Bethlehem University
Faculty of science
Biotechnology Master Program

Sub-genotyping and Drug Resistance Prediction in Palestinian Hepatitis B Virus (HBV) isolates

By Zakeih Hussein Abdelnabi

In Partial Fulfillment of the Requirements for the Degree of Master of Science in Biotechnology

December, 2012

The undersigned hereby certify that they have read and recommend to the Faculty of Scientific Research and Higher Studies at the Palestine Polytechnic University and the Faculty of Science at Bethlehem University for acceptance a thesis entitled:

Sub-genotyping and Drug Resistance Prediction in Palestinian Hepatitis B Virus (HBV) isolates

By Zakeih Hussein Abdelnabi

In partial fulfillment of the requirements for the degree of Master of Science in Biotechnology

Graduate Advisory Committee:

Dr. Maysa Azzeh, Al-Quds University	Date
Prof. Dr. Mazin Qumsiyeh, Bethlehem University	Date
Prof. Dr. Wolfram Gerlich, Justus Liebig University, Germany	Date

Approved for the Faculties

Dean of Faculty of Scientific Research and Higher studies Palestine Polytechnic University	Dean of Faculty of Science Bethlehem University

Sub-genotyping and Drug Resistance Prediction in Palestinian Hepatitis B Virus (HBV) isolates

Hepatitis B virus (HBV) is a double-stranded DNA virus of the hepadnaviridae family. Samples with different HBsAg (serology marker for HBV infection) index were subjected to real time PCR of the x gene, a non structural gene of the HBV DNA. Forty samples with viral load above 10^5 copies/ml were subjected to genotyping and drug resistance analysis. The forward primer was in the S gene, while the reverse primer was in the overlapping polymerase (Pol)/reverse transcriptase (RT) gene region. The sequence in the S gene reflects the genotype and subgenotype, while the sequence in the pol/RT gene reflects the region of possible drug resistance. The sequencing results showed that HBV D1 was the most prominent subgenotype among Palestinians carrying HBV. Variable mutations existed within the S gene, but five patients were presented with four known escape mutations including the common G145R and D144E. Circulation of such mutant HBV strains is the leading cause for vaccine failure. Furthermore, a ratio of 4.25 of non-synonymous to synonymous mutations in the S gene indicated a strong selection effect on HBV strains circulating in the studied Palestinian patients, which may be due to immune selection. Although all patients were naïve, with the exception of one, variable mutations were found in the sequences of the HBV polymerase gene (the target of the anti viral drugs). None of these mutations was reported earlier to associate with drug resistance. However, few of them can evoke resistance if occurring in combination with other mutations. One of main alarming result presented here was the infection of children with maternal HBV strain, despite vaccination. This consequence demands a re-thinking of the current Palestinian health strategy in testing pregnant women. Identifying specific genotypes of pathogens in the different geographic areas with high prevalence may lead to further steps in optimizing vaccines, diagnostic tools, choice of drugs and control measures according to the predominant local strains. The study presented here is the first comprehensive research addressing genotypes and mutation analysis of HBV virus S gene and polymerase in Palestine and one of the unique ones in the entire Arab world.

Key words: Hepatitis B, genotype, subgenotype, non-synonymous mutations, synonymous mutations, drug resistance.

دراسة النمط الجيني و مقاومة الدواء عند فيروس التهاب الكبد الفيروسي (ب) في فلسطين

فيروس التهاب الكبد ب (HBV) هو فيروس ينتمي لعائلة Hepadenoviridae ، وهو يحتوي سلاسل مزدوجة من المادة الوراثية DNA , و يتم التشخيص الإصابة بهذا الفيروس بإيجابية المستضد السطحي . (HBS Ag). في هذه الدراسة تم استخدام عينات مختلفة المؤشر للمستضد السطحي (HBS Ag Index)، وقد تم استخدام تقنية real time PCR على الجين (X). هو جين غير هيكلي من الحمض النووي ل HBV. وفي البحث المختبري وجدنا أربعين عينة تحتوي على 10^5 نسخة جينية من الفيروس/ مل خضعت للتحليل الجيني وتحليل مقاومة الدواء . لبلمرة المنطقة المنشودة من الحمض النووي DNA لفيروس HBV , وقمنا بتصميم (forward primer) وهو نقطه الانطلاق لبلمرة الحمض النووي في الجين (S)، و نقطة انطلاق عكسية (reverse primer) في المنطقة المتداخلة بين الجين (S) و الجين (RT).

كما وجدنا أن تسلسل الجينات في الجين (S) يعكس النمط الجيني وأنواعه genotype وsubgenotype، في حين أن تسلسل الجينات في(RT) يعكس منطقة مقاومة الدواء. وأظهرت النتائج أن سلالة (HBV-D1) كانت الأبرز بين الفلسطينيين حاملي فيروس HBV، وعثرنا على طفرات متغيرة variable mutations داخل الجين (S) في خمسة مرضى، اكتُشف لديهم 4 طفرات معروفة ب (الطفرات الهاربة) (escape mutations) أهمها G145R وD144E. إن انتشار هذا النوع من الطفرات في سلالات HBV يعد السبب الأساسي لفشل اللقاح. علاوة على ذلك،فان نسبة الطفرات غير المرادفة non-synonymous mutations إلى الطفرات المرادفة synonymous mutation تساوي 4.25 مما يشير إلى أن حدوث طفرات في جين (S) له تأثير قوي على الاختيار المناعي (immune selection) للسلالات المنتشرة في المرضى الفلسطينيين في هذه الدراسة. ورغم أن جميع المرضى بهذا الفيروس لم يعالجوا من قبل - باستثناء واحدة- إلا أنه تم العثور على طفرات متغيرة في جين ال RT.وعلى الرغم من عدم التسجيل سابق عن علاقة أي من هذه الطفرات بالمناعة الدوائية ،فأن بعض الطفرات قد تسبب المناعة الدوائية إذا تصادف وجودها مع طفرات أخرى.من النتائج المقلقة في هذه الدراسة انتقال سلالة هذا الفيروس من الأمهات المصابات ب HBV إلى أطفالهن على الرغم من التطعيم . هذه النتيجة تتطلب إعادة التفكير في إستراتجية وزارة الصحة الفلسطينية الحالية في اختبار الإصابة لدى النساء الحوامل .

والأمر يتطلب تحديد النمط الجيني و مسببات الأمراض في المناطق الجغرافية المختلفة ذات معدل الانتشار الفيروسي المرتفع لنتمكن من تصنيع لقاحات وأدوات تشخيص و أدوية وتدابير رقابة أكثر ملائمة للسلالات المحلية السائدة.هذه الدراسة هي أول بحث شامل يتناول الأنماط الجينية وتحليل الطفرات الجينية لكل من جين S وجين RT في فيروس HBV في فلسطين، كما أنها دراسة رائدة على مستوى العالم العربي وبخاصة أن النتائج التي وصلنا إليها جديرة بالاهتمام من قِبَل المؤسسات الصحية المحلية و تمثل خطوة أولى في إنشاء قاعدة معلومات جينية لمسببات الأمراض المنتشرة في فلسطين.

Declaration

I declare that the Master Thesis entitled “Sub-genotyping and Drug Resistance Prediction in Palestinian Hepatitis B Virus (HBV) isolates
“Is my own original work, and hereby certify that unless stated, all work contained within this thesis is my own independent research and has not been submitted for the award of any other degree at any institution, except where due acknowledgment is made in the text.

Name and signature: Zakeih Abdelnabi
Date: 2012

Statement of Permission To Use

In presenting this thesis in partial fulfillment of the requirements for the joint master degree in biotechnology at the Palestine Polytechnic University and Bethlehem University, I agree that the library shall make it available to borrowers under rules of the library. Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgement of the source is made.

Permission for extensive quotation from, reproduction, or publication of this thesis may be granted by my main supervisor, or in his absence, by the Dean of Higher Studies when, in the opinion of either, the proposed use of the material is for scholarly purposes. Any copying or use of the material in this thesis for financial gain shall not be allowed without my written permission.

Signature: Zakeih Abdelnabi

Date: 2012

Dedication

I dedicate those pure souls of my dear grandfather Dr. Hafez Abdelnabi and my grandmother Abdelqader Shaheen.

I dedicate my thesis to my parents, for their prayers that were always helping me, for their endless support, their love and patience.

To my husband, who encouraged me to pursue the master degree on first place, for his endless support and efforts during this critical stage.

To my sisters (Ala', Asma', Doa', Malak and Abrar) and brothers (Anas and Issa) for their support and love.

To my grandmother, for her support and wisdom advices.

To all of my family and my husband’s family, for their encouragement.

To all my friends, specially Wurood Al-Ja’bari and Suha Abu Sneineh.

Acknowledgement

I would like to express my deep and sincere gratitude to my supervisor Dr. Maysa Azzeh for facilitating this work on first place. I would also like to thank her suggestions, guidance, encouragement, valuable advices and support throughout this study.

I also wish to express my warm and sincere thanks to Dr. Zaidoun Salah for his efforts.

I express my special thanks to Dr. Moien Kanaan for his assistance, support and professional administrative facilitation.

My deep gratitude goes to all staff of the Virology Laboratory at the medical Research Center, Al-Quds University, especially Niveen Saleh and Lina Qurei.

Finally, I would like to thank my friends and colleagues especially Orayb Sharha.

Table content :

List of Figures:

List of Tables:

List of abbreviations:

HBV	Hepatitis B virus
HBsAg	Hepatitis B surface Antigen
HAV	Hepatitis A virus
HCV	Hepatitis C virus
HDV	Hepatitis D virus
HEV	Hepatitis E virus
HBcAg	Hepatitis B core antigen
anti-HBs	antibody to the surface antigen of the hepatitis B virus
ORFs	Open Reading Frames
C	Core protein
P	Polymerase
S	Surface antigen
cccDNA	covalently closed circular DNA
pgRNA	pregenomic RNA
RC	Relaxed Circular
IFN-α	Alpha Interferon
ALT	Alanine aminotransferase
RT	Reverse Transcriptase
Pol	Polymerase

1. INTRODUCTION

1.1 Historical background

Viral hepatitis is an inflammation of the liver caused by viral infection. Over a thousand years ago, hepatitis A virus was recognized causing liver disease transmitted by a fecal-oral route (Seeger et al, 2007). In the 19th and early 20th centuries a new type of hepatitis appeared to be transmitted from blood and body fluids, identified later as hepatitis B (Seeger et al, 2007).

In 1885, Lurman documented the existence of a second form of hepatitis which was later designated hepatitis B. He noticed that 15% of shipyard workers in Bremen, Germany had developed jaundice after they had received smallpox vaccine prepared from human lymph (MacCallum, 1946; Hasegawaet al., 1991; Touzé et al., 2002). In 1937, an outbreak of jaundice among children, who received measles vaccine prepared from one batch of human serum, was observed (McNalby, 1939). This observation was confirmed experimentally using human serum containing yellow fever vaccine in volunteers (Findlay and MacCallum, 1937; Franco et al., 1992). Finally, Fox et al. (1942) defined human serum as the vehicle for hepatitis transmission.

According to the differences observed between infectious hepatitis (Hepatitis A) and serum derived hepatitis, MacCallum introduced another definition for the second type of viral hepatitis which he called hepatitis B. This term has been adopted by the World Health Organization (WHO) (WHO, 1977).

While some studies contributed to understanding the seroepidemiology of hepatitis A and B (Murray, 1955; Krugman and Giles, 1973) the first specific trace of a human hepatitis virus was discovered by Blumberg and colleagues in the sixties. Blumberg and colleagues were studying sera from transfused individuals to identify and track genetic differences in human populations. Blumberg was particularly interested in antibodies binding human serum proteins. During those studies, a new antigen was identified in serum from an Australian Aborigine and was named accordingly "Australia" antigen (Blumberg et al., 1965). In 1968, different scientists identified the "Australia" antigen to be specific for hepatitis B patients' sera, which was later designated the term "Hepatitis B Antigen" (HBsAg) (Prince, 1968; Okochi and

Murakami,1968).Blumberg received the Nobel Prize in Medicine in 1976, for his discovery of HBsAg. This discovery had a significant benefit for blood banks; they could now screen for infected blood, leading to a decline in cases of post transfusion hepatitis *(*Blumberg, 1977).

Currently we know that at least 5 viruses belonging to different viral families can cause hepatitis in humans. Hepatitis A virus (HAV) is a single stranded RNA virus and belongs to the Picornaviridae family, genus hepatovirus. Hepatitis B virus (HBV) a partially double stranded DNA virus and belongs to the Hepadnaviridae family, genus orthohepadnavirus. Hepatitis C (HCV) is a positive stranded RNA virus of the Flaviviridae family, genus hepacivirus. Hepatitis D virus (HDV) is a single stranded circular RNA virus classified as the type species of its own. Hepatitis E virus (HEV) is a positive stranded RNA virus of the family Hepeviridae (***www.ictvdb.org)***.

Over 300 million people are infected with HBV, ranging from asymptomatic carriers to patients with severe health consequences, giving HBV, among all hepatitis viruses, a global importance (Beasley, 1988; Beasley et al., 1981; Lau et al.,1993).

1.2 Classification of HBV

Hepatitis B virus (HBV) belongs to the *Hepadnaviridae* family and the virus has partially double stranded DNA (Seeger and Mason, 2000). The term "Hepadna" comes from the fact that the virus infects the liver referred to with "hepa" and it is a DNA virus referred to with "dna". Hepadnaviruses contain two genera; ortho-hepadnaviruses which infect mammals like humans, apes and some monkey species, e.g. woolly monkeys (Lanford et al.,1998) and avihepadnaviruses which infect birds likeducks (Mason et al. 1980) herons (Sprengel et al., 1988), and geese (Chang et al., 1999). HBV strains of humans and apesform one virus species within the genus orthohepadnavirus and this is the prototype of the *hepadnaviridae* family (Fauquet et al., 2005).

1.3 Structure of HBV

There are three different known particle forms associated with HBV: the Dane particle (called after its discoverer), 20-nm spheres and 20-nm diameter filaments (Kaplan et al.,1973;Seeger et al., 2007**)**. These forms were characterized from HBsAg positive sera and coexist during infection in different quantities (Bayeet al.,1968; Daneet al.,1970; Robinson and Lutwick,1976). However, the only infectious form of HBV is the Dane particle (Seeger et al., 2007).

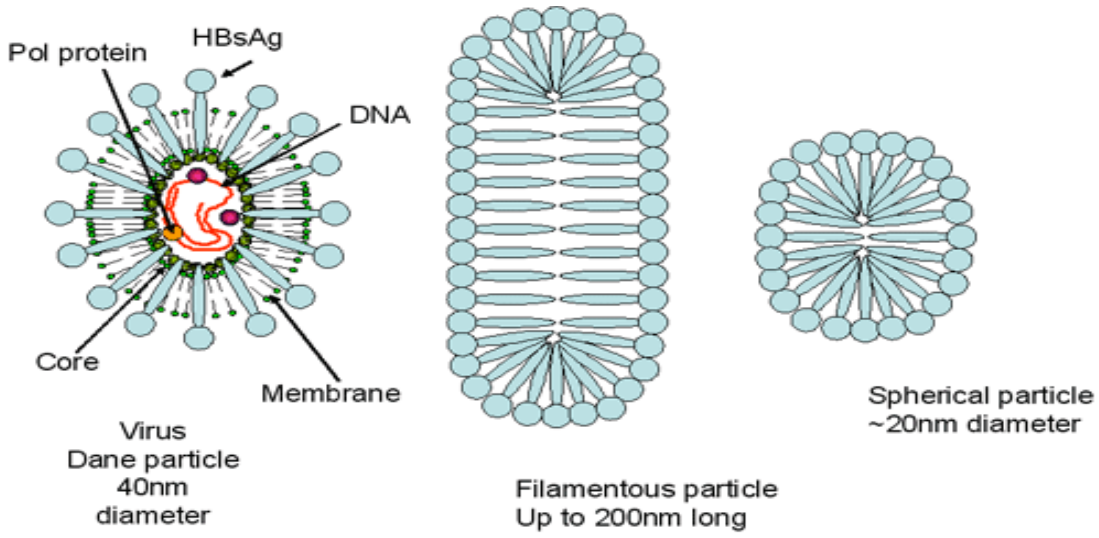


Figure 1.1: HBV particle forms. Dane particle resembles the infectious form of HBV; filamentous and spherical particles are produced during infection and are mainly composed of HBsAg. From http://pathmicro.med.sc.edu/virol/hep-bstruct.gif

The Dane particle has a lipoprotein envelope, which surrounds the viral capsid protein, the core (c). The core protein is a phosphoprotein known as HBcAg (Robinson et al., 1974; Summers et al., 1975), which encapsidates the viral DNA. A glycoprotein containing lipid bilayer forms the envelope of the Dane particle; this is called hepatitis B surface antigen (HBsAg). HBsAg is the main marker for HBV infection (Hoofnagle et al., 1973), against which neutralizing antibodies (anti-HBs) are directed (Hoofnagle et al., 1981). Interestingly, HBsAg is the only viral component of the 20-nm spheres and filaments, beside host-derived lipids (Gavilanes

et al., 1982; Peterson, 1981; Koff and Galambos, 1987). Therefore, these particles are noninfectious, but immunogenic and can induce neutralizing anti-HBs antibody response. Indeed, such purified particles served initially as HBV vaccine before the development of recombinant HBsAg preparations (Koff and Galambos, 1987).

