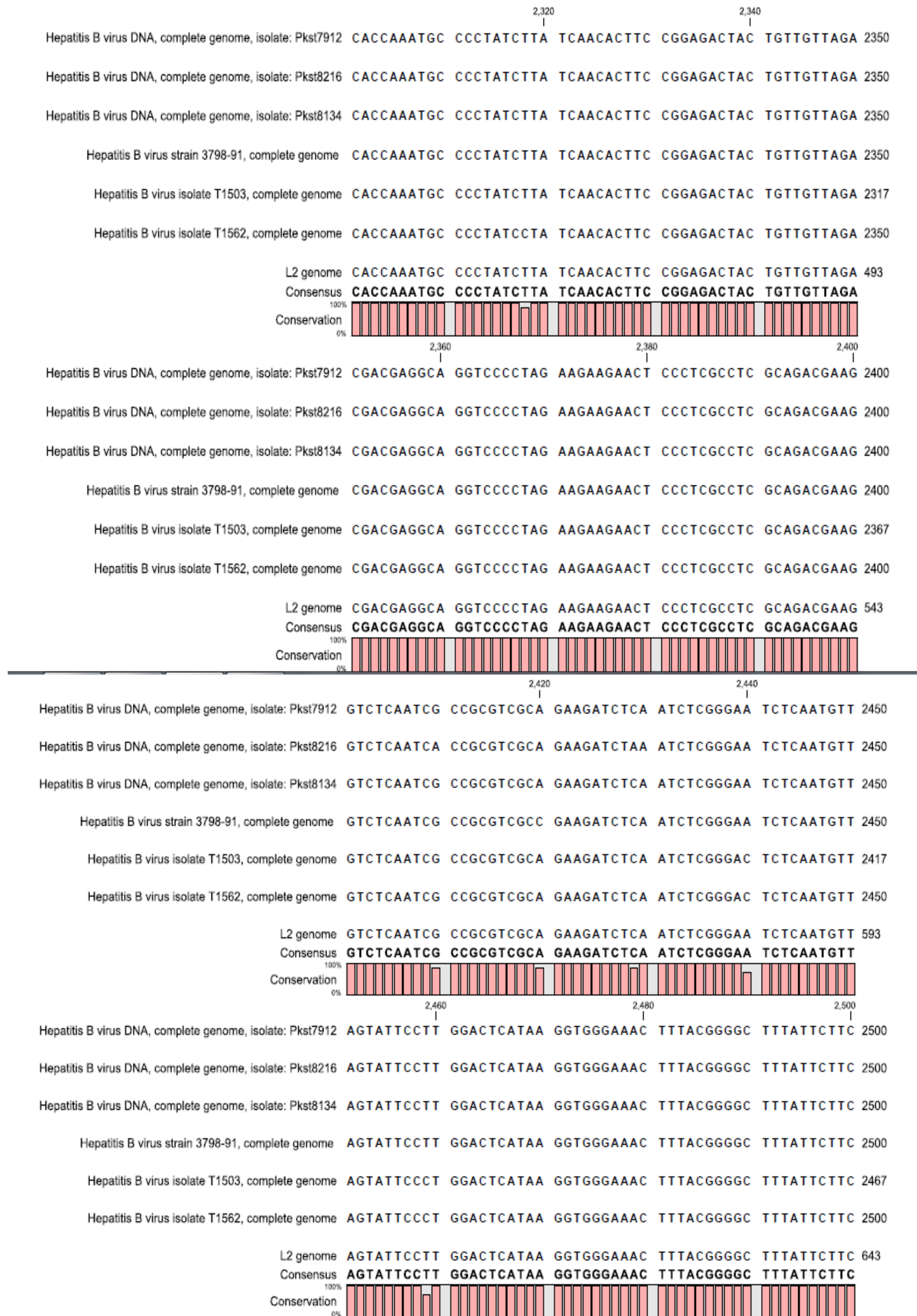
100%
Conservation
0%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Hepatitis B virus DNA, complete genome, isolate: Pkst8134 CACCAAATGC CCCTATCTTA TCAACACTTC CGGAGACTAC TGTTGTTAGA 2350
Hepatitis B virus strain 3798-91, complete genome CACCAAATGC CCCTATCTTA TCAACACTTC CGGAGACTAC TGTTGTTAGA 2350
Hepatitis B virus isolate T1503, complete genome CACCAAATGC CCCTATCTTA TCAACACTTC CGGAGACTAC TGTTGTTAGA 2317
Hepatitis B virus isolate T1562, complete genome CACCAAATGC CCCTATCCTA TCAACACTTC CGGAGACTAC TGTTGTTAGA 2350
L2 genome CACCAAATGC CCCTATCTTA TCAACACTTC CGGAGACTAC TGTTGTTAGA 493
Consensus CACCAAATGC CCCTATCTTA TCAACACTTC CGGAGACTAC TGTTGTTAGA
100%
Conservation
0%
2,360
2,380
2,400
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Hepatitis B virus DNA, complete genome, isolate: Pkst8216 CGACGAGGCA GGTCCCCTAG AAGAAGAACT CCCTCGCCTC GCAGACGAAG 2400
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Hepatitis B virus strain 3798-91, complete genome CGACGAGGCA GGTCCCCTAG AAGAAGAACT CCCTCGCCTC GCAGACGAAG 2400
Hepatitis B virus isolate T1503, complete genome CGACGAGGCA GGTCCCCTAG AAGAAGAACT CCCTCGCCTC GCAGACGAAG 2367
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100%
Conservation
0%
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2,440
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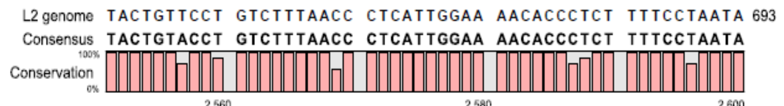
Hepatitis B virus DNA, complete genome, isolate: Pkst8216 GTCTCAATCA CCGCGTCGCA GAAGATCTAA ATCTCGGGAA TCTCAATGTT 2450
Hepatitis B virus DNA, complete genome, isolate: Pkst8134 GTCTCAATCG CCGCGTCGCA GAAGATCTCA ATCTCGGGAA TCTCAATGTT 2450
Hepatitis B virus strain 3798-91, complete genome GTCTCAATCG CCGCGTCGCC GAAGATCTCA ATCTCGGGAA TCTCAATGTT 2450
Hepatitis B virus isolate T1503, complete genome GTCTCAATCG CCGCGTCGCA GAAGATCTCA ATCTCGGGAC TCTCAATGTT 2417
Hepatitis B virus isolate T1562, complete genome GTCTCAATCG CCGCGTCGCA GAAGATCTCA ATCTCGGGAC TCTCAATGTT 2450
L2 genome GTCTCAATCG CCGCGTCGCA GAAGATCTCA ATCTCGGGAA TCTCAATGTT 593
Consensus GTCTCAATCG CCGCGTCGCA GAAGATCTCA ATCTCGGGAA TCTCAATGTT
100%
Conservation
0%
2,460
2,480
2,500
Hepatitis B virus DNA, complete genome, isolate: Pkst7912 AGTATTCCTT GGACTCATAA GGTGGGAAAC TTTACGGGGC TTTATTCTTC 2500
Hepatitis B virus DNA, complete genome, isolate: Pkst8216 AGTATTCCTT GGACTCATAA GGTGGGAAAC TTTACGGGGC TTTATTCTTC 2500
Hepatitis B virus DNA, complete genome, isolate: Pkst8134 AGTATTCCTT GGACTCATAA GGTGGGAAAC TTTACGGGGC TTTATTCTTC 2500
Hepatitis B virus strain 3798-91, complete genome AGTATTCCTT GGACTCATAA GGTGGGAAAC TTTACGGGGC TTTATTCTTC 2500
Hepatitis B virus isolate T1503, complete genome AGTATTCCCT GGACTCATAA GGTGGGAAAC TTTACGGGGC TTTATTCTTC 2467
Hepatitis B virus isolate T1562, complete genome AGTATTCCCT GGACTCATAA GGTGGGAAAC TTTACGGGGC TTTATTCTTC 2500
L2 genome AGTATTCCTT GGACTCATAA GGTGGGAAAC TTTACGGGGC TTTATTCTTC 643
Consensus AGTATTCCTT GGACTCATAA GGTGGGAAAC TTTACGGGGC TTTATTCTTC
100%
Conservation
0%

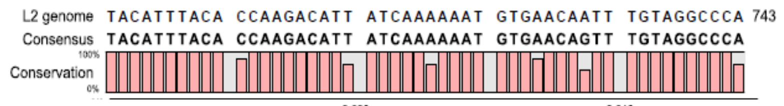
	2,520 / 2,540	
Hepatitis B virus DNA, complete genome, isolate: Pkst7912	TACTGTCCCG GTCTTTAACC CTCATTGGAA AACACCCTCT TTTCCTAATA	2550
Hepatitis B virus DNA, complete genome, isolate: Pkst8216	TACTGTACCT GTCTTTAATC CTCATTGGAA AACACCCTCT TTTCCTAATA	2550
Hepatitis B virus DNA, complete genome, isolate: Pkst8134	TACTGTACCT GTCTTTAATC CTCATTGGAA AACACCCACT TTTCCTAATA	2550
Hepatitis B virus strain 3798-91, complete genome	TACTGTACCT GTCTTTAATC CTCATTGGAA AACACCCTCT TTTCCTAATA	2550
Hepatitis B virus isolate T1503, complete genome	TACTGTACCT GTCTTTAACC CTCATTGGAA AACACCATCT TTTCCCAATA	2517
Hepatitis B virus isolate T1562, complete genome	TACTGTACCT GTCTTTAACC CTCATTGGAA AACACCATCT TTTCCCAATA	2550
L2 genome	TACTGTTCCT GTCTTTAACC CTCATTGGAA AACACCCTCT TTTCCTAATA	693
Consensus	**TACTGTACCT GTCTTTAACC CTCATTGGAA AACACCCTCT TTTCCTAATA**	