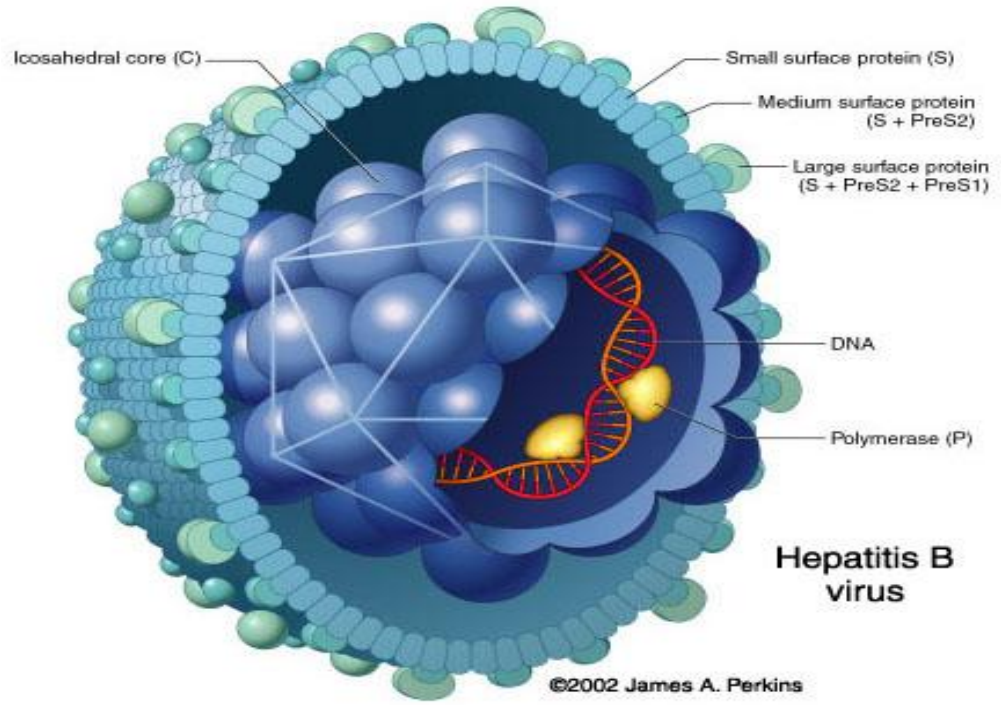


Figure 1.2: **Structure of HBV virus.**A cartoon resembling the 3 dimensional structure of HBV virus.From the Hepatitis B Database, © James A Perkins. http://www.ibibiobase.com/projects/hepatitis/hepatitis-aB.htm

1.4 Genome structure of HBV

HBV DNA is only 3.2 kb long and therefore considered as one of the smallest known viral genomes (Summers et al., 1975). The characteristic feature of the HBV lies in the lack of symmetry in its partial double stranded DNA (Seeger et al., 2007). The DNA contains a minus strand that is complementary to viral mRNAs, and a partially completed plus strand (Summers et al., 1975; Lutwick and Robinson, 1977). The

viral reverse transcriptase is covalently bound to the minus strands via a phospho-tyrosine bond. The minus strand encodes all known viral transcripts, which a recapped m7G and polyadenylated. The minus strand contains a total of four open reading frames (ORFs) which are fully or partially overlapped. These ORFs are responsible for the transcription and expression of different HBV proteins (Tiollais et al, 1985). The four genes encoded by the HBV genome are the core protein (C), the polymerase (P), the three polypeptides of the surface antigen (S) and transactivator of the viral transcription (X).

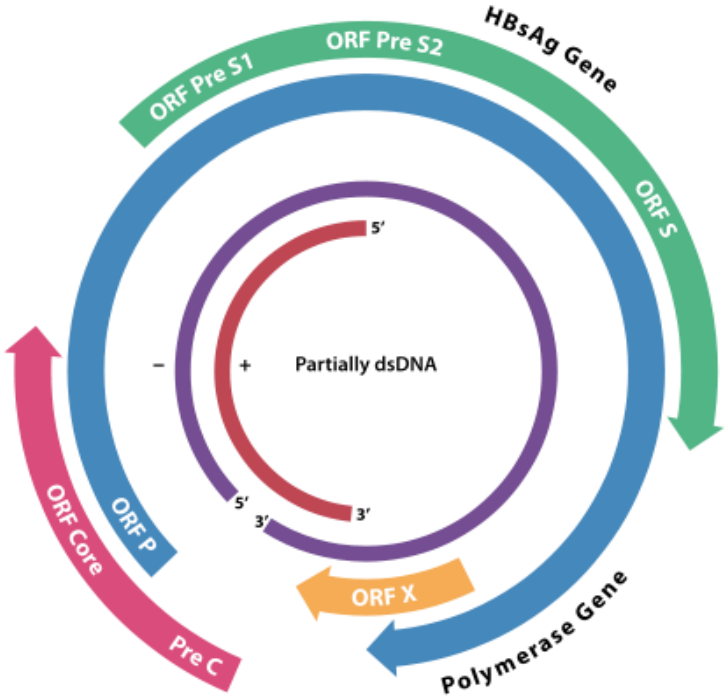


Figure 1.3: The HBV genome organization. Minus and plus strands (see above) are indicated in purple and red. The overlapping gene region between the S gene and the viral polymerase are illustrated.
http://en.wikipedia.org/wiki/File:HBV_Genome.svg

1.5 HBV DNA and replication

The nucleocapsid contains a circular viral DNA of about 3,000 bp, as well as an endogenous DNA polymerase activity that synthesized virus DNA when virions were treated with nonionic detergent and incubated in the presence of dNTPs (Kaplan et al., 1973; Robinson et al., 1974; Robinson, 1976). Summers showed that the circular conformation was maintained by a short cohesive overlap between the 5′ ends of the two DNA strands and that the circle was only partially double stranded, one strand remain incomplete. This strand was completed, and the single-stranded gap partially filled in by the endogenous DNA polymerase reaction (Summers et al., 1975). HBV has a unique replication system via reverse transcription, which is an error-prone enzyme resulting in a large number of nucleotide substitutions during replication leading to high genetic variability of HBV if the virus is put under selection pressure (Seeger et al., 2007).

Recent studies reported that HBV virion interacts with the hepatocyte surface Heparansulfateproteoglycans, which functions as primary attachment receptor for HBV (Schulze et al., 2007; Leistner et al., 2008). The PreS domain of the viral large surface protein plays an essential role in further binding and mediation of endocytosis. Amyristate moiety linked to the N-terminal glycine of PreS1 and the cysteine-rich antigenic loop of the S domain were identified to be critical for HBV infectivity (Le Seyec, 1999).

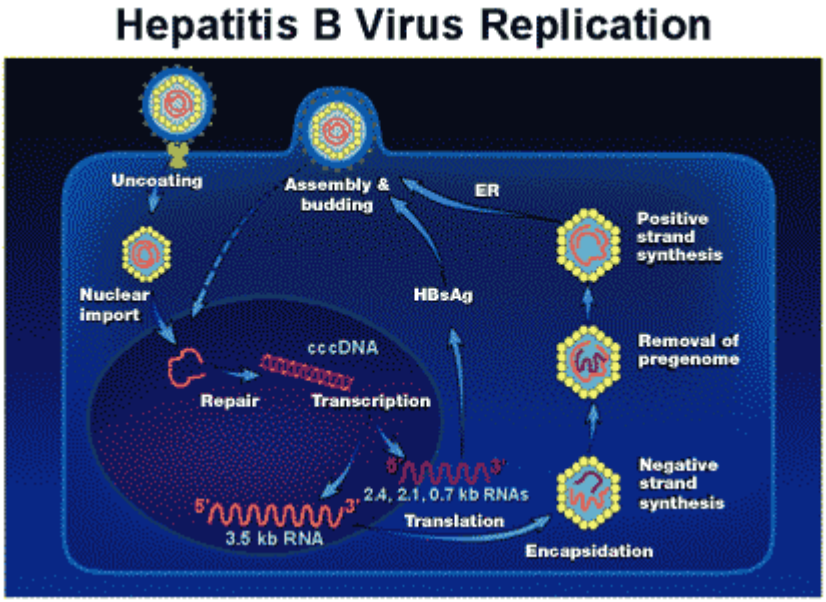


Figure 1.4: An illustration of HBV replication cycle. Hepatitis B is a DNA virus, which uses the reverse transcriptase activity of its polymerase for replication. The virus enters the cell via endocytosis and the viral capsid is released into the cytoplasm. The viral DNA is then transferred to the nucleus and transformed into closed circular DNA (cccDNA), which is the template for transcription of viral mRNAs. Hereby cellular RNA polymerase transcribes 4 viral mRNAs. The viral reverse transcriptase replicates the viral DNA from the largest mRNA, the pregenomic RNA (pgRNA) for capsid core proteins and viral DNA polymerase. Progeny viruses are produced, which can either be released or used for further production of viral RNA and viruses. http://micro-writers.egybio.net/blog/?p=13

In the cytoplasm, virions are uncoated and nucleocapsids are transported to the cell nuclear pore where the viral DNA is released to the nucleoplasm (Kann et al., 1997, Rabe et al., 2006). The relaxed circular (RC) viral DNA is converted, inside the host cell nucleus, into a plasmid-like covalently closed circular (ccc) DNA, which functions as the template for the transcription of the viral mRNAs for translation of viral proteins (Rall et al, 1983). Accordingly, several genomic and subgenomic RNAs are transcribed by cellular RNA polymerase. Pregenomic RNA (pgRNA) is the genomic transcript, which is translated to core and polymerase. These three components assemble to immature capsids where the reverse transcription process takes place to produce the new RC-DNA genomes. The DNA-containing mature

nucleocapsids can either function as progenitor for further intracellular cccDNA amplifications, or be enveloped and released from the cell as mature progeny virions (Seeger et al., 2007).

Regarding the other mRNAs, 3.5, 2.4, 2.1, and 0.7 kb RNAs transcripts coding for pre-C/C (C=capsid), PreS/S, and x viral proteins, are produced (Gough, 1983; Enders et al., 1985; Treinin and Laub, 1987; Seeger et al., 2007).

The core protein (C) is essential and forms the capsid. The precore (pre-C) gene encodes the HBeAg, which is a marker of partial immune tolerance against HBV. The pre-C protein does not play a role in viral replication, as HBV mutants defective for HBeAg were detectable in individuals who are chronically infected (Brunetto, 1999). On the other hand, pre-C protein seems to play a role in the regulation of the immune response against HBV (Saito et al., 1985).

The Pre-S/S coding region encodes three glycoproteins; the HBsAg (S protein), M protein (PreS2 protein) and L protein (PreS1). S (24 or 27 KD), M (33 or 36 kD) and L (39 or 42 KD) refer to small, medium and large based on the molecular weight of these proteins. HBsAg is most abundant in virions, spheres and filamentous structures representing the classical HBsAg discovered by Blumberg (see above). PreS2 is the larger form of HBsAg and represents about 10% to 15% of total envelope proteins and so far was not associated with specific function in HBV infection (Bruss and Ganem, 1991). PreS1 represents only 1% to 2% of total surface proteins in virions and is a ligand for the viral receptor (Heermann et al., 1984; Le Seyec, 1999).

The polymerase (P) gene encodes the viral DNA polymerase. While the N-terminus of the viral polymerase encodes the terminal protein (TP) domain, its C-terminus encodes for the reverse transcriptase (RT), whose coding region is overlapped by the Pre-S/S gene.

HBx or x is the smallest gene which overlaps the C-terminal portion of the polymerase. x protein was found to be essential for efficient viral infection and replication (Chen et al., 1993; Zoulim and Seeger,1994; Zhang et al.,2001) and is considered a transactivator for cellular promoters with specific binding sites (Seeger et al., 2007).

1.6 HBV Genotypes and serotypes

HBsAg is divided into four major serotypes according to the antigenic determinants of HBsAg; *adw*, *ayw*, *adr* and *ayr* (Norder et al., 1994; Ken-ichi et al., 1995, Okamoto et al., 1988). The subtype determinant w can be divided further into subdeterminants w1 to w4 (Sandler et al., 1978). Furthermore, HBV is classified into eight or nine genotypes (A-I) according to the overall nucleotide sequence variation (Norder et al. 2004, Yu et al. 2010). The DNA variations in each case however, account for least 8% of the DNA sequence of the complete genome (Norder et al., 2004; Yu et al., 2010). The genotypes are further-more divided into different subgenotypes. Subgenotypes differ at least by 4%in their S gene nucleotide sequences from each other and are referred to with numbers (Ying et al., 2011).

Table 1.1: Overview of the 8 major genotypes of HBV (Locarnini, 2004)**.** The 8 major known genotypes are classified using alphabet A-H. The global distribution of these genotypes is given in the last column. Recently another genotype; I, was proposed (Yu et al., 2010). [a]PreS1=108 or 119 AA; BCP, basal core promoter mutations; ND, not described; common= up to 50% of isolates; uncommon=<10% of isolates; very common=most isolates.

			HBV Proteins			Frequency of Mutation		
Genotype	Subtype	Genome Length (nt)	PreS1[a]	Pol	Core	PC	BCP	Global Distribution
A	adw2, ayw1	3221	119	845	185	Uncommon (C1858)	Common	Western Europe, United States, Central Africa, India
B	adw2, ayw1	3215	119	843	183	Common (T1858)	Common	Japan, Taiwan, Indonesia, China, United States
Bj	adw2, aywl	3215	119	843	183	Common	Uncommon	Japan
Ba	adw2, aywl	3215	119	843	183	Low	Uncommon	China, Taiwan, Indonesia, Vietnam
C	adw2, adr, ayr	3215	119	843	183	Common T/C1858	Common	East Asia, Taiwan, Korea, China, United States, Japan, Polynesia
D	ayw2, ayw3	3182	108	832	183	Common T1858	Common	Mediterranean region, India, United States
E	ayw4	3212	118	842	183	ND	ND	West Africa
F	adw, ayw	3215	119	843	183	Uncommon (C1858)	ND	Central and South America, Polynesia
G	adw2	3248	108	842	195	Very common (insertion)	ND	United States, Europe
H	adw	3215	119	843	183	ND	ND	Central and South America

1.7 HBV laboratory diagnostics

HBsAg is measurable in the blood circulation before and during an acute HBV infection and in chronic infections (Figure 1.5). Once HBsAg has become undetectable, the patient will become immune but she or he may become an occult HBV carrier because the HBV viral DNA may still persist in the liver in the form of CCC-DNA and/or viral DNA integrated in the host genome (Seeger et al., 2007).

HBeAg is a serologic marker of active HBV infection, as it is produced shortly after infection initiation (Figure 1.5). In this period the HBV viral load is high ($\geq 10^6$). Once HBeAg becomes undetectable, anti-HBe antibodies will circulate in the blood, indicating the end of active viral replication and the beginning of clinical resolution of both transient and chronic infections. However, many chronically infected patients may be HBeAg negative and anti-HBe positive.

Anti-HBcIgM circulation in the blood is the first host immunologic sign of acute HBV infection and is detectable soon after HBeAg (Figure 1.5). Once the infection is resolved, anti-HBcIgM disappears while anti-HBcIgG is highly persistent, and can be measured in the blood circulation for decades after the clinical resolution of the infection (occult infection).

Antibodies directed against HBsAg (Anti-HBs) are used as a marker to detect the level of protection against HBV. Anti-HBs are detectable in the blood circulation of patients, who cleared the HBV infection (Figure 1.5). Furthermore, anti-HBs are also the marker used to detect the protection against HBV in vaccinated individuals.

The positive or negative detection of one or more of these markers indicates the level of HBV infection or protection against HBV.