Conservation (100% / 0%)



	2,560 / 2,580 / 2,600	
Hepatitis B virus DNA, complete genome, isolate: Pkst7912	TACATTTACA CCAAGACATT ATCAAAAAAT GTGAACAATT TGTAGGCCCA	2600
Hepatitis B virus DNA, complete genome, isolate: Pkst8216	TACATTTACA CCAAGACATT ATCAAAAAAT GTGAACAATT TGTAGGCCCA	2600
Hepatitis B virus DNA, complete genome, isolate: Pkst8134	TACATTTACA TCAAGACATT ATCAAAAAAT GTGAACAGTT TGTAGGCCCA	2600
Hepatitis B virus strain 3798-91, complete genome	TACATTTACA CCAAGACATT ATCAAAAAAT GTGAACAGTT TGTAGGCCCA	2600
Hepatitis B virus isolate T1503, complete genome	TACATTTACA CCAAGACATC ATCAACAAAT GTGAACAGTT TGTAGGCCCT	2567
Hepatitis B virus isolate T1562, complete genome	TACATTTACA CCAAGACATC ATCAACAAAT GTGGACAGTT TGTAGGCCCT	2600
L2 genome	TACATTTACA CCAAGACATT ATCAAAAAAT GTGAACAATT TGTAGGCCCA	743
Consensus	**TACATTTACA CCAAGACATT ATCAAAAAAT GTGAACAGTT TGTAGGCCCA**	

Conservation (100% / 0%)

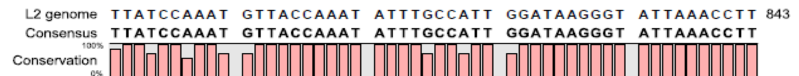


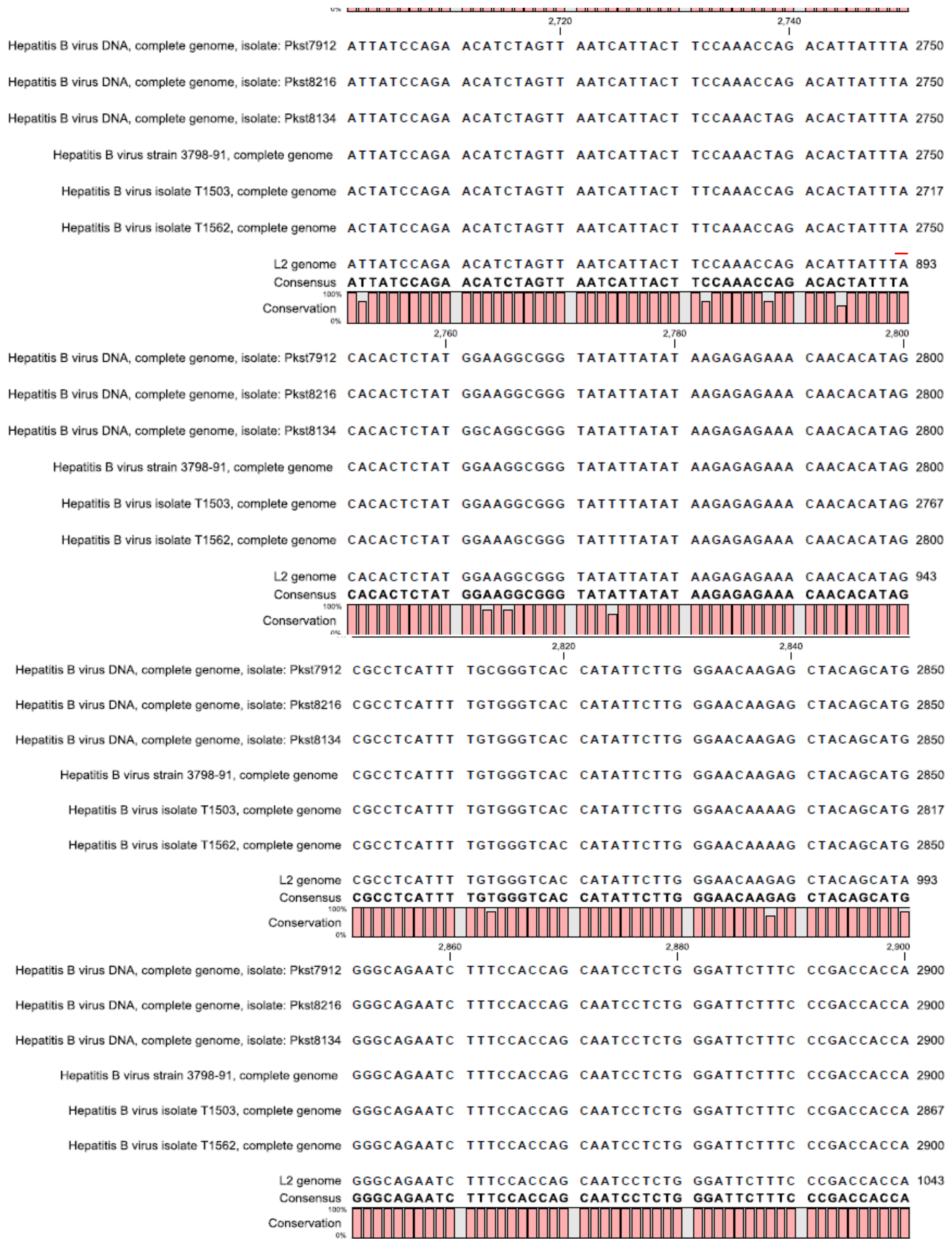
	2,620 / 2,640	
Hepatitis B virus DNA, complete genome, isolate: Pkst7912	CTCACAGTCA ATGAGAAAAG AAGACTACAA TTGATTATGC CTGCTAGGTT	2650
Hepatitis B virus DNA, complete genome, isolate: Pkst8216	CTCACAATCA ATGAGAAAAG AAGACTGCAA TTGATTATGC CTGCTAGGTT	2650
Hepatitis B virus DNA, complete genome, isolate: Pkst8134	CTCACAGTTA ATGAAAAAAG AAGATTGCAA TTGATTATGC CTGCTAGGTT	2650
Hepatitis B virus strain 3798-91, complete genome	CTCACAGTTA ATGAGAAAAG AAGATTGCAA TTGATTATGC CTGCTAGGTT	2650
Hepatitis B virus isolate T1503, complete genome	CTTACTGTCA ATGAGAAAAG AAGATTGCAA TTGATTATGC CTGCTAGGTT	2617
Hepatitis B virus isolate T1562, complete genome	CTTACTGTCA ATGAGAAAAG AAGATTGCAA TTGATTATGC CTGCTAGGTT	2650
L2 genome	CTTACAATCA ATGAGAAAAG AAGACTGCAA TTGATTATGC CTGCTAGGTT	793
Consensus	**CTCACAGTCA ATGAGAAAAG AAGATTGCAA TTGATTATGC CTGCTAGGTT**	