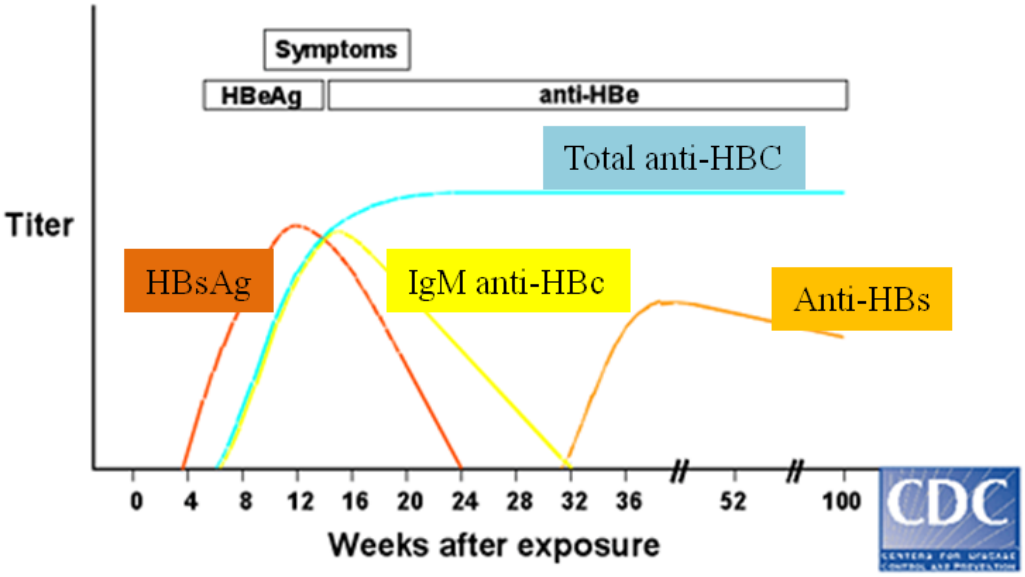


Figure 1.5: Markers of acute resolving HBV infection. Titer of serological markers used for HBV diagnostics are shown against time scale. HBsAg is the first detectable serologic marker upon HBV infection. HBeAg is produced during the acute phase of infection. Symptoms are clear during the phase of acute infection, which is also marked with the detection of IgM anti-HBc and anti-HBc. Anti HBc antibodies remain detectable during the late phase of infection. At this late phase, infected person can become immune due to natural infection, if it is accompanied by detectable levels of anti-HBs. CDC, USA

Beside the serological markers mentioned above, detection of HBV DNA is becoming common for accurate diagnosis. The most common method for DNA detection is based on Taqman detection of the x gene (see methods for Taqman, see above for x gene). Hereby, $>10^4$ genome copies/ml serum or plasma is considered critical for a possible risk of liver cirrhosis (Chu et al.,2002; Cacciola et al.,2000; Lindh et al., 2000). Typical HBV carriers with no detectable HBsAg (occult infection) have $\leq 10^3$ copies/ml and are considered of lower risk of liver cirrhosis (Seeger et al., 2007).

1.8 Vaccination against HBV

Engerix B produced by GlaxoSmithKline Biologicals, Rixensart, Belgiumis the HBV vaccine used worldwide and consists of the S protein of HBsAg. Engerix B contains

purified small HBs protein of the virus obtained by culturing genetically engineered Saccharomyces cerevisiae cells, which carry and express the S gene of HBV. The vaccine is administered by the intra-muscular route in the deltoid muscle and is given regularly given in two initial injections 1 month apart, followed by a booster at 6 months. Although it is generally accepted that vaccinees with an anti-HBs titer >10 IU/ml are considered immune, some European recommendations consider anti-HBs titers below 100IU/ml as a risk for infection (Gunson et al., 2003). 95% of healthy children or young adults produce >10 IU/ml, i. e. protective levels of anti-HBs, but 5% of people remain non-responders (Alter, 2003; Damme, 2001; Lavanchy, 2004).

The HBV vaccine is associated with rare side effects, most commonly pain or soreness at the injection site. Neurologic disorders such as multiple sclerosis, Guillain-Barré syndrome, and transverse myelitis have not been causally linked to the HBV vaccine (Ascherio et al., 2001; Confavreux et al., 2001).

Anti-HBs titers decline by time to undetectable levels, immunity against clinical disease persists for years, suggesting the existence of animmunologic memory (Alter, 2003; Damme, 2001; Lavanchy, 2004). International vaccination programs, which started in 1991, have led to a substantial decrease of HBV infection incidences worldwide (Seeger et al., 2007). However there are specific cases, which cannot be overcome by simple regular vaccination. Babies born to women, who are HBsAg positive, need to receive passive immunization (HBIG) before vaccination directly after birth with the first vaccine (Alter, 2003; van Damme, 2001; Lavanchy, 2004). Mother to child transmission occurs often, either in utero or through exposure to blood or blood contaminated fluids at or around birth. Such perinatal transmission is believed to account for 35% to 50% of hepatitis B carriers (Yao, 1996). The risk of perinatal transmission is associated with the HBeAg status of the mother. If a mother is positive for both HBsAg and HBeAg 70% to 90% of her children become chronically infected (Stevens et al, 1975; Akhter et al., 1992). If a mother is positive for the HBsAg but negative for HBeAg, the risk of transmission is significantly lower (Okada et al., 1976; Beasley et al., 1981; Aggarwal and Ranjan, 2004).

People, who are at higher risk of infection are suggested to booster or renew vaccination, i.e. health worker. Some studies suggested that health worker and people in high epidemic areas should keep anti-HBs level >100IU/ml (Seeger et al., 2007).

The recently introduced third-generation preS1/preS2/S vaccine (Sci-B-Vac™) was shown to produce a rapid onset of anti-HBs in healthy children and newborns (Madalinski et al., 2001; 2004). It was also shown to be more effective in the non-responder group. Sci-B-Vac is currently produced and approved in different countries; mainly in Asia (http://www.scigenltd.com).

1.9 HBV therapy

Treatment of HBV infection depends on different parameters (Cronberg et al., 2011):

1- Status of HBV markers (HBsAg, HBeAg, anti-HBc, anti-HBc-IgM)
2- HBV viral load
3- Liver function enzymes (ALT; Alanine Transaminase, AST; Aspartate transaminase)
4- Immune status of the infected patient
5- Presence of liver cirrhosis

An acute hepatitis B infection does not necessarily require therapy as 90-95% of acute HBV infection in adults resolves the infection and develop immunity (Yu et al., 2011). Children are at much higher risk for chronic infection. Up to 90% of infected young children will fail to clear the virus and go on to develop chronic infection. If the acute infection is extremely severe, fulminant hepatitis will develop. A chronic HBV infection does not resolve and may be life shortening at some stages. Chronic hepatitis B infection may require treatment because of the increasing risk of liver cirrhosis and hepatocellular carcinoma. These complications are dependent on the parameters mentioned above, but basically on the viral load in serum of the patients (Block et al. 2003, Cougot et al. 2005, Chen et al. 2006, Iloeje et al 2006).

1.9.1 Treatment with alpha interferon(IFN-α)

IFN-α is considered most suitable in HBeAg positive or negative patients with high ALT levels (Cronberg et al., 2008). HBV genotype is critical, as it was shown that genotypes A and B have a higher treatment success than genotype C and D (Perillo, 2009). IFN-α stimulates the immune system (T-cell) of the patient, inhibits viral maturation and has an anti-fibrotic effect, which controls the infection (Seeger et al. 2007).

IFN-α is prescribed subcutaneously three times weekly for at least 6 months (Seeger et al., 2007). A less frequent administration of therapy can be achieved with the recently introduced pegylatedIFN-α (Pegasys®) (Cronberg et al., 2008).HBeAgserconversion and decrease of viral titer occurs in about 40% of either IFN-α or pegylated IFN-α treated patients (Lau et al.,2005; Janssen et al., 2005; Perrillo, 2009).

Unfortunately, interferon may lead to serious side effects, which include flu like symptoms, depression, hair loss and leucopenia (Perillo, 2009; Seeger et al., 2007). Therefore, it is advised to monitor the therapy success by quantitation of HBsAg in patients selected for this therapy (Moucari, 2009; Brunetto 2009).

1.9.2 Treatment with nucleoside analogues

As mentioned above, nearly 60% of patients do not respond to IFN therapy and require treatment with reverse transcriptase inhibitors (nucleoside analogues), which are administered orally. Nucleoside analogues compete with the cellular nucleotide triphosphates dNTPs during HBV DNA replication via reverse transcription, which leads to interruption of viral replication activity (Ghany and Liang 2007).

Lamivudine (Zeffix®), Entecavir (Baraclude®) and Telbivudine (Sebivo®) are the currently known nucleoside analogues used to treat HBV infection. Lamivudine is a nucleoside analogue of cytidine in which the 3 'carbon atom of the ribose is replaced by a sulfur atom (Figure 1.6). Thus hydroxyl is no longer provided for the chain extension (Ghany and Liang 2007). It was originally developed for the treatment of HIV, but also shows activity against the reverse transcriptase of HBV.

Entecavir is a cyclopentane derivative (Figure 1.6) and nucleoside analogue of guanosine, originally developed for herpes treatment. The required dose is smaller by a factor of 100 than lamivudine (Ghany and Liang 2007). **Telbivudine (Sebivo®)** (β-L-2′-deoxythymidine) is an orally bioavailable L-nucleoside with potent and specific anti-HBV activity (Standring et al., 2001). Preclinical toxicologic experiments showed that telbivudine had no mutagenic or carcinogenic effects suggesting it may be appropriate for men and women in their reproductive years (Bridges, 2006) and for prenatal therapy of HBV infected gravids. Telbivudine was more efficient in reducing HBV viral load than lamivudine and evoked less resistance lamivudine (Lai et al., 2004; Lai et al., 2005).

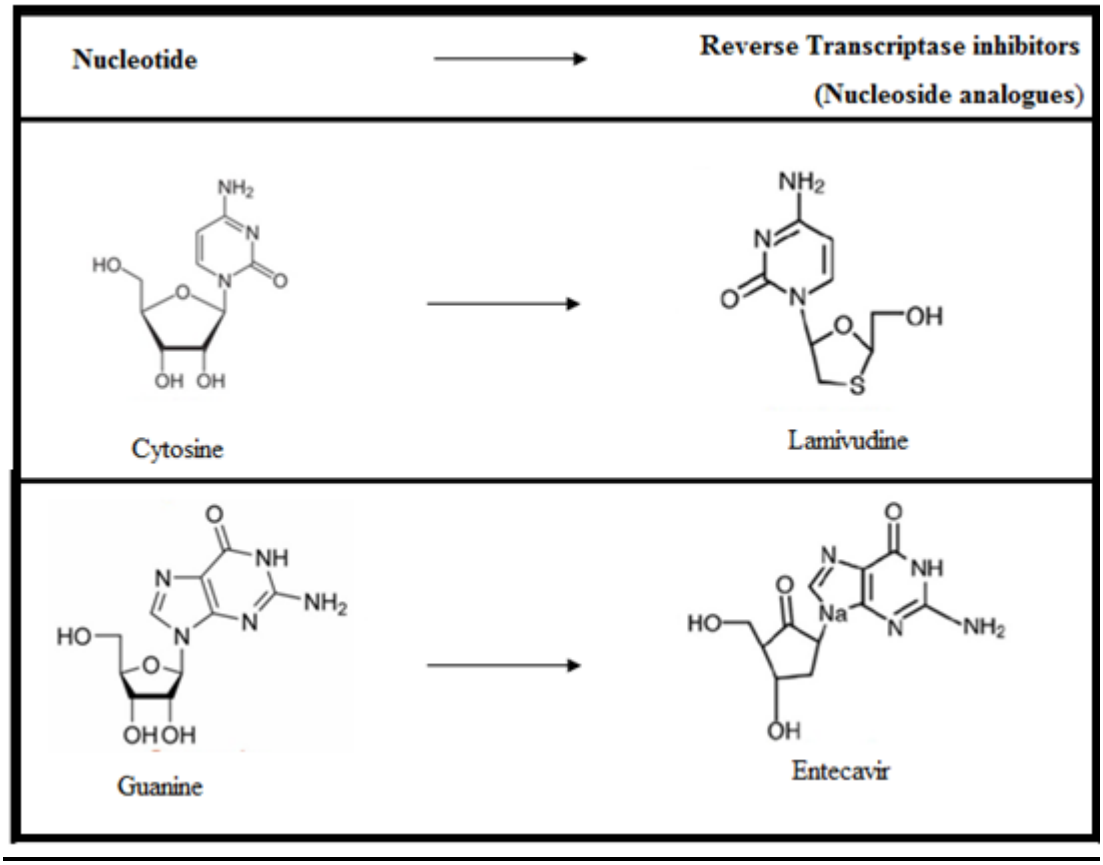


Figure 1.6: Chemical structure of clinically relevant nucleoside analogues. Shown are the respective naturally occurring nucleosides and modified analogues (modified from Ghany and Liang 2007).

1.9.3 Treatment with nucleotide analogues

Adefovir (Hepsera®), which also emerged from the HIV research, was an alternative to lamivudine and was recommended for lamivudine-resistant HBV variants. Adefovir became an approved treatment for HBV in the United States in September 2002 and in the European Union in March 2003.

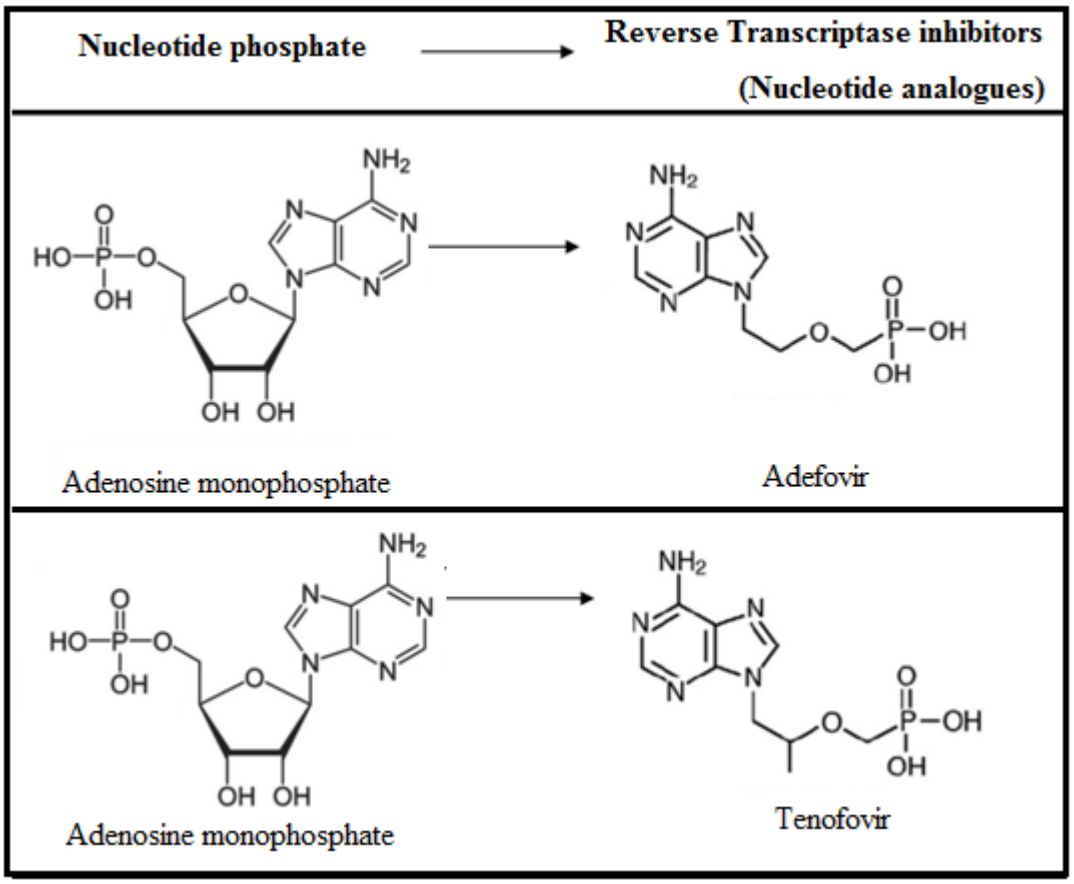


Figure 1.7: Chemical structures of clinically relevant nucleotide analogues. Shown are the naturally occurring nucleotides and the resulting modified analogues (modified from Ghany and Liang, 2007).

Adefovir is an acyclic phosphonate (Figure 1.7), which acts as a nucleotide analogue of adenosine (DeClercq et al. 2005). However, it turned out to be a sub-optimal treatment option, as it does not evolve the desired effect in all patients, beside the fact that it was shown to be nephrotoxic, even in small amounts. **Tenofovir** disoproxil (Viread®) is the last approved drug (2008) against HBV. It provides a slightly

modified form of adefovir with an additional methyl group (Figure 1.7), making it less nephrotoxic than adefovir (Reynaud et al. 2009).

1.10 Mutations in HBV genome

Viral reverse transcriptase (RT) is an error prone-enzyme, as it lacks a proof reading function producing HBV mixture of mutants and wild type. Therefore mutations can occur either naturally or may be evoked during antiviral therapy (Ghany and Liang, 2007). Interestingly, the frequency of HBV mutation is as high as $1.4\text{-}3.2 \times 10^{-5}$ nt substitutions per base per cycle (Okamoto, 1987; Girones and Miller 1989; Nowak et al., 1996; Locarnini, 2004; Seeger et al., 2007). Taking into consideration that the HBV genome is only ~3200bp, each single base can be subjected to change each day (Ghany and Liang, 2007). This mutation rate is 10 times higher than that known for other DNA viruses and is almost as high as that known for HIV and retroviruses (Gojobori et al., 1990, Locarnini, 2004).

1.10.1 Mutations in the Basal Core Promoter, Precore, and Core Genes

There are two major groups of mutations identified to be involved in reduction or block of HBeAg. The first includes a translational stop-codon mutation in the precore gene; precore stop-codon mutation (Lok et al., 1994). This precore stop-codon mutation occurs mainly in HBV genotypes B, D, E, and G and in some strains of genotype C, but rarely in genotypes A or F or in certain strains of HBV genotype C. The second group of mutations affects the basal core promoter, which results in a transcriptional reduction of precore and core mRNA, which is mainly found in genotype A–infected individuals (Hunt et al., 2000). HBeAg-negative status but active liver disease is the main parameters related to core gene mutations.

1.10.2 Mutations in the X Gene

Mutations in the x region may affect the basal core promoter and enhancer II regulatory elements which play a role in replication. X gene is affected in case of some specific core promoter mutation, due to reading frame overlap. Other mutations

in the basal core promoter may lead to production of shortened x proteins, which affect the transactivation activity of HBx antigen (Gunther et al., 1999).

1.10.3 Mutations in the S Gene and "vaccine escape mutants"

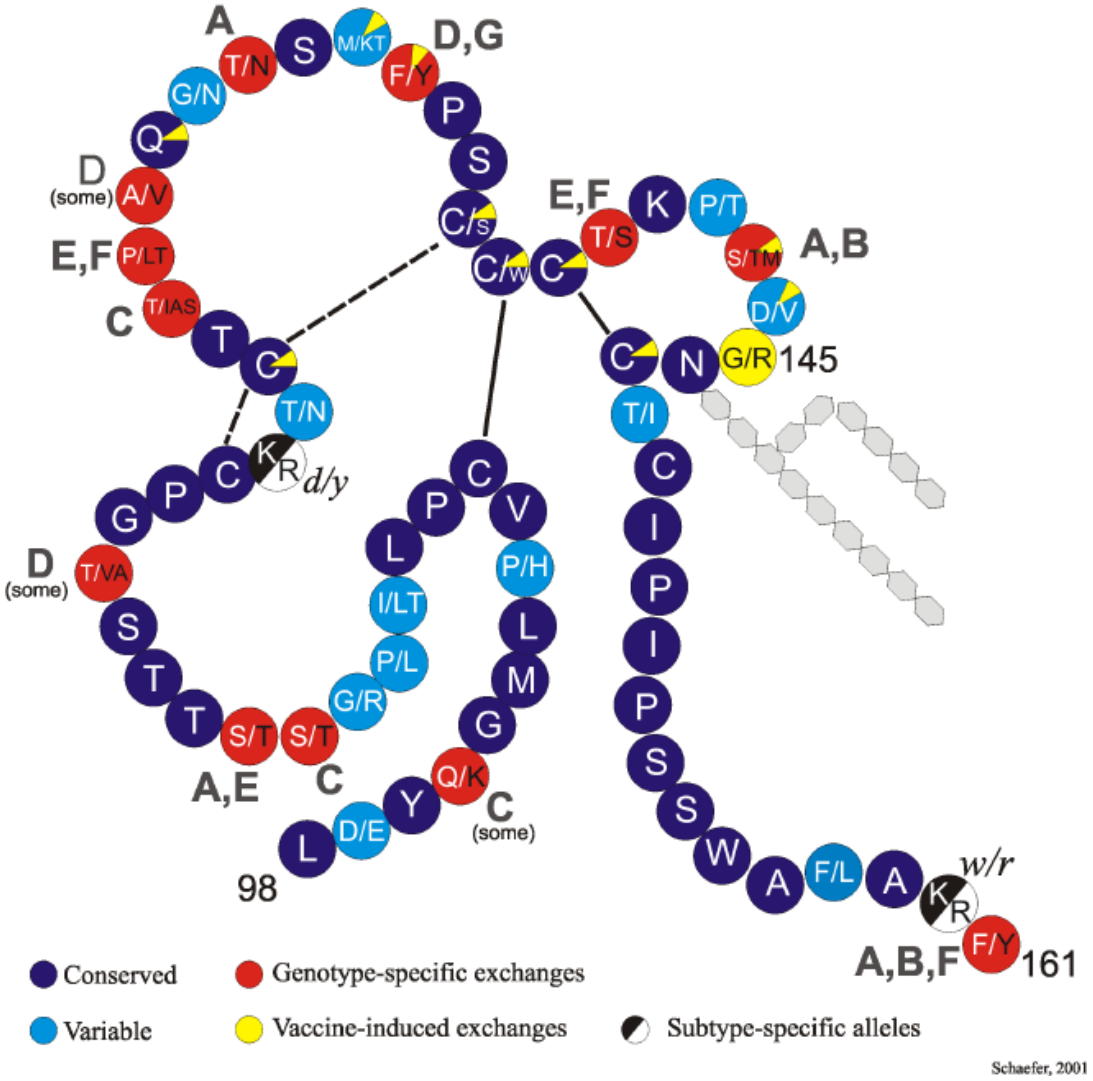
Genomic analysis studies of HBV genome from different patients showed that the Pre-S gene reveals the highest heterogeneity of all other genes. Heterogeneity is evoked by point mutations, deletions and by genetic recombination with pre-S genes of different HBV strains (Gunther et al., 1999; Locarnini, 2004).

Hepatitis B vaccines contain mainly the major or small HBsAg protein; they induce an immune response to the major hydrophilic region, located at amino acids 99 to170 (Locarnini, 2004). This anti-HBs response produces protective immunity. However, HBsAg may co-occur with the corresponding anti-HBs antibody in HBV carriers.

A common antigenic "a" determinant in the S-gene product is highly conserved and marked by one or two loops conformed by amino acids 124-147 (Figure1.8) (Brown et al., 1984; Guerrero et al., 1988; Howard et al., 1988). Interestingly, HBV vaccinees who seroconverted to anti-HBs, may evoke HBV mutants encoding HBsAg without this conformational antigenic determinant "a" (Carman et al., 1990; Hino et al., 1995; Karthigesu et al., I994; Okamoto et al., 1992). Such mutants can escape the host immune responses, and are therefore called "escape mutants". Such escape mutants were also shown to occur naturally even without such artificial immune pressure (Yamamoto et al., 1994).

Vaccinees, who develop chronic HBV infection, are presented with detectable levels of serum HBsAg, which lacks the group-specific "a" determinant (Seeger et al., 2007). The best known HBV escape mutant, associated with vaccine failure, occurs on amino acid 145, which changes glycine to arginine or lysine (SG145R) (Carman et al., 1990; Fujii et al., 1992; Harrison et al., 1991; Hino et al., 1995; McMahon et al., 1992; Okamoto et al., 1992; Yamamoto et al., 1994).Other escape mutations are Lys-141 to Glu-or Ile-141 (sK141E/I) (Karthigesu et al., 1994; McMahon et al., 1992) and Asp-I44 to Ala-144 (sD144A) (Harrison et al., 1994). Amino acid conversion

at position 126, from Ile or Thr in the wildtype to Asn or Ser has also been reported also (Okamoto et al., 1992; Yamamoto et al., 1994).



<u>Figure 1.8:</u> Scheme of Amino acids in the "a" determinant of the S gene of HBV. The "a" determinant loop (124-147) of the S gene is illustrated within the S gene using different colors to verify the status of amino acid; conserved, variable, escape mutants, genotype and subgenotype specific AA exchanges. Scheme was designed by Dr. Schaefer and kindly provided by Dr. Glebe, Institute of Medical Virology, School of Medicine, University of Giessen, Germany.

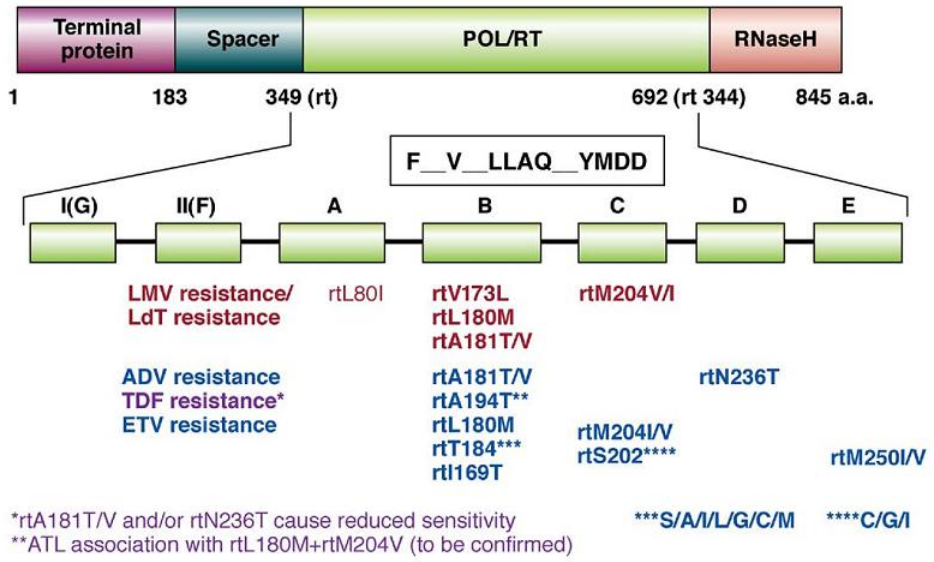
Anti-HBs produced upon vaccination does not necessarily guarantee a fully obstruction of HBV infection, but they do play a major role in slowing down viral

spread from infected to uninfected hepatocytes until the cellular immune response can destroy the infected cells (Seeger et al., 2007). This is why vaccine escape mutants are rare, specifically in healthy people. A study performed on chimpanzees revealed that immunization with the known recombinant HBV vaccine, despite challenge with G145R mutant, provides a true protection against escape mutants (Ogata et al., 1999). However, unvaccinated chimpanzees challenged with G145R escape mutant developed infection (Ogata et al., 1999).

Young children and immunocompromised people are at higher risk to develop escape mutants. Unfortunately, some diagnostic kits for HBsAg failed to detect escape HBsAg mutants (Coleman et al., 1999). 2% of the children of HBsAg-positive mothers, or with HBsAg-positive family contacts were shown to develop HBsAg positivity (Zanetti et al., 1988; Carman et al., 1990).

1.10.4 Antiviral drug resistance

An HBV mutation, which occurs during replication, may result in either synonymous (not associated with an amino acid change) or non-synonymous change (associated with an amino acid change). A drug resistance mutation is defined to be able to induce an amino acid change that decreases the susceptibility to an antiviral drug. The different HBV antiviral drugs induce different rates of drug resistance as well as different mutations in the polymerase (pol)/reverse transcriptase (RT) reading frame (Figure 1.9). These mutations occur on different domains of the pol/RT gene and are specific for each drug (Stuyver et al.,2001; Angus et al.,2003). Hereby primary drug resistant mutations are differentiated from secondary mutations. In the first case, amino acid substitutions lead to reduced susceptibility to an antiviral, while in the second case, amino acid substitutions restore functional defects in the viral polymerase activity (i.e., replication fitness). Secondary mutations follow the primary drug resistance.



<u>Figure 1.9:</u> Mutations in the polymerase/RT gene causing primary antiviral drug resistance. Summary of known polymerase gene mutations conferring resistance to nucleos(t)ide analogs. rtM204V/I mutations causing resistance to lamivudine (LMV) and telbivudine (LdT) are located in the YMDD (see above) motif within the C domain of the polymerase. rtM204V/I may be associated with compensatory mutations (rtL180M and/or rtV173L) in the B domain. rtA181V/T mutations in the B domain cause resistance to adefovir (ADV) and can also evoke decreased susceptibility to LMV and LdT. A combination of mutations in the B, C, or D domains evokes resistance to entecavir (ETV). Some mutations' associations are to be confirmed. From Zoulim and Locarnini (2009).

Lamivudine is still the drug used widely worldwide and is the only drug made available by the Palestinian Ministry of Health for HBV patients. Lamivudine evokes the highest resistance rate among HBV antivirals with a yearly rate of 14-32%, becoming 70% after four years of treatment (Lai et al., 2003). Mutations causing lamivudine resistance are located within the tyrosine-methionine-aspartate-aspartate (YMDD) motif of the viral pol/RT reading frame. M204V/I/S (methionine to valine, isoleucine or serine substitution) is a primary mutation caused by lamivudine on the YMDD motif, which results in decreasing lamivudine sensitivity more than 100-fold (Stuyveret al., 2001). Secondary or compensatory mutations can co-occur with the M204V/I and are located on the A or B domains of the pol/RT reading frame. L180M

(leucine to methionine substitution) is the most common mutation, V173L (valine to leucine substitution) and L80V/I and other mutations are also such compensatory mutations caused by lamivudine (Figure 1.9) (Ono et al., 2001; Stuyver et al., 2001).

Entecavir, the most effective antiviral against HBV evokes a very low mutation rate, undetectable after one year of treatment and less than 1% after 2 years of treatment (Colonno et al., 2006a and Colonno et al., 2006b). But this is the case only for naïve patients; this rate is much higher if entecavir is given after lamivudine treatment (Colonno et al., 2006a). The reason for this is that the lamivudine resistance mutations M204V/I and L180M are less sensitive for Entecavir (Ladner et al., 1997; Lampertico et al., 2005; Colonno et al., 2006a). Different mutations were found to be associated with entecavir resistance in two patterns: M250V+I169T+M204V+L180M and 184G+S202I+M204V+L180M (Ghany and Liang 2007). This means that entecavir resistance cannot be evoked by single mutations but rather a combination of mutations. For example in vitro studies showed that the occurrence of the single mutation rtM250V results in low resistance rate to entecavir, which increases >250 fold in the presence of the lamivudine rtM204V mutation (Tenney et al., 2004).

Telbivudine seems so far to cause only the M204I mutation (Ghany and Liang 2007).
Adefovir causes a lower rate of resistance compared to lamivudine with only 2% after 2 years and 29% at 5 years of treatment (Yang et al., 2002; Hadziyannis et al., 2006). The known mutations associated with adefovir resistance are rtN236T and rtA181V located in the B domain (Figure 1.9) (Angus et al., 2003; Borroto-Esodaet al., 2006). Similar to entecavir, adevovir resistance is also a result of combined mutations on different domains of the HBV polymerase (Bartholomeusz et al., 2004; Bartholomeusz et al., 2006). Interestingly, mutations rtN236T and A181V are associated with only a 5- to 10-fold decrease in sensitivity to adefovir in vitro assays (Angus et al., 2003; Villeneuve et al., 2003; Locarnini et al., 2004). While the rtN236T mutant remains sensitive to lamivudine, telbivudine, and entecavir (Brunelle et al., 2005; Angus et al., 2003), the rtA181T/V mutation is associated with reduced susceptibility to lamivudine, telbivudine, and entecavir, but is still sensitive to tenofovir (Villet et al., 2006; Qi et al., 2006).

Tenofovir is effective against the primary lamivudine-resistant virus (Van Bommel et al., 2006; Delaney et al., 2006). The rtA194T mutation was reported to confer resistance to tenofovir (Figure 1.9) (Delaney et al., 2006; Sheldon et al., 2006).

1.11 Research significance of this thesis

Genotyping has a wide range of aspects in identification of pathogens, prediction of outbreaks and drug resistance, besides the molecular epidemiology aspect of pathogens. Using recent exact methods of molecularbiology and bioinformatics, this current research established a solid profile of the HBV virus strains circulating among Palestinians. This profile provides two basic pieces of data regarding the HBV genome isolated from Palestinian patients; one is specific to the genotypes and subgenotypes of HBV, the other one is specific to the gene targeted by HBV antivirals; the HBV polymerase (Pol)/reverse transcriptase (RT).

No documented data or previous studies have been reported on HBV among patients in Palestine and to the best of our knowledge this study is the first to address this issue. Therefore, the main objective of this study was to genotype and sub-genotype HBV strains circulating among Palestinians and to predict drug resistance in these HBV isolates based on the DNA/amino acid sequences.

1.12 Research objectives

1- To identify the most prevalent HBV genotype and sub-genotype in Palestine
2- To detect the existence of drug resistance causing mutations in the Palestinian isolates.
3- To establish a genetic profile for HBV circulating among Palestinians.

2. Material and methods

2.1 Patients samples and confidentiality

Samples used in this study belonged to patients who were tested for HBV viral load at the Virology Laboratory, Medical Research Center (MRC), Al-Quds University, Jerusalem, Palestine, where this research took place. Samples were from Al-Makassed Islamic Charity Hospital, Jerusalem, Medicare Laboratories, Palestine or were sent directly to the Virology Laboratory by physicians. Patient's names' were substituted by codes. Age, sex and residency of patients were recorded after patients' oral or written approval. If the sample was archived like in the case of most samples from Al-Makassed, residency and age were taken from patient's medical file based on Al-Makassed Hospital approval (following standard ethics in research protocols).