Conservation (100% / 0%)

	2,660 / 2,680 / 2,700	
Hepatitis B virus DNA, complete genome, isolate: Pkst7912	TTATCCAAAT GTTACCAAAT ATTTGCCATT GGATAAGGGT ATTAAACCTT	2700
Hepatitis B virus DNA, complete genome, isolate: Pkst8216	TTATCCAAAT GTTACCAAAT ATTTGCCATT GGATAAGGGT ATTAAACCTT	2700
Hepatitis B virus DNA, complete genome, isolate: Pkst8134	CTATCCCAAT GTTACCAAAT ATTTGCCATT GGATAAGGGT ATTAAACCTT	2700
Hepatitis B virus strain 3798-91, complete genome	TTATCCAAAT GTTACCAAAT ATTTGCCATT GGATAAGGGT ATTAAACCTT	2700
Hepatitis B virus isolate T1503, complete genome	TTACCCTAAC TTTACCAAAT ATTTACCTTT AGATAAGGGT ATTAAACCTT	2667
Hepatitis B virus isolate T1562, complete genome	TTACCCTAAC TTTACCAAAT ATTTACCTTT AGATAAGGGT ATTAAACCTT	2700
L2 genome	TTATCCAAAT GTTACCAAAT ATTTGCCATT GGATAAGGGT ATTAAACCTT	843
Consensus	**TTATCCAAAT GTTACCAAAT ATTTGCCATT GGATAAGGGT ATTAAACCTT**	

Conservation (100% / 0%)