2.2 Sample selection

The 200 samples used for viral load testing in this study were positive for HBsAg as shown in the test request for HBV DNA. Most of the samples came from Al-Makassed hospital, where HBsAg testing was performed on the AxSym machine (Abbott, Germany) using the original Abbott kit (HBsAgV2). The same test method is used at the Medicare laboratories, from where other samples are originated. This HBsAg test is qualitative but the strength of the signal is recorded by an index number which was revealed from the AxSym system. In some cases, an HBeAg test (HBe 2.0, AxSym, Abbott, Germany) had also been performed and its result was indicated in the HBV real time PCR test request. HBeAg was determined mainly in patients with symptomatic acute HBV infection. Therefore, HBeAg data was available only in 9 patients and all of them were HBeAg positive.

2.3 Sample storage

Samples received as serum samples were stored at -20 °C and delivered on ice to the Virology Laboratory. In few cases, blood samples were drawn from patients at the Virology Laboratory. Serum was separated and frozen until DNA extraction.

2.4 HBV DNA extraction

Samples with HBV viral load mainly above 1000 copies/ml were identified by real time PCR assays and subjected to genotyping and drug resistance analysis, HBV DNA was extracted from the serum using the QIAamp DSP DNA Blood Mini Kit (Qiagen, Hilden, Germany). The extraction steps were performed according to the manufacturer's instruction as follows:

1. 20 µl Qiagen Proteinase K were pipetted into the bottom of a 1.5 ml microcentrifuge tube.
2. 200 µl serum were added to the microcentrifuge tube. If the sample volume was less than 200 µl, PBS was added to adjust the amount.
3. 200 µl buffer AL were added to the sample, mixed by pulse-vortexing for 15s.
4. The mixture was incubated at 56ºC for 10 min followed by a short spin down.
5. 200 µl ethanol (96-100 %) were added to the sample, mixed by vortexing and briefly centrifuged to remove drops from the inside of the tube lid.
6. The mixture from step 5 was transferred carefully to the QIAamp Mini spin column (in a 2ml collection tube) without wetting the rim. The cap was closed and the column in the collection tube was centrifuged at 8000 rpm for 1 min. Thereafter, the QIAamp Mini spin column was placed in a clean 2ml collection tube, and the tube containing the filtrate discarded.
7. 500 µl Buffer AW1 were added to the QIAamp Mini spin column without wetting the rim and centrifuged at 8000 rpm for 1 min. The filtrate was discarded.
8. The QIAamp Mini spin column was replaced in a clean 2 ml collection tube, 500 µl Buffer AW2 were added and. Filtrate was discarded and centrifuging step was repeated.
9. Finally, the QIAamp Mini spin column was placed in a clean microcentrifuge tube, 200 µl elution buffer was added to the column and centrifuged at 14000 rpm for 3 min. The filtrate in this step contains the extracted DNA.

2.5 HBV DNA amplification

2.5.1 HBV viral load

Real-time PCR was performed using an ABI Real Time PCR 7500 system (Applied Biosystems, USA). All positive controls, negative controls and test samples were tested in duplicate. A total of 25-µl reaction mixture consisted of 5µl control (positive control; standard, negative control; ultra pure water) or sample DNA, 12.5 µl TaqMan universal master mix (Applied Biosystems), 1 µl of each primer (forward and reverse, each at 10 pmol/µl), 0.4 µl probe (100nmol/µl) and 5.1 µl ultra pure water. The amplification reaction started with 2 min at 50°C, followed by 10 min at 95°C and final 45 cycles as following: 95°C for 15s and 60°C for 1 min.

The primer pair (XF:5'-GAC GTC CTT TGT **Y**TA CGT CCC GTC- 3', XR: 5'- TGC AGA GGT GAA GCG AAG TGCACA- 3') and probe (FAM 5'- ACG GGG CGC ACC TCT CTT TAC GCG G-3' –MGBNFQ) were specific to the x gene of the HBV virus.

Validated complete genome HBV-DNA (Clonit, Italy) at 10^6 genome copies/µl was serially diluted in ultra pure water down to 10^1 copies/µl and utilized as standard in all RT-PCR assays. To rule out the possibility of carry over between samples, some samples were subjected to sequencing analysis. For this, the PCR product of six different HBV positive samples was purified employing the MinElute PCR purification Kit (cat. no. 28004, Qiagen, Germany). Sequencing was performed at the Heredity Laboratory of the Bethlehem University using the ABI PRISM 3130 Genetic Analyzer. The sequencing PCR reaction was performed with the forward primer in each reaction and the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, USA, cat no. 4337451-100).

2.5.2 Amplification of the S/RT gene region

We have received validated primer sequences from the Institute of Medical Virology, School of Medicine, University of Giessen, Germany, kindly provided by Dr. Dieter Glebe (see Table 2.1). However, during this work, we have noticed that some samples, despite viral load above 10^6 genome copies/ml did not reveal a high yield

and clear PCR product. Therefore, we designed another primer pair. For this, HBV sequences were downloaded into the Clone Manger Program and aligned. The primer pair was selected from the least variable regions of S gene and the overlapping polymerase RT gene. Basically, two new reverse primers in the RT gene region and as lightly extended forward primer in the S region were designed (see Table 2.1). These primer pairs covered additional 123 or159bp of the RT gene region. A plasmid (pcDNA3-1820, kindly provided by Dr. Glebe) containing the full length genome of HBV was used as positive control. The plasmid DNA received was eluted in dH2O, transformed into competent DH5α E. coli, selected for ampicillin resistance and finally purified using a Maxi prep kit (Qiagen). The plasmid DNA was titered using HBV viral load assay. 10^9 copies/ml were used in each reaction as positive DNA control.

The following GI numbers representing different HBV genotypes were use in the alignment and primer design: HBV genotype A (295367581), HBV genotype B (315539191), HBV genotype C (315539231, 315539196, 315539216), HBV genotype D (14887248, 315539176, 315539186, 315539226) and HBV genotype G (62006071).

Table 2.1: Primers used to amplify the reverse transcriptase (rt) domain of the polymerase gene and S gene of HBV. K= G or T, D=A, G or T according to IUPAC nucleotide code.

Primer (location on gene)	**Primer sequences**	**Source**
S6 sense (374-388)	5'-tggatgtgtctgcggc-3'	IMV-JLUG
S6 antisense (971-995)	5'-ckttgaacadactttccaatcaatag-3'	IMV-JLUG
S374 (374-390)	5'-tggatgtgtctgcggcgt-3'	Our design
P1154 (1136-1154)	5'-gcaacggggtaaaggttca-3'	Our design
P1117(1099-1117)	5'-gccttgtaagttggcgag-3'	Our design

2.5.2.1 PCR conditions

The calibrated PCR reaction of 20µl included 5 µl extracted DNA, 1.2µl of each primer (10 pmol/µl), 0.25µl polymerase enzyme (Advantage®2 polymerase mix, Cat.No.639201, Clonetech, CA, USA), 1µl dNTPs (40mM, Cat. No. U1511, Promega, WI, USA), 2 µl of the provided polymerase buffer and 9.35µl dH_2O. The reaction was carried out in a special PCR tube (0.2 ml Axygen Inc., USA) using Swift™ MaxPro Thermal Cyclers (ESCO Global, USA). The PCR reaction started with a single hot start step for 3min at 95ºC followed by 40cycles in the following order, 30 seconds at 95ºC (denaturation), 45 seconds at 58 ºC (annealing) and 1min at 68ºC (extension) respectively. An additional extension step was performed for another 5min at 68 ºC, to assure the complete extension of the amplified product. The reaction was then cooled down to 4ºC and either stored at -20ºC or used for analysis.

2.6 Detection of PCR product

To detect the amplified gene product of the PCR reactions, agarose gel electrophoresis was used to separate the PCR product of expected 620bp (S6 primer)/780 bp (S374+P1154). 1 % agarose (Amresco) gel was prepared in 1x TAE (10 x TAE = 400nM Tris-HCL, pH 8.3; 200mM Na-Acetat; 20 mM EDTA). The agarose was boiled until it was well dissolved, ethidium bromide or SYBER green (Invitrogen, Cat. No. S33102, safe DNA gel stain) was added when agarose suspension had cooled down to 40ºC, carefully mixed, poured into the agarose gel casting system (BioRad, UK or Cleaver**,** U.S.A) and a comb for the generation of sample pockets was inserted. 2 µl (10%) of each PCR product was filled into one gel pocket along with the DNA size control (2 µl from 100 bp marker (gene ruler express DNA ladder, Fermentas, Cat No. SM1558). After running the electrophoresis (100 mv for 30min) using a Bio Rad power supply, the migrated DNA bands in the agarose gel were visualized under UV light. A digital image of the gel was taken using a gel documentation system (Pharmacia Biotech).

2.7 PCR Purification

To purify the positive PCR product, we used either the Qiagen kit (Qiagen, see below) or the Antarctic Phosphatase kit (M0289L New England BioLabs, MA, USA). This step is essential to get rid of PCR components and concentrate the desired PCR product for sequencing purposes or further analysis.

Antarctic Purification kit was used as follows:

1- 1/10 volume of Antarctic phosphatase restriction buffer were added to 1-5µg of DNA.
2- 1 µl of Antarctic Phosphatase enzyme was added and mixed.
3- Mixture was incubated for 15 minutes at 37°C followed by further incubation for 5 minutes at 65°C.
4- 3 µl purified DNA was analyzed on a gel to verify the amount of eluted DNA. The result was photographed using the gel documentation system (Pharmacia Biotech). This photo reflects the DNA quality and quantity and was sent to the Bethlehem Geneticcenter along with the remaining 7 µl and the forward primer AdhexF2 for sequencing.

The MinElute PCR purification Kit (Cat. No. 28004, Qiagen) was employed to purify the desired PCR product in some cases, where Antarctic Phosphatase was not efficient.

1. 5 volumes of Buffer PB was added to 1 volume of the PCR reaction mix. In our case, 100 µl of buffer PB were added to 18 µl PCR reaction. The color of the mixture is expected to turn yellow; otherwise Sodium acetate should be added.

2. To bind DNA, the sample mixture (from step 1.) was applied to the MinElute column and centrifuged at 17,900 g for 1 min.

3. The flow-through was discarded and 350 µl Buffer PE (wash buffer) was added to the MinElute column and centrifuged for 1 min at maximum speed (17,900 g).

4. Flow-through was discarded and centrifugation step repeated.

5. For elution of the bound DNA, the MinElute column was placed in a clean 1.5 ml microcentrifuge tube. 10 µl Elution Buffer (10 mM TrisHCl, pH 8.5) or water was added to the center of the column's membrane. After 1 min standing at RT, the column/microcentrifuge tube was centrifuged for 1 min at 17,900 g.

6. 3 µl purified DNA was analyzed on a gel to verify the amount of eluted DNA. The result was photographed using the gel documentation system (Pharmacia Biotech). This photo reflects the DNA quality and quantity and was sent to the Bethlehem Heredity Center along with the remaining 7 µl and the forward primer AdhexF2 for sequencing.

2.8 Sequencing of PCR product

Purified PCR product obtained as explained in 2.7 was sequenced at the Heredity Laboratory of the Bethlehem University. Sequencing was performed using sequencer machine ABI PRISM 3130 Genetic Analyzer. The sequencing PCR reaction was performed once with the specific reverse/forward primer and BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, USA, cat no. 4337451-100).

2.9 HBV Sequence analysis

With the exception of one single case, sequences from each, forward and reverse primer was used in the sequence analysis of each PCR product belonging to each patient.

2.9.1 General overview of sequencing results

The S/RT gene sequences were readable using the Chromas lite program, Technelysium Ltd (www.technelysium.com.au/chromas.html). Using the NCBI blast database (http://blast.ncbi.nlm.nih.gov/Blast.cgi), sequences were compared with other known published HBV genotypes. The HBV genotypes with the highest maximal identification with the Palestinian samples' sequences were documented for

initial orientation of available Palestinian genotypes. The accurate sequence analysis was performed using the DNASTAR program, see below.

2.9.2 Correction of HBV Sequences

Although the received sequences were subjected to an overview sequence analysis using the NCBI blast machinery, each single nucleotide was verified for the detailed DNA analysis using the MegAlign of the DNASTAR gene analysis program (DNASTARInc.,Madison, WI, USA). For this, each forward and reverse sequence was aligned with the reference genotype they present. The program shows nucleotide differences in red. Each of this red colored nucleotide is double checked in the Chromas chromatogram. If the peak of the nucleotide is clear, no corrections performed and it means it is a true mutation. If the peak shows another nucleotide, the wrong nucleotide was replaced by the correct one, which in most cases is in accordance with the reference sequence. In other cases, some nucleotides were not in the sequence read out and it shows a gap in the MegAlign alignment. The chromatogram was checked, if these nucleotides were clearly represented by peaks, they were added. Generally, the first 30 nucleotide region at the 5' and sometimes some nucleotides at the 3' showed very bad readouts and/or mixed peaks in the chromatogram. These regions are trimmed, as they cannot be correctly identified.

2.9.3 HBV Reference sequences

In order to identify the HBV sequences revealed from Palestinian samples published complete S/RT gene sequences were used as a reference. For this, complete HBV genomes representing different genotypes were downloaded into the MegAlign using the MegAlign program searching machinery (Lasergene version 8, DNASTAR Inc., Madison, WI, USA). The complete genomes representing different D1, D3 and A2 subgenotypes were then trimmed to keep the S/RT region present. Each reference gene was divided into S (nt155-nt830) reference and RT (nt130-nt1162) reference genes based on the nucleotides presenting the regions. These reference sequences were saved under the name of the subgenotype they present to be loaded into the

MegAlign program for further analysis. The GI accession number and the country of each sequence were recorded, see below.

The following sequences were used as D1 references: 50982394 Turkey 2, 87295370 India, 196051019 China, 297595194 Egypt 2, 314912594 Sudan 1, 341830691 Turkey, 364505188 Syria 1, 375493437 Egypt 1. The following sequences were used as D3 references: 341830677 Turkey, 371941924 Haiti and Africa, 323367003 Serbia, 399923494 Caribbean Island, 324604922 Pakistan, 261292086 Canada). The following sequences were used as A2 references: 18389985 Korea, 1155012 Göttingen/Germany, 59802797 Atlanta, 261288752Poland, 375268579 Japan, 282921785 Argentina, 395992324 Caribbean Island of Martin, 387538382 Serbia.

2.9.4 Accurate sequence analysis of Palestinian human HBV

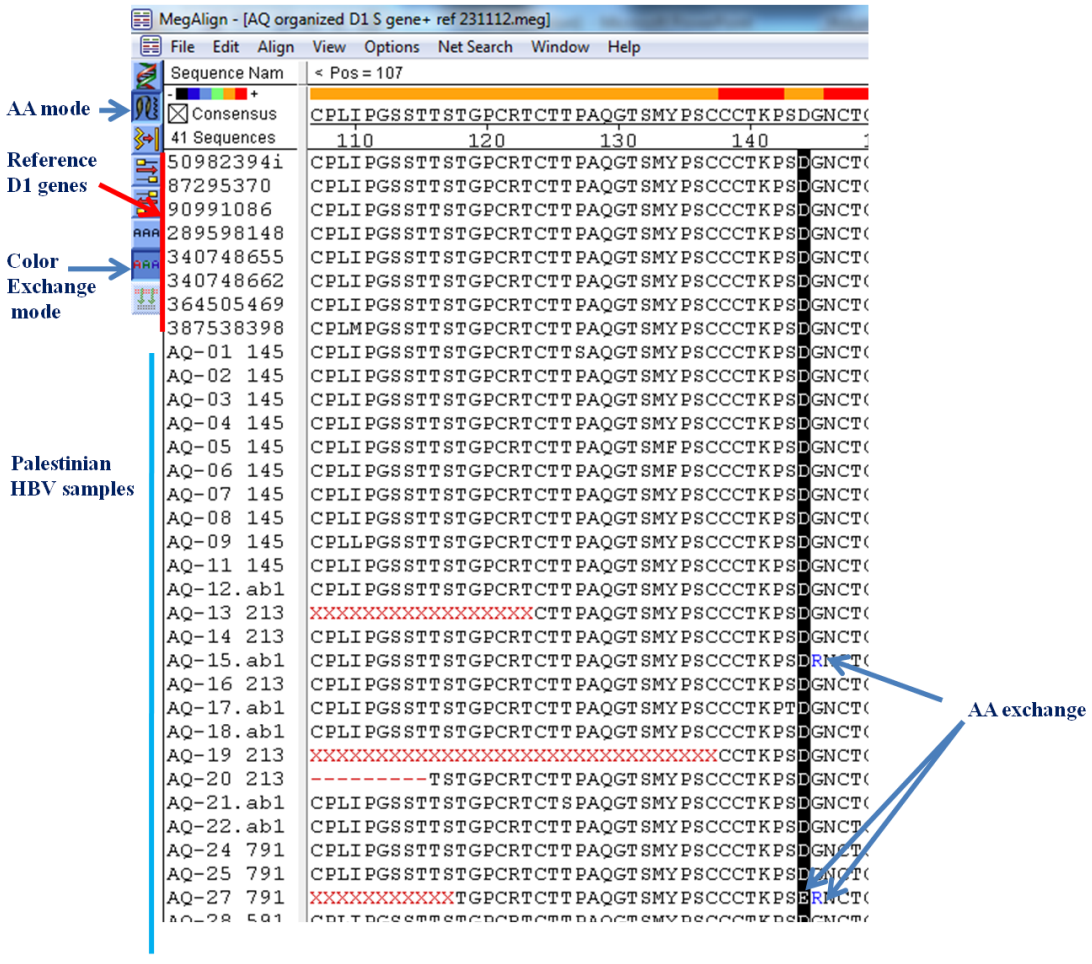
2.9.4.1 Sequence alignment

Using the DNAStar program, a phylogenetic tree showing the relevance of the different sequences to each other was established using the percent identity and divergence of the sequences calculated by the program. Finally, the confirmation of this analysis was ensured by adding the calculated bootstrapping value to the phylogenetic tree. All alignments were made using the Clustal W method and the following alignment conditions:

Pairwise alignment was always slow accurate with gap penalty 10; gap length 0, 10 and DNA weight matrix IUB. As for the multiple alignment gap penalty was also 10, gap length 0.20. These parameters were suggested by the program manager to fulfill the most ideal sequence alignment. Hereby the Clustal W method aligns sequences using the method of Thompson et al. (1994). Clustal W method was designed to create more accurate alignments than Clustal V when alignments include highly divergent sequences. Gap penalty is the amount deduced from the alignment score for each gap in the alignment. Gaps of the different sizes carry the same penalty, gap length penalty is the value deducted from the alignment score after first multiplying it by the length of gaps. Longer gaps have a greater penalty than shorter gaps.