2,720
2,740
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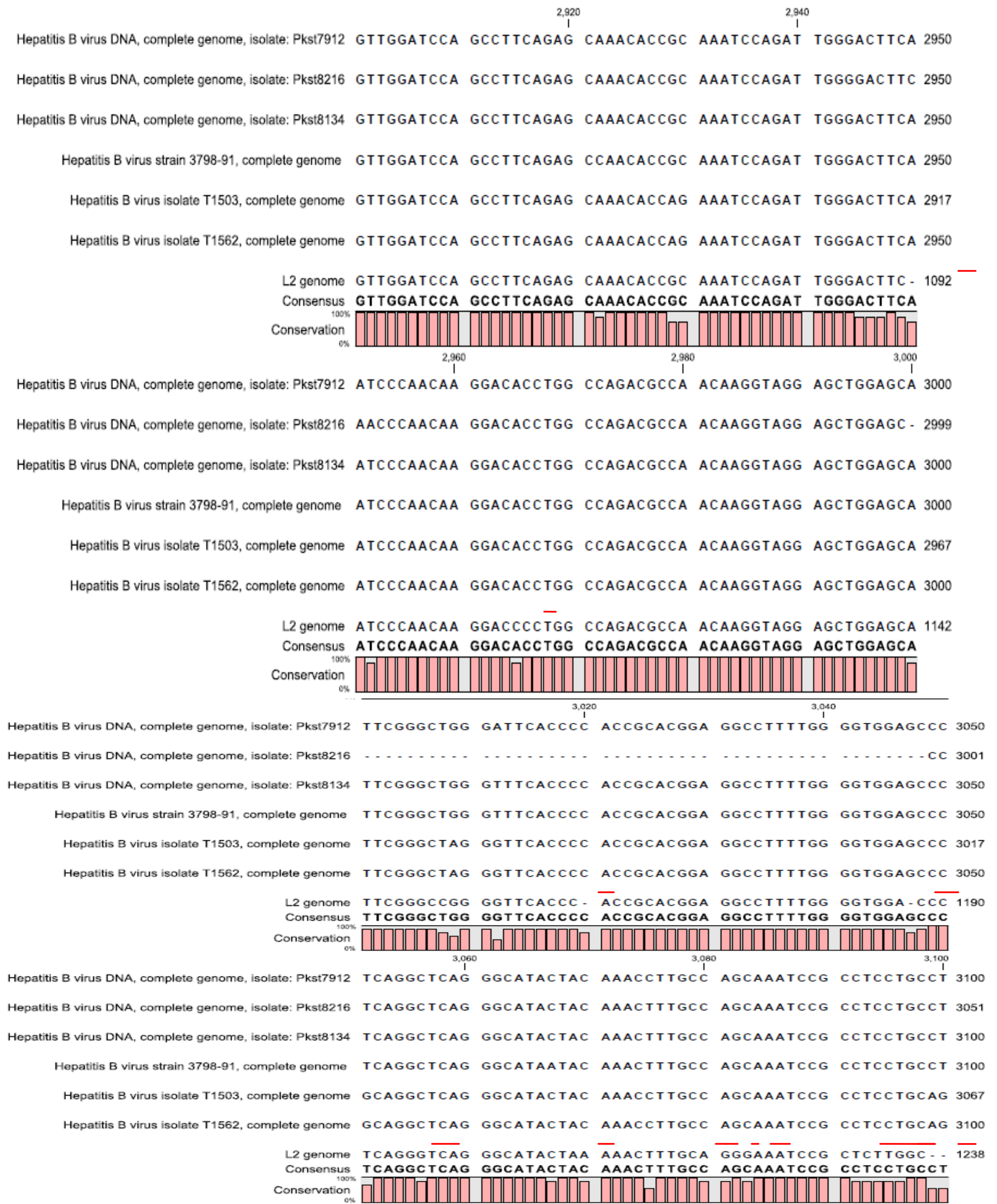
Hepatitis B virus DNA, complete genome, isolate: Pkst8216 ATTATCCAGA ACATCTAGTT AATCATTACT TCCAAACCAG ACATTATTTA 2750
Hepatitis B virus DNA, complete genome, isolate: Pkst8134 ATTATCCAGA ACATCTAGTT AATCATTACT TCCAAACTAG ACACTATTTA 2750
Hepatitis B virus strain 3798-91, complete genome ATTATCCAGA ACATCTAGTT AATCATTACT TCCAAACTAG ACACTATTTA 2750
Hepatitis B virus isolate T1503, complete genome ACTATCCAGA ACATCTAGTT AATCATTACT TTCAAACCAG ACACTATTTA 2717
Hepatitis B virus isolate T1562, complete genome ACTATCCAGA ACATCTAGTT AATCATTACT TTCAAACCAG ACACTATTTA 2750
L2 genome ATTATCCAGA ACATCTAGTT AATCATTACT TCCAAACCAG ACATTATTTA 893
Consensus ATTATCCAGA ACATCTAGTT AATCATTACT TCCAAACCAG ACACTATTTA
Conservation
2,760
2,780
2,800
Hepatitis B virus DNA, complete genome, isolate: Pkst7912 CACACTCTAT GGAAGGCGGG TATATTATAT AAGAGAGAAA CAACACATAG 2800
Hepatitis B virus DNA, complete genome, isolate: Pkst8216 CACACTCTAT GGAAGGCGGG TATATTATAT AAGAGAGAAA CAACACATAG 2800
Hepatitis B virus DNA, complete genome, isolate: Pkst8134 CACACTCTAT GGCAGGCGGG TATATTATAT AAGAGAGAAA CAACACATAG 2800
Hepatitis B virus strain 3798-91, complete genome CACACTCTAT GGAAGGCGGG TATATTATAT AAGAGAGAAA CAACACATAG 2800
Hepatitis B virus isolate T1503, complete genome CACACTCTAT GGAAGGCGGG TATTTTATAT AAGAGAGAAA CAACACATAG 2767
Hepatitis B virus isolate T1562, complete genome CACACTCTAT GGAAAGCGGG TATTTTATAT AAGAGAGAAA CAACACATAG 2800
L2 genome CACACTCTAT GGAAGGCGGG TATATTATAT AAGAGAGAAA CAACACATAG 943
Consensus CACACTCTAT GGAAGGCGGG TATATTATAT AAGAGAGAAA CAACACATAG
Conservation
2,820
2,840
Hepatitis B virus DNA, complete genome, isolate: Pkst7912 CGCCTCATTT TGCGGGTCAC CATATTCTTG GGAACAAGAG CTACAGCATG 2850
Hepatitis B virus DNA, complete genome, isolate: Pkst8216 CGCCTCATTT TGTGGGTCAC CATATTCTTG GGAACAAGAG CTACAGCATG 2850
Hepatitis B virus DNA, complete genome, isolate: Pkst8134 CGCCTCATTT TGTGGGTCAC CATATTCTTG GGAACAAGAG CTACAGCATG 2850
Hepatitis B virus strain 3798-91, complete genome CGCCTCATTT TGTGGGTCAC CATATTCTTG GGAACAAGAG CTACAGCATG 2850
Hepatitis B virus isolate T1503, complete genome CGCCTCATTT TGTGGGTCAC CATATTCTTG GGAACAAAAG CTACAGCATG 2817
Hepatitis B virus isolate T1562, complete genome CGCCTCATTT TGTGGGTCAC CATATTCTTG GGAACAAAAG CTACAGCATG 2850
L2 genome CGCCTCATTT TGTGGGTCAC CATATTCTTG GGAACAAGAG CTACAGCATA 993
Consensus CGCCTCATTT TGTGGGTCAC CATATTCTTG GGAACAAGAG CTACAGCATG
Conservation
2,860
2,880
2,900
Hepatitis B virus DNA, complete genome, isolate: Pkst7912 GGGCAGAATC TTTCCACCAG CAATCCTCTG GGATTCTTTC CCGACCACCA 2900
Hepatitis B virus DNA, complete genome, isolate: Pkst8216 GGGCAGAATC TTTCCACCAG CAATCCTCTG GGATTCTTTC CCGACCACCA 2900
Hepatitis B virus DNA, complete genome, isolate: Pkst8134 GGGCAGAATC TTTCCACCAG CAATCCTCTG GGATTCTTTC CCGACCACCA 2900
Hepatitis B virus strain 3798-91, complete genome GGGCAGAATC TTTCCACCAG CAATCCTCTG GGATTCTTTC CCGACCACCA 2900
Hepatitis B virus isolate T1503, complete genome GGGCAGAATC TTTCCACCAG CAATCCTCTG GGATTCTTTC CCGACCACCA 2867
Hepatitis B virus isolate T1562, complete genome GGGCAGAATC TTTCCACCAG CAATCCTCTG GGATTCTTTC CCGACCACCA 2900
L2 genome GGGCAGAATC TTTCCACCAG CAATCCTCTG GGATTCTTTC CCGACCACCA 1043
Consensus GGGCAGAATC TTTCCACCAG CAATCCTCTG GGATTCTTTC CCGACCACCA
Conservation