2.9.4.2 Mutation analysis of Palestinian HBV samples

For accurate mutation analysis of Palestinian HBV samples, samples aligned with reference genes in MegAlign DNAStar program were eye inspected. The program does color the nucleotides in case of point mutations in the DNA mode precisely in red and can therefore be easily tracked. This is not exactly the case in the amino acid mode, therefore eye inspection was necessary. Once an amino acid exchange was inspected, the entire amino acid column was marked, of the reference and the samples (Figure 2.1).



<u>Figure 2.1:</u> Amino acid (AA) MegAlign example of the S gene of Palestinian samples representing D1 subgenotype. Palestinian samples (marked bright blue) are aligned with reference published S gene sequences from the region (marked red). AA exchanges are pointed to with blue arrows. The AA exchange examples given here is D144E and G145R.

Once the AA being checked is marked, the program was switched to the DNA mode to check the point mutation responsible for the amino acid exchange (Figure 2.2). The same region marked in the AA mode is marking now the DNA code. The mutation was identified as point mutation C to A causing the AA exchange from D (Aspartic acid) to E (Glutamic acid), please compare marked region in figure 2.1 to marked region in figure 2.2.

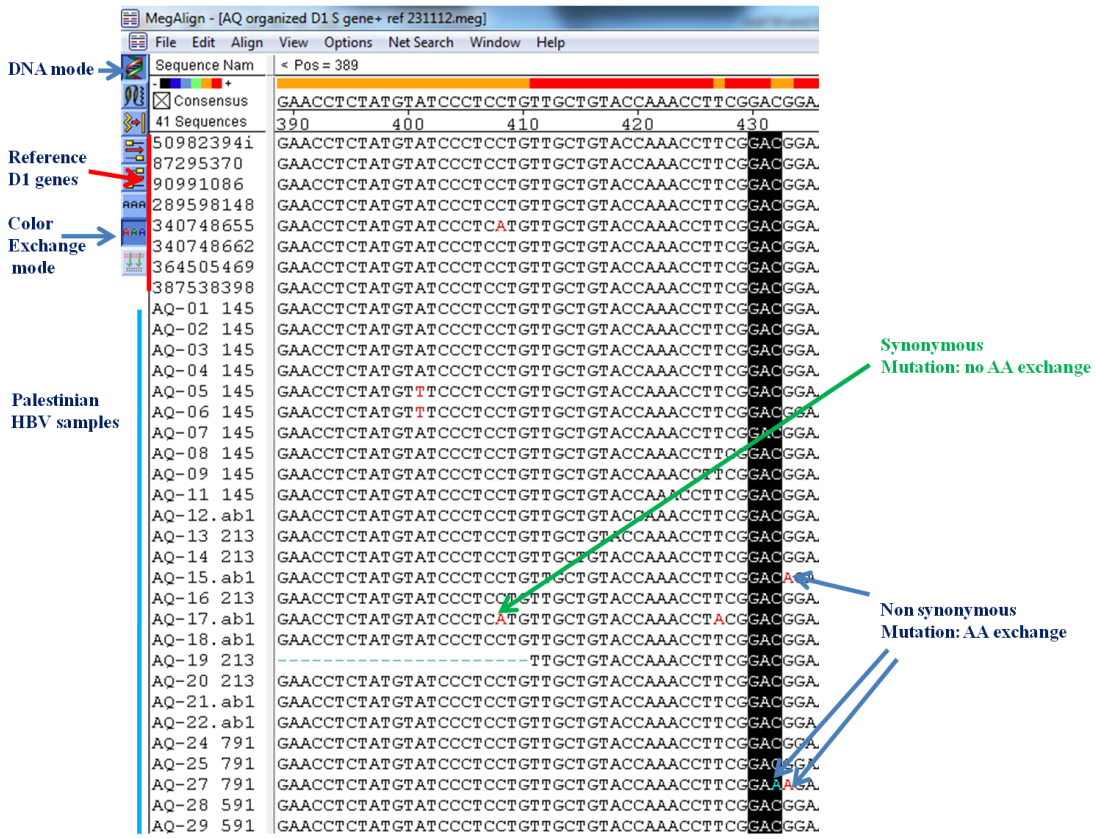


Figure 2.2: DNA nucleotide (nt) MegAlign example of the S gene of Palestinian samples representing D1 subgenotype. Palestinian samples (marked bright blue) are aligned with reference published S gene sequences from the region (marked red). Non-synonymous exchanges are pointed to with blue arrows (which cause the AA exchanges D144E and G145R). An example of synonymous mutation is pointed to with green arrow.

Furthermore synonymous mutations were also eye inspected in the DNA mode of MegAlign. Nucleotides are marked red if they do not match most nucleotides in the other genes at the same position (see figure 3.2, green arrow). In this case a point mutation exchanged the genetic code TCC with TCA, both encoding Serine.

2.9.5 Tracking and characterizing mutations in S and RT genes

Mutations identified using the MegAlign program was further characterized to find out whether they are functionally essential or not. For this, each mutation was basically blasted using Pubmed searching machinery and/or Google searching machinery using the mutation and HBV as searching words (i.e. D144E HBV). Once a publication had identified and characterized the mutation, the publication was marked as a reference. In case of the S gene, genotype specific, escape mutation and variable mutations were differentiated. In case of the RT gene region, the influence of the mutation on drug susceptibility was the main concern in this study. Beside accredited publications, some websites specialized in HBV mutations were also used either to confirm our work or to look for yet unpublished mutations, see below.

1- Stanford University HIV drug resistance database

http://hivdb.stanford.edu/HBV/HBVseq/development/HBVseq.html

2- Max Planck Institut für Informatik

http://hbv.bioinf.mpi-inf.mpg.de/index.php

3. RESULTS

3.1 HBV detection

200 HBsAg positivesamples (see 2.2) were subjected to real time PCR analysis of the HBV X gene (see 2.5.1). HBeAg status was known for 9 samples, which were tested positive for HBeAg. HBeAg was unknown for all other samples. HBV viral loadresults on all samples ranged between 0 and 10^{12} HBV genome copies/ml. Viral loads for HBeAg positive samples were between 10^{7} and 10^{12} HBV genome copies/ml respectively.

3.1.1 Relationship between HBsAg and HBV viral load

The index indicating the signal strength of the HBsAg test was known for 150 patients. Viral load was tested negative for 10% of the samples and was <100 genome copies/ml for 17% of the samples. 100 patients (50%) of the 200 samples had a viral load above 10^{5} HBV genome copies/ml and these were subjected to genotyping analysis. Of these, 54 samples showed a well-definedPCR product of the S/RT gene amplificationwith one sharp band in the gel electrophoresis. However, 14 samples showed unspecific PCR products besides the desired band. Although they were subjected to gel extraction of the correct PCR band, we were informed that the sequencing failed due to low DNA concentration. 40 samples showed clear specific PCR product and high quality sequences, which were good enough for the genetic analysis. Data of these 40 patients are summarized in table 3.1 below. Out of the 40 samples, AQ-31 was the only patient reported to have received lamivudine, the sample was taken few months after treatment. The patient received another course of treatment for 3 months. After that the HBV viral load dropped down to 10^{8}.

Table 3.1: Data of the 40 patients subjected to S/RT genetic analysis. Each patient was given a code, which substitutes the name. AQ refers to Al-Quds. Age, patient's residence; Gender, HBeAg and viral load are given. Bold/underlined codes refer to mother/child patients. HBeAg positive samples are marked red.

Patient	Age	Region	Gender	HBeAg	Viral load Copy/ml
AQ-01	67	S	Male	Unknown	10^9
AQ-02	34	M	Male	Unknown	10^{10}
AQ-03	32	M	Male	Unknown	10^8
AQ-04	28	N	Male	Unknown	10^8
AQ-05	68	N	Female	Unknown	10^8
AQ-06	40	M	Male	Unknown	10^7
AQ-07	49	S	Female	Unknown	10^6
AQ-08	22	M	Female	Positive	10^9
AQ-09	38	M	Male	Unknown	10^7
AQ-10	47	N	Male	Positive	10^7
AQ-11	30	M	Female	Unknown	10^8
AQ-12	68	M	Female	Unknown	10^7
AQ-13	47	M	Female	Unknown	10^6
AQ-14	64	S	Male	Unknown	10^6
AQ-15	36	M	Male	Unknown	10^6
AQ-16	40	M	Male	Unknown	10^{10}
AQ-17	35	N	Male	Unknown	10^6
AQ-18	27	N	Male	Unknown	10^7
AQ-19	67	N	Male	Unknown	10^6
AQ-20	36	M	Female	Unknown	10^6
AQ-21	80	M	Male	Unknown	10^6
AQ-22	44	N	Male	Unknown	10^{11}
AQ-23	49	S	Male	Unknown	10^8
AQ-24	23	N	Female	Unknown	10^{11}
AQ-25	8 months	N	Male	Unknown	10^{10}
AQ-26	40	M	Female	Unknown	10^5
AQ-27	42	M	Female	Positive	10^7
AQ-28	42	M	Female	Positive	10^9
AQ-29	25	M	Male	Positive	10^{12}
AQ-30	55	S	Male	Positive	10^9
AQ-31	50	S	Female	Positive	10^{10}
AQ-32	3	M	Female	Unknown	10^{11}
AQ-33	9	M	Female	Unknown	10^8
AQ-34	62	S	Male	Unknown	10^9
AQ-35	28	M	Female	Positive	10^7
AQ-36	34	S	Male	Positive	10^7
AQ-37	5	M	Male	Unknown	10^{10}
AQ-38	61	M	Male	Unknown	10^8
AQ-39	35	N	Female	Unknown	10^9
AQ-40	3y	N	Female	Unknown	10^9

3.2 Residency of patients subjected to genetic analysis

Patients subjected to genetic analysis were from all over the West Bank with 27.5% from southern Palestine, 45% from Jerusalem and Ramallah districts and 27.5% from northern Palestine (Figure 3.1). The patients were between 8 months and 80 years old, distributed between 57.5 % males to 43.5 % females.

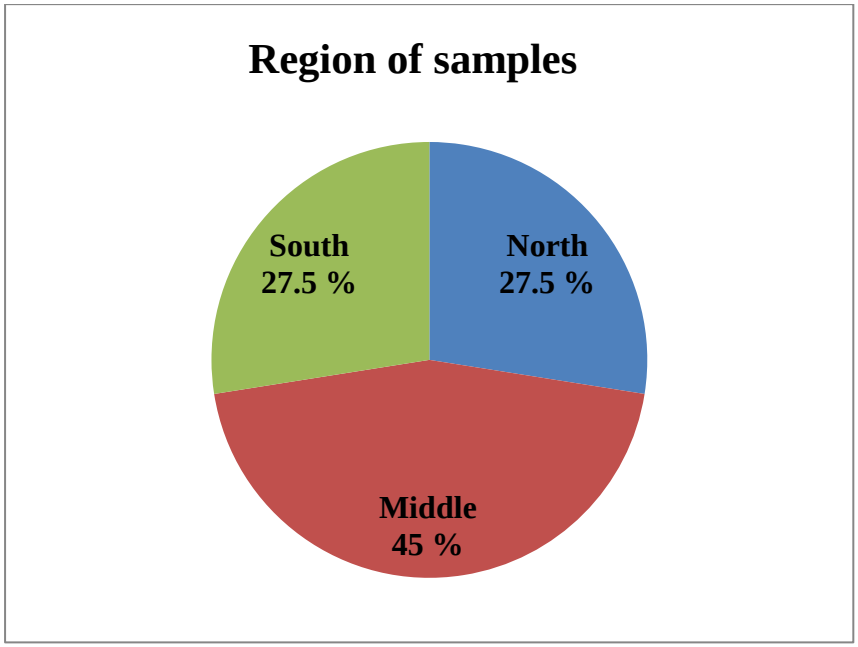


Figure 3.1: Residency of HBV patients subjected to genotyping analysis in this work. The three main districts of Palestine were divided into South (Hebron and Bethlehem districts), North (Nablus, Tulkarem, Qalqilya, Salfit, Jenin and Tubas districts) and Middle (Jerusalem and Ramallah districts). One sample from Gaza was included within the south section.

3.3 Amplification of S and RT gene

Due to the variability in the S region, the sequence of different genotypes was taken into consideration in primer design (see 2.5.2). The result of the primer and PCR condition calibration is shown in figure 3.2, samples amplification is shown in figure

3.3. Either mix A or mix B were used for the amplification, the nested PCR did not have any advantage on the PCR yield.

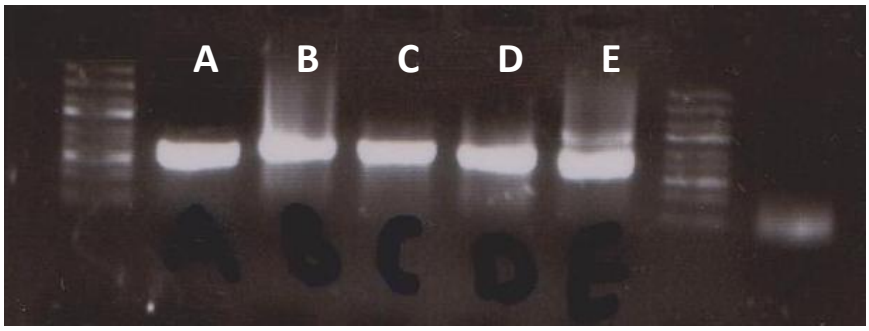


Figure 3.2: Calibration of PCR amplification of the S and RT genes. Mix A: (S6+S6 antisense), mix B (S374+P1154), mix C (S6 sense+P1195)+mix B, mix D (S6 sense+P1117)+mix B, mix E (mix A+ mix B).

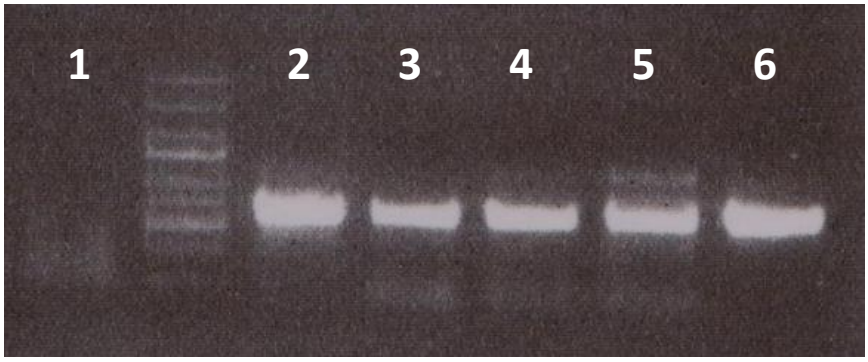


Figure 3.3: Amplification of S/RT gene using mix A. An example of S/RT amplification in HBV positive samples (viral load above 10^6). 1, dH_2O negative control, 2-5 samples from DNA of different patients, 6, positive control.

3.4 Sequence analysis of S gene

The sequences revealed for the S gene region were not equally in length for all Palestinian isolates, however all of them covered the "a" determinant of the S gene

(AA 124-146), while 82.5% covered the S gene loop (AA98-161). The length of S region covered by each sample is listed in table 3.2.