2,920 2,940
Hepatitis B virus DNA, complete genome, isolate: Pkst7912 GTTGGATCCA GCCTTCAGAG CAAACACCGC AAATCCAGAT TGGGACTTCA 2950
Hepatitis B virus DNA, complete genome, isolate: Pkst8216 GTTGGATCCA GCCTTCAGAG CAAACACCGC AAATCCAGAT TGGGGACTTC 2950
Hepatitis B virus DNA, complete genome, isolate: Pkst8134 GTTGGATCCA GCCTTCAGAG CAAACACCGC AAATCCAGAT TGGGACTTCA 2950
Hepatitis B virus strain 3798-91, complete genome GTTGGATCCA GCCTTCAGAG CCAACACCGC AAATCCAGAT TGGGACTTCA 2950
Hepatitis B virus isolate T1503, complete genome GTTGGATCCA GCCTTCAGAG CAAACACCAG AAATCCAGAT TGGGACTTCA 2917
Hepatitis B virus isolate T1562, complete genome GTTGGATCCA GCCTTCAGAG CAAACACCAG AAATCCAGAT TGGGACTTCA 2950
L2 genome GTTGGATCCA GCCTTCAGAG CAAACACCGC AAATCCAGAT TGGGACTTC- 1092
Consensus GTTGGATCCA GCCTTCAGAG CAAACACCGC AAATCCAGAT TGGGACTTCA
100%
Conservation
0%
2,960 2,980 3,000
Hepatitis B virus DNA, complete genome, isolate: Pkst7912 ATCCCAACAA GGACACCTGG CCAGACGCCA ACAAGGTAGG AGCTGGAGCA 3000
Hepatitis B virus DNA, complete genome, isolate: Pkst8216 AACCCAACAA GGACACCTGG CCAGACGCCA ACAAGGTAGG AGCTGGAGC- 2999
Hepatitis B virus DNA, complete genome, isolate: Pkst8134 ATCCCAACAA GGACACCTGG CCAGACGCCA ACAAGGTAGG AGCTGGAGCA 3000
Hepatitis B virus strain 3798-91, complete genome ATCCCAACAA GGACACCTGG CCAGACGCCA ACAAGGTAGG AGCTGGAGCA 3000
Hepatitis B virus isolate T1503, complete genome ATCCCAACAA GGACACCTGG CCAGACGCCA ACAAGGTAGG AGCTGGAGCA 2967
Hepatitis B virus isolate T1562, complete genome ATCCCAACAA GGACACCTGG CCAGACGCCA ACAAGGTAGG AGCTGGAGCA 3000
L2 genome ATCCCAACAA GGACCCCTGG CCAGACGCCA ACAAGGTAGG AGCTGGAGCA 1142
Consensus ATCCCAACAA GGACACCTGG CCAGACGCCA ACAAGGTAGG AGCTGGAGCA
100%
Conservation
0%
3,020 3,040
Hepatitis B virus DNA, complete genome, isolate: Pkst7912 TTCGGGCTGG GATTCACCCC ACCGCACGGA GGCCTTTTGG GGTGGAGCCC 3050
Hepatitis B virus DNA, complete genome, isolate: Pkst8216 ---------- ---------- ---------- ---------- --------CC 3001
Hepatitis B virus DNA, complete genome, isolate: Pkst8134 TTCGGGCTGG GTTTCACCCC ACCGCACGGA GGCCTTTTGG GGTGGAGCCC 3050
Hepatitis B virus strain 3798-91, complete genome TTCGGGCTGG GTTTCACCCC ACCGCACGGA GGCCTTTTGG GGTGGAGCCC 3050
Hepatitis B virus isolate T1503, complete genome TTCGGGCTAG GGTTCACCCC ACCGCACGGA GGCCTTTTGG GGTGGAGCCC 3017
Hepatitis B virus isolate T1562, complete genome TTCGGGCTAG GGTTCACCCC ACCGCACGGA GGCCTTTTGG GGTGGAGCCC 3050
L2 genome TTCGGGCCGG GGTTCACCC- ACCGCACGGA GGCCTTTTGG GGTGGA-CCC 1190
Consensus TTCGGGCTGG GGTTCACCCC ACCGCACGGA GGCCTTTTGG GGTGGAGCCC
100%
Conservation
0%
3,060 3,080 3,100
Hepatitis B virus DNA, complete genome, isolate: Pkst7912 TCAGGCTCAG GGCATACTAC AAACCTTGCC AGCAAATCCG CCTCCTGCCT 3100
Hepatitis B virus DNA, complete genome, isolate: Pkst8216 TCAGGCTCAG GGCATACTAC AAACTTTGCC AGCAAATCCG CCTCCTGCCT 3051
Hepatitis B virus DNA, complete genome, isolate: Pkst8134 TCAGGCTCAG GGCATACTAC AAACTTTGCC AGCAAATCCG CCTCCTGCCT 3100
Hepatitis B virus strain 3798-91, complete genome TCAGGCTCAG GGCATAATAC AAACTTTGCC AGCAAATCCG CCTCCTGCCT 3100
Hepatitis B virus isolate T1503, complete genome GCAGGCTCAG GGCATACTAC AAACCTTGCC AGCAAATCCG CCTCCTGCAG 3067
Hepatitis B virus isolate T1562, complete genome GCAGGCTCAG GGCATACTAC AAACCTTGCC AGCAAATCCG CCTCCTGCAG 3100
L2 genome TCAGGGTCAG GGCATACTAA AAACTTTGCA GGGAAATCCG CTCTTGGC-- 1238
Consensus TCAGGCTCAG GGCATACTAC AAACTTTGCC AGCAAATCCG CCTCCTGCCT
100%
Conservation
0%