Table 3.2: The S gene region covered by each Palestinian sample. The nucleotide position on the HBV genome and the AA covered are shown. The subgenotype of each sample is listed:Red=D1, blue=D3 and violet=A2

Patient	S gene region (nt)	S gene region (aa)	Subgenotype
AQ-01	434-830	94-226	D1
AQ-02	416-830	88-226	D1
AQ-03	416-830	88-226	D1
AQ-04	416-830	88-226	D1
AQ-05	416-830	88-226	D1
AQ-06	410-830	86-226	D1
AQ-07	413-830	87-226	D1
AQ-08	416-830	88-226	D1
AQ-09	377-830	75-226	D1
AQ-10	413-830	87-226	D3
AQ-11	413-830	87-226	D1
AQ-12	374-830	74-226	D1
AQ-13	524-830	124-226	D1
AQ-14	458-830	100-226	D1
AQ-15	377-830	75-226	D1
AQ-16	374-830	74-226	D1
AQ-17	377-830	75-226	D1
AQ-18	473-821	107-223	D1
AQ-19	566-830	138-226	D1
AQ-20	500-734	116-195	D1
AQ-21	380-830	76-226	D1
AQ-22	383-830	77-226	D1
AQ-23	548-827	132-225	A2
AQ-24	431-830	93-226	D1
AQ-25	416-830	88-226	D1
AQ-26	380-830	76-225	A2
AQ-27	506-830	118-226	D1
AQ-28	416-830	88-226	D1
AQ-29	380-830	76-226	D1
AQ-30	437-830	95-226	D1
AQ-31	416-830	88-225	A2
AQ-32	398-830	82-226	D1
AQ-33	401-830	83-226	D1
AQ-34	377-830	75-226	D1
AQ-35	428-830	92-226	D1
AQ-36	425-830	91-226	D1
AQ-37	377-830	75-226	D1
AQ-38	374-830	74-226	D1

AQ-39	416-830	88-226	**D1**
AQ-40	416-830	88-226	**D1**

3.4.1 HBV genotypes

The genetic analysis of the 40 samples based on the S gene sequences revealed that 37 (92.5%) of the samples represented the D genotype, while only 7.5% belonged to A genotype (figure 3.3). Further genetic analysis of the D genotype revealed that 97% belonged to D1, while 3% (one sample) represented D3 subgenotype respectively (figure 3.4). The genotypes representing A belonged to subgenotype A2.

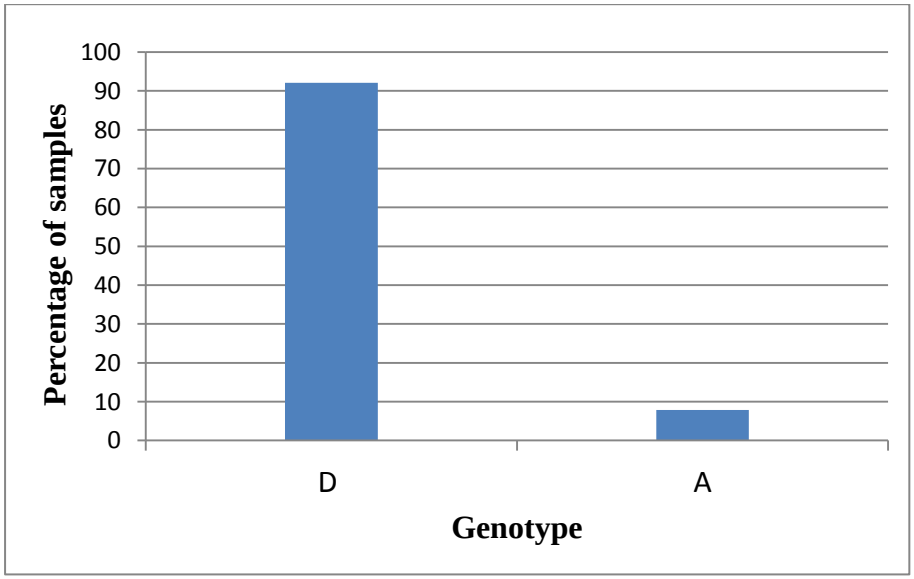


Figure 3.4; Distribution of HBV genotypes in Palestinian HBV patients and carriers. The total number of samples (40) was calculated as 100%, the percentage of each genotype was based according to the number of samples of each genotype.

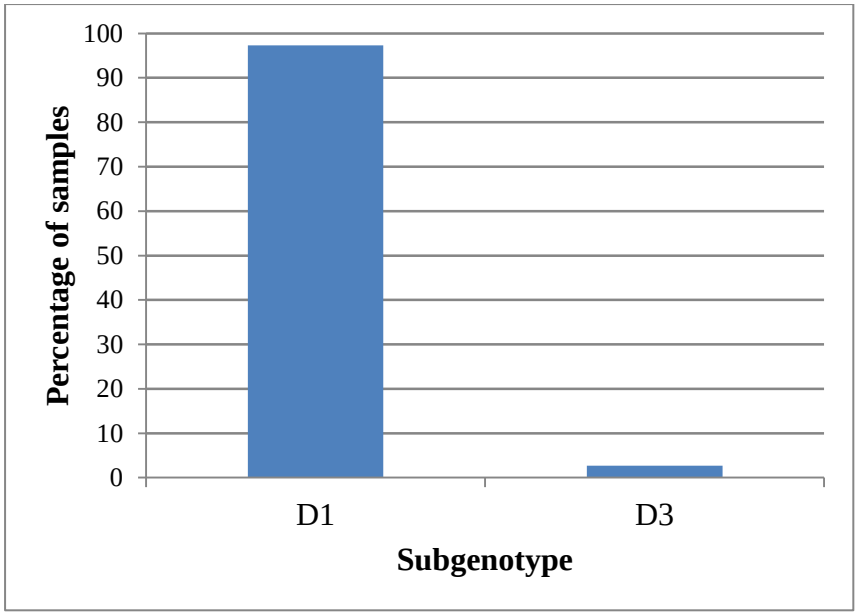
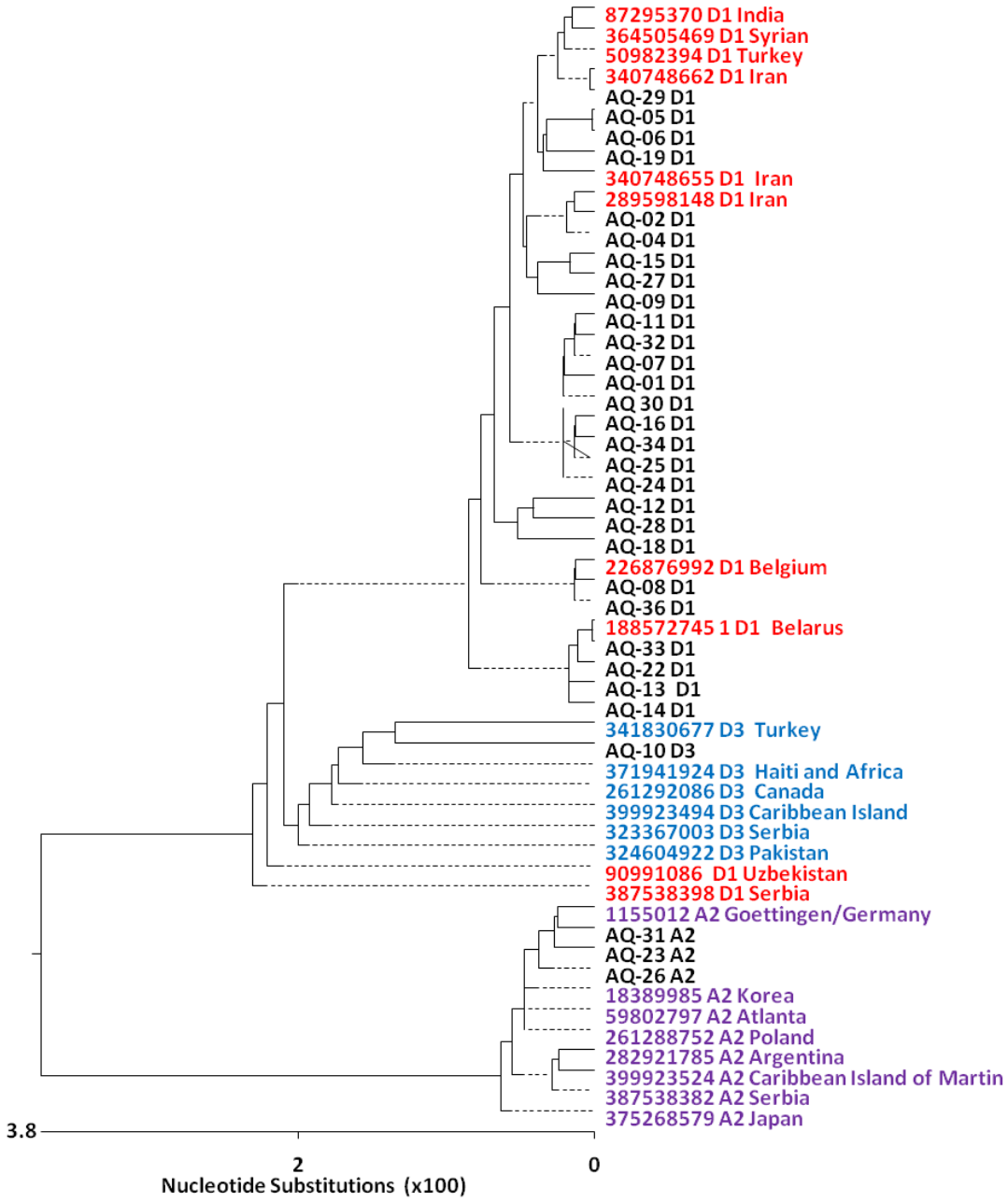


Figure 3.5; Distribution of D subgenotypes in Palestinian HBV patients and carrier. The total number of samples representing D genotype was calculated as 100%, the percentage of each subgenotypes was based according to the number of samples representing each subgenotype.

3.4.2 Phylogenetic trees

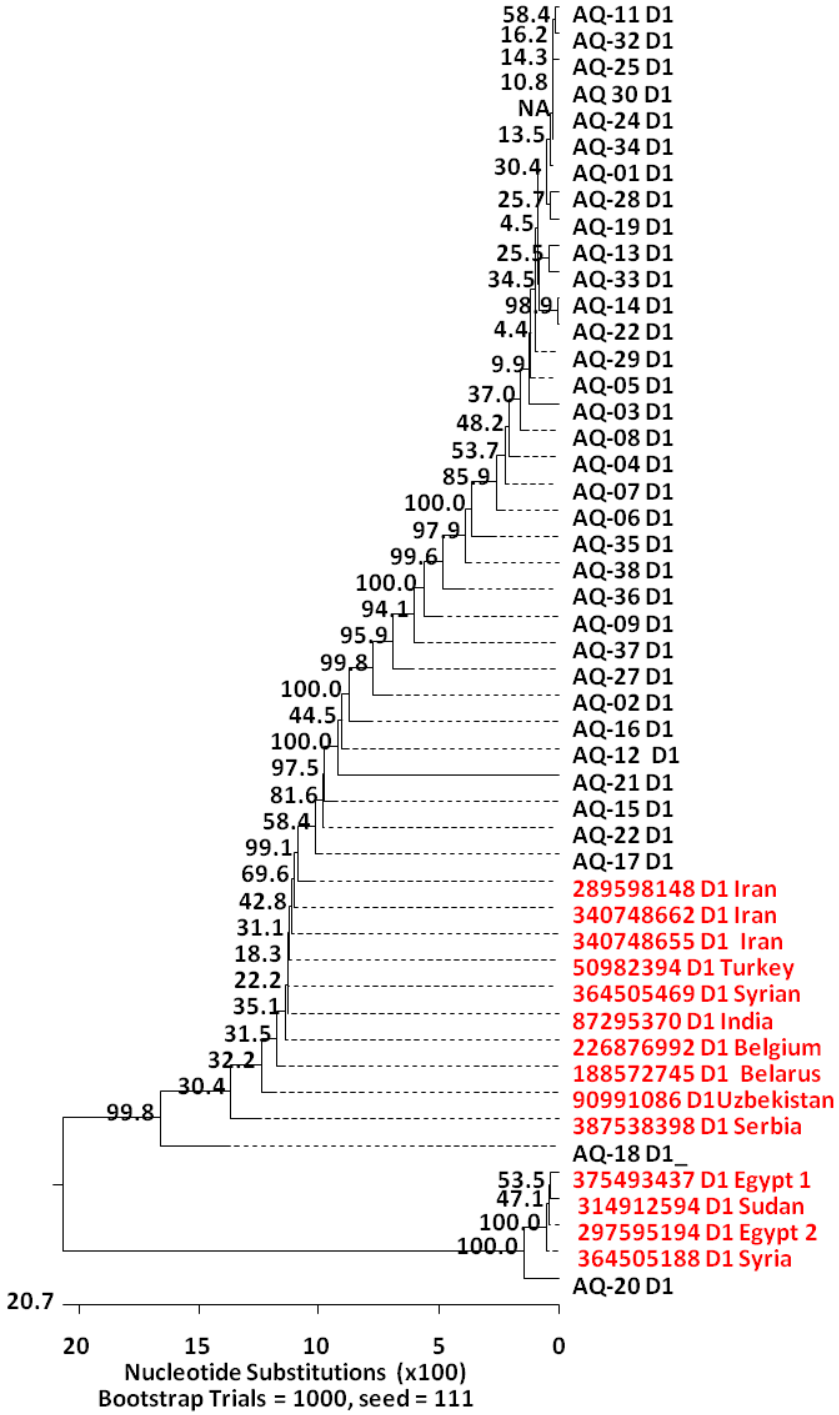
To verify the different genotypes and subgenotypes found among Palestinians, HBV subgenotypes from the region were downloaded into the DNAstar program. A general genetic tree is shown in figure 3.6. The published subgenotypes are represented in different colors for better clarification. D1 subgenotype is the most common in Palestinian HBV samples and clustered clearly among published regional D1 subgenoypes (Figure 3.6) from Iran, Turkey and Syria. The Palestinian D3 sample clustered nicely within the branches of the D3 references. The three Palestinian A2 samples clustered perfectly within the A2 references.



<u>Figure 3.6:</u> Phylogenetic tree of Palestinian subgenotypes. The tree represents the genetic distribution of Palestinian subgenotypes among regional and international subgenotypes. Red=D1, blue=D3 and violet=A2. The Phylogenetic tree was generated using the neighbor joining method (MegAlign-DNASTAR program). Reference genes are referred to with the GI accession number followed by subgenotype and the country, where the genotype was isolated. The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree indicate the number of substitution events.

3.4.3 Phylogenetic analysis of Palestinian D1 subgenotypes

To further elucidate the most common Palestinian subgenotype D1, another Phylogenetic analysis was performed on the S gene for D1 subgenotypes, compared to regional D1 subgenotypes. In this case bootstrap values were calculated and added to the genetic tree using the DNASTAR program. As clearly represented in figure 3.7, most of the Palestinian samples clustered together on one main branch, which also included the regional references from Iran, Turkey and Syria. One single Palestinian sample clustered on the second main branch with regional references from Egypt and Sudan.



<u>Figure 3.7:</u> Phylogenetic tree of Palestinian D1 HBV subgenotypes. The phylogenetic tree was generated using the neighbor-joining method (MegAlign-DNASTAR program). The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree indicate the number of substitution events used to generate the bootstrap value. Palestinian D1 Samples are in black. Reference genes are in red and referred to with the GI accession number, subgenotype and the country of origin.

3.4.4 Phylogenetic analysis of Palestinian D3 subgenotypes

There was one single Palestinian isolate representing the D3 subgenotype. As clearly represented in the Phylogenetic tree (figure 3.8), the AQ-10 is on one main branch, while all of the reference genes clustered together on the second main branch.

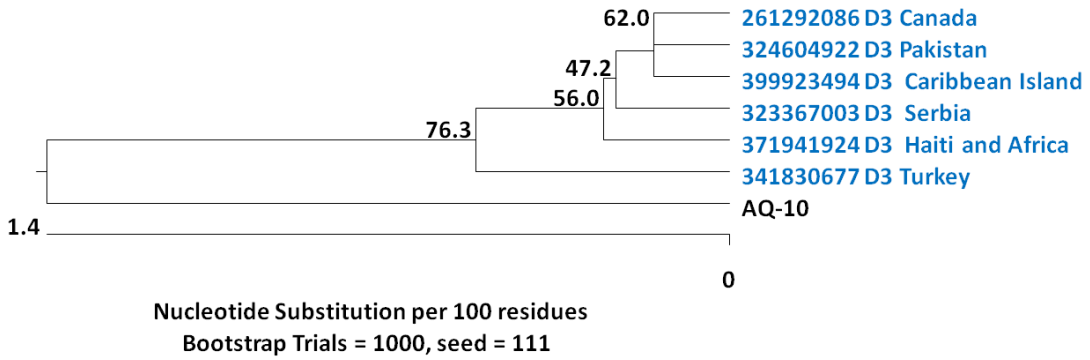


Figure 3.8: Phylogenetic tree of Palestinian D3 HBV subgenotypes. The phylogenetic tree was generated using the neighbor-joining method (MegAlign-DNASTAR program). The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree indicate the number of substitution events used to generate the bootstrap value. The Palestinian D3sample is in black. Reference genes are in blue and referred to with the GI accession number, subgenotype and the country of origin.