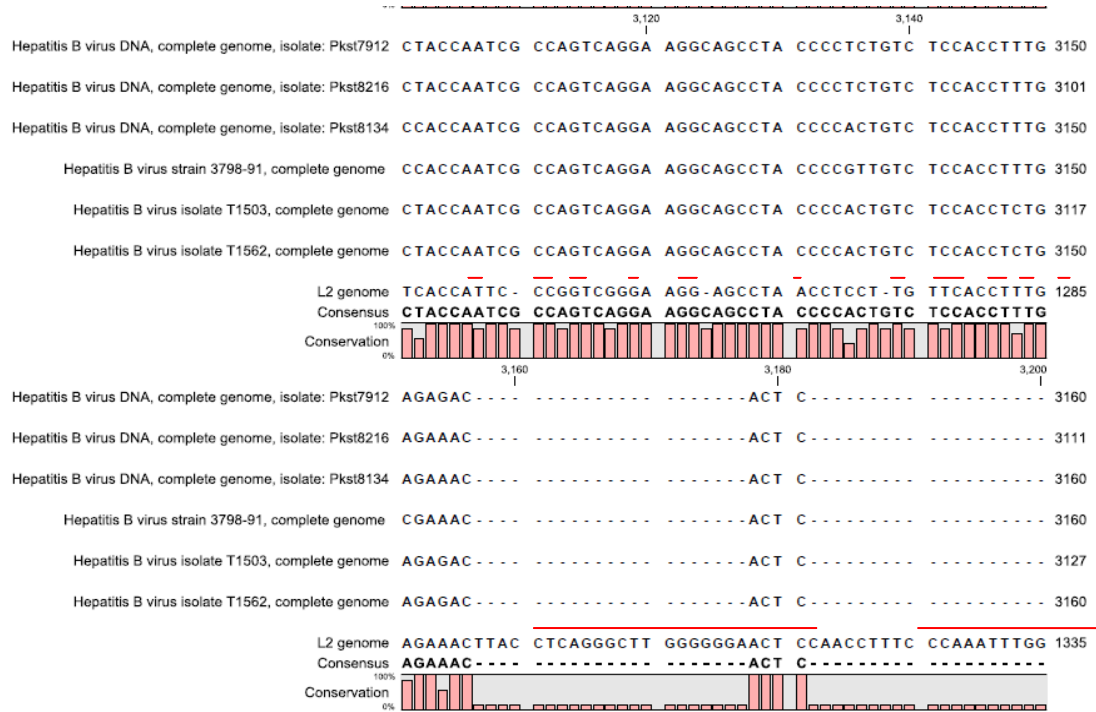


Figure 4.5: Alignment of sample sequence and reference sequence using CLC workbench.

Chapter 5

DISCUSSION

Since the discovery of HBV in 1960's, enormous progress has been made in elucidating its molecular characteristics, etiology and phylogenetic evolution. Despite advancements in development of an effective vaccine, annually more then 360 million people continue to be chronically infected with this virus and are at danger of developing Hepatocellular carcinoma (Fan *et al.,* 2011). In spite of the high HBV endemicity in Asia there is scarcity in the availability of data from this continent especially in Pakistan. Sequence data on complete HBV genomes isolated from HBV chronic patients are particularly rare (Mumtaz K *et al.,* 2011). Till date, genetic plasticity in whole genome variants has not been characterized in the HBV infected group of Pakistan population. The drastic changes in HBV pool in Pakistan due to HBV vaccine are quite conflicting and debatable issues. There is little data regarding random population statistics for HBV complete genome and its gene products. It is also note worthy that for a developing country like Pakistan, reverse transcriptase inhibitor Lamivudine has been used extensively to treat patients continuously for the duration of several years. This has lead to increased frequency of progeny viruses with genetic alterations in Surface and Polymerase ORF (Khokhar *et al.,* 2005).

Sequence analysis of various genes from HBV genome, have lead to some very important and interesting findings related to viral pathogenesis and persistence.

The most important selective force during the ordinary course of HBV infection appears to be the immune response (Tong *et al*., 2005). The growing knowledge of the function of the precore region product in stimulating serum HBeAg led to search for changes in the precore region sequence of HBV DNA in the circulation of carriers after they had seroconverted to anti-HBe (Kreutz, 2002). The maturity of anti-HBe antibody in hepatitis B patients usually correlates with decline of HBV viremia. As an end result, escape mutants of anti-HBe are selected. Many factors have been linked to highly replicative hepatitis B virus. Among these, viral factors are very important in immunomodulation. Precore/core region is linked to important areas of viral pathogenesis (Brunetto*et al.,* 1999; Kock*et al.,* 2004; McMillan *et al*., 1996). There are reports supporting the fact that these resistant mutants can spread to and harmfully affect the vaccinated individuals (Sayan and Akhan, 2011; Villet*et al.,* 2008). The global distribution of HBV genotypes and sub genotypes has been established, and it is becoming apparent that mutations associated with specific genotypes also have distinct global distribution pattern and may affect the clinical outcome of infection within a specific populations (Kao *et al*., 2000; Kramvis and Kew, 2005).

This study was therefore designed with the particular aims to identify the status of variants in HBV genomes especially vaccine escape mutants so that the information can be exploited by the personnel involved in pharmaceutical companies (development of drugs/inhibitors for reverse transcriptase of HBV), healthcare (diagnostic errors of HBV and occult infections) as well as the authorities (design future policies) for the proper management of Hepatitis B virus

infections in Pakistan. Through full-length HBV genomic analysis, it is possible to explore whether there are substantial nucleic acid sequence variations between HBV isolates from acute, chronic patients and hepatocellular carcinoma patients (HCC). And by further cell transfection studies, functional differences between these HBV isolates might also be exposed (Takahashi *et al*., 1999).

CONCLUSION

The present study was planned to successfully characterize full length genomes from Pakistani isolates of chronic HBV infected population. As a consequence mutations in different viral genes including Surface gene (involved in pathogenicity and immunogenicity) can be explored. Similarly viral replication activity can be monitored by mapping mutations in polymerase and precore/core gene. Exclusively large no of complete genome sequences of HBV can help in predicting the actual role of X gene in development of hepatocellular carcinoma.

FUTURE PROSPECTS

A more comprehensive analysis of complete HBV genome on a large population set is necessary to better describe the course of infection in HBV patients. In addition more sequences need to be obtained in order to depict a true picture of HBV circulating strains in Pakistani population. The study could be enhanced to include mutants that are linked exclusively to fulminant, cirrhotic/non-cirrhotic hepatitis and hepatocellular carcinoma patients of Pakistan. Determination of the genotypes and subtypes of the HBV can provide molecular epidemiological data from Pakistani population, which further leads towards, devising new strategies to combat immune escape variants of HBV. This could further lead to better antiviral treatment options and new diagnostic assays can be designed to under stand the stage of the disease.

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