3.4.5 Phylogenetic analysis of Palestinian A2subgenotypes

There were three Palestinian samples representing the A2subgenotype. Figure 3.9 shows theses samples on one of the main branches, AQ-23 and AQ-26 clustered together on a sub-branch, while AQ-31 clustered on another sub-branch.

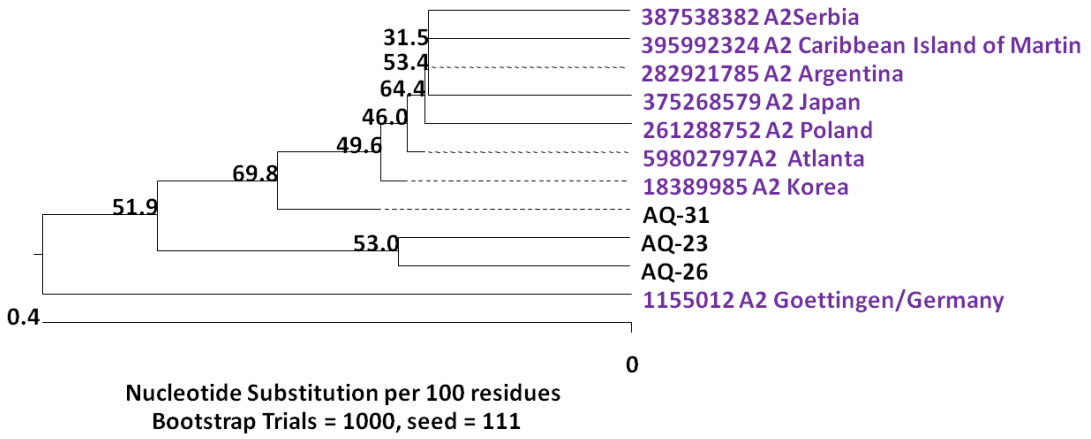


Figure 3.9: Phylogenetic tree of Palestinian A2 HBV subgenotypes. The phylogenetic tree was generated using the neighbor-joining method (MegAlign-DNASTAR program). The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree indicate the number of substitution events used to generate the bootstrap value. The Palestinian A2 Samples are in black. Reference genes are in purple and referred to with the GI accession number, subgenotype and the country of origin.

3.5 Mutation analysis of the S gene

The amino acid sequences and the DNA sequences of the S gene in each sample were subjected to a mutation analysis using published S gene of subgenotype D1 as a reference (see 2.9.3). The sizes of the S gene sequences for each Palestinian isolate revealed are listed in table 3.2. The S gene mutations detected in Palestinian D1 genotype are summarized in table 3.3. Subgenotype D1 has the HBsAgantigen subtype formula ayw2 whichcharacterized by the amino acids R122, P127 and K160. Hereby 17 mutations were found all over the 36 Palestinian D1 subgenotypes. Some mutations were localized in the "a" determinant, others downstream the S gene region.

Seven different synonymous mutations were found in the 36 D1 subgenotypes, three of which were referred to as polymorphism as they occurred in more than 10% of the samples, see table 3.4.

Table 3.3: Non-synonymous mutations in the S region of Palestinian D1 subgenotypes. The AA exchange is presented by position on the S gene; the nucleotide mutation causing the AA exchange is presented by the position on HBV genome reference gene (GI 87295370). This work was performed using the MegAlign DNASTAR program.

S Non-synonymous mutations(Nucleotide position) D1	Amino acid Mutation (AA position)	Occurrence in samples
410:A/T	I86F	1
429:T/C	I92T	1
482:A/C	I110L	1
484:T/G	I110L	1
531: C/G	T126S	1
533: C/T	T127S	1
555:A/T	Y134F	2
581:T/A	S143T	1
586: C/A	D144E	2
587: G/A	G145R	3
720:C/T	T189I	1
753:A/C	Y200F	1
765:G/A	S204R	1
771:A/T	Y206L	1
772:G/T	Y206L	1
774:G/A	S207N	2
784:T/A	S210R	1
791:T/A	L213I	3
791:T/A	L213F	1

Table 3.4: Synonymous mutations in the S region of Palestinian D1 subgenotypes. The position of nucleotide mutation is shown as well as the unaffected AA position on the S gene (nt position is based on HBV genome reference gene GI 87295370). This work was performed using the MegAlign DNASTAR program. Exchanges marked with (*) are considered polymorphisms due to their prevalence in >10 % of the patients.

Sgenesynonymousmutations (nucleotide position) D1	**Amino acid (AA position)**	**Occurrence in samples**
457:A/G	Q101Q	2
493:T/(A,C,G)	S113S	11*
499:T/(C,A)	T115T	7*
538:T/A	A123A	2
562:C/A	S136S	1
619:C/T	S155S	4*
784:T/C	S210S	1

The only Palestinian D3 sample, AQ-10 was presented by different mutations (see table 3.5). The only mutation in the "a" determinant of the S gene is T125M, which was reported earlier for genotype D (see discussion). All other mutations are outside the "a" determinant loop. Y200F, S204R and S207N are common between the only D3 Palestinian sample and few Palestinian D1 samples. Three synonymous mutations were found in the S sequence of the D3 sample, presented in table 3.6. Synonymous mutation S136S was common between the Palestinian D1 sample and the only D3 sample.

Table 3.5: Non-synonymous mutations in the S region of Palestinian D3 subgenotype. The AA exchange is presented by position on the S gene; the nucleotide mutation causing the AA exchange is presented by the position on HBV genome reference gene (GI 341830677). This work was performed using the MegAlign DNASTAR program.

S gene Non synonymous mutations (Nucleotide position) D3	Amino acid Mutation (AA position)	Occurrence in samples
528: C/T	T125M	1
753: A/T	Y200F	1
762: C/A	P203Q	1
766: T/A	S204R	1
770:T/A, 771: A/C	Y206T	1
774: G/A	S207N	1

Table 3.6: Synonymous mutations in the S region of Palestinian D3 subgenotype. The position of nucleotide mutation is shown as well as the unaffected AA position on the S gene (nt position is based on HBV genome reference gene GI 341830677). This work was performed using the MegAlign DNASTAR program.

SHBsynonymousmutations (nucleotide position) D3	Amino acid (AA position)	Occurrence in samples
532:T/C	T126T	1
562:C/A	S136S	1
616:A/G	S154S	1

Regarding the mutations found in the S gene of the 3 A2 Palestinian genotypes, L209V was the only amino acid exchange found, due to a T to G point mutation. Two synonymous mutations were found in one of the three samples at position L84L (406: C/T) and L94L (436: A/G). Nucleotide position was defined based on GI1155012, Göttingen/Germany reference A2 genotype.

3.6 Mutations in the RT gene

The sequences' lengths revealed for the RT gene regions are listed in table 3.7 (see below). Hereby the AA position in the RT gene and the nucleotide position within the HBV genome are given in the list.

The sequence of each sample was subjected to mutation analysis of the RT gene region. Hereby the RT gene region of each of the Palestinian subgenotypes was aligned with reference genes in MegAlignprogram. The analysis was made for D1, D3 and A2 separately using reference genes presenting each genotype (see 2.9.3). Non-synonymous and well as synonymous mutations were identified using MegAlign.

All mutations listed in the following tables were verified using Pubmed publications and the websites mentioned in 2.9.5 (see discussion). None of the mutations was known yet to be responsible for drug resistance.The most common RT mutation in Palestinian D1 subgenotypes was Y135S, followed by N248H, F122I and H124Y (see figure 3.10). A total of 23 mutations were found in the RT gene region of the Palestinian D1 isolates.

Table 3.7: The RT gene region covered by each Palestinian sample. The nucleotide position on the HBV genome and the AA covered are shown. The subgenotype of each sample is listed:Red=D1, blue=D3 and violet=A2

Patient	RT region (nt)	RT region (aa)	
AQ-01	435-991	103-288	**D1**
AQ-02	418-916	97-263	**D1**
AQ-03	415-991	96-288	**D1**
AQ-04	415-991	96-288	**D1**
AQ-05	418-991	97-288	**D1**
AQ-06	409-985	94-286	**D1**
AQ-07	415-985	96-286	**D1**
AQ-08	418-988	97-287	**D1**
AQ-09	376-952	83-275	**D1**
AQ-10	415-991	96-288	**D3**
AQ-11	412-991	95-288	**D1**
AQ-12	523-982	132-285	**D1**
AQ-13	523-994	132-289	**D1**
AQ-14	451-994	108-289	**D1**
AQ-15	490-991	121-288	**D1**
AQ-16	376-904	83-259	**D1**
AQ-17	376-847	83-240	**D1**
AQ-18	436-1090	103-321	**D1**
AQ-19	565-994	146-289	**D1**
AQ-20	502-733	125-202	**D1**
AQ-21	382-856	85-243	**D1**
AQ-22	430-847	101-240	**D1**
AQ-23	547-994	140-289	**A2**
AQ-24	430-994	101-289	**D1**
AQ-25	415-994	96-389	**D1**
AQ-26	379-949	84-274	**A2**
AQ-27	505-943	126-272	**D1**
AQ-28	418-1099	97-824	**D1**
AQ-29	379-1093	84-322	**D1**
AQ-30	436-1090	103-321	**D1**
AQ-31	415-994	96-289	**A2**
AQ-32	400-1069	91-308	**D1**
AQ-33	403-1051	92-308	**D1**
AQ-34	376-1114	83-329	**D1**
AQ-35	427-970	100-281	**D1**
AQ-36	424-961	99-278	**D1**
AQ-37	376-943	83-272	**D1**
AQ-38	376-970	83-281	**D1**
AQ-39	415-934	96-269	**D1**
AQ-40	415-934	96-269	**D1**

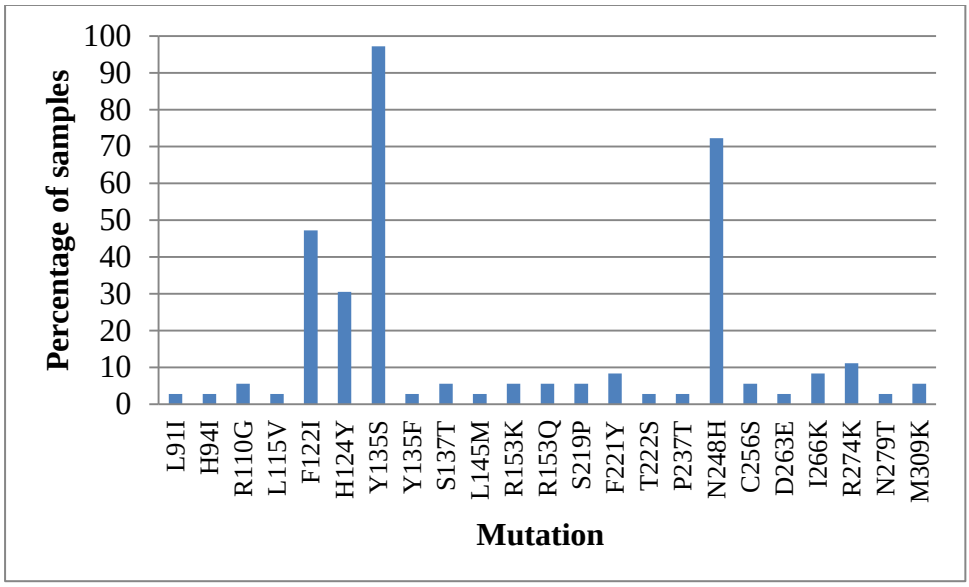


Figure 3.10: Percentage of mutation occurring in the RT gene of D1 genotypes. The percentage of mutations among D1 subgenotypes was calculated using the total number of mutations as 100%. The frequency of occurrence of each mutation in the total D1 subgenotypes is presented in table 3.7 respectively.

The non-synonymous RT gene mutations in the Palestinian samples are listed in table 3.8, along with the nucleotide positions on the HBV genome. Most mutations were caused by single nucleotide substitutions. However, F122I mutation was caused by three different nucleotide substitutions at position 493. Another two mutations; H124Y and S219P were caused by two different nucleotide substitutions at positions 499 and 784 respectively.

Eleven synonymous gene mutations occurred in the RT gene region of the Palestinian D1 samples, presented in table 3.9. L169L was the most common synonymous mutation, occurring in four samples. Mutation V286V was found in three samples, while V142V, Q215Q, V253V and L260L (position 907) were found in two different samples each. L260L was actually found in 2 samples due to nucleotide substitution on position 907 and in another single sample, due to nucleotide substitution on position 909 respectively.

Table 3.8: Non-synonymous mutations in the RT region of Palestinian D1 subgenotypes. The AA exchange is presented by position on the RT gene; the nucleotide mutation causing the AA exchange is presented by the position on HBV genome reference gene (GI 87295370). This work was performed using the MegAlign DNASTAR program.

RT Non-synonymous mutations (Nucleotide position) D1	Amino acid Mutation (AA position)	Occurrence in samples
400:C/A	L91I	1
410:A/T	H94I	1
457:A/G	R110G	2
472:T/G	L115V	1
493:T/(A,C,G)	F122I	15
499:T/(C,A)	H124Y	9
533:A/C	Y135S	35
533:A/T	Y135F	1
538:T/A	S137T	2
562:C/A	L145M	1
586:C/A	R153K	2
587:G/A	R153Q	2
784:T/(C,A)	S219P	2
791:T/A	F221Y	3
793:A/T	T222S	1
823:C/A	P237T	1
871:A/C	N248H	24
895:T/A	C256S	2
918:T/A	D263E	1
926:T/(G,A)	I266K	3
950:G/A	R274K	4
965:A/C	N279T	1
1055:T/A	M309K	2

Table 3.9: Synonymous mutations in the RT region of Palestinian D1 subgenotypes. The position of nucleotide mutation is shown as well as the unaffected AA position on the RT gene. The nucleotide is presented by the position on HBV genome reference gene (GI 87295370). This work was performed using the MegAlign DNASTAR program. Exchanges marked with (*) are considered polymorphisms due to their prevalence in >10 % of the patients.

RT synonymous mutations (nucleotide position) D1	Amino acid (AA position)	Occurrence in samples
555:A/T	V142V	2
619:C/T	L168L	4*
720:C/T	H117H	1
774:G/A	Q215Q	2
853:A/C	R242R	1
888:C/A	V253V	2
906:A/C	S259S	1
907:T/(A,C)	L260L	2
909:G/A	L260L	1
969:G/A	R280R	1
987:C/(G,T,A)	V286V	3

Seven non-synonymous mutations were found in the RT region of the only Palestinian D3 isolate (table 3.10). Hereby L145M, C256S and I266K were common with D1 mutations in the RT region. Six synonymous mutations were found in the RT region of the only Palestinian D3 isolate (table 3.11). Synonymous mutations Q215 and R280 were common between D3 and D1.

Table 3.10: Non-synonymous mutations in the RT region of Palestinian D3 subgenotypes. The AA exchange is presented by position on the RT gene; the nucleotide mutation causing the AA exchange is presented by the position on HBV genome reference gene (GI 341830677). This work was performed using the MegAlign DNASTAR program.

RT Non-synonymous mutations (Nucleotide position) D3	Amino acid Mutation (AA position)	Occurrence in samples
532:T/C	Y135H	1
562:C/A	L145M	1
616:A/G	I163V	1
766:T/A	S213T	1
770:T/A	V214D	1
895:T/A	C256S	1
926:T/A	I266K	1

Table 3.11: Synonymous mutations in the RT region of Palestinian D1 subgenotypes. The position of nucleotide mutation is shown as well as the unaffected AA position on the RT gene. The nucleotide is presented by the position on HBV genome reference gene (GI 87295370). This work was performed using the MegAlign DNASTAR program.

RT synonymous mutations (nucleotide position) D3	Amino acid (AA position)	Occurrence in samples
528:C/T	H134H	1
753:A/T	V208V	1
762:C/A	A211A	1
774:G/A	Q215Q	1
852:G/A	K241K	1
969:G/A	R280R	1

Out of the five non-synonymous mutations detected in the RT gene region of the three Palestinian A2 samples;I253V and L217 were present in all, see table 3.12. Non-synonymous R274K was the only common mutation between Palestinian A2 and D1 subgenotypes.

Table 3.12: Non-synonymous mutations in the RT region of Palestinian A2 subgenotypes. The AA exchange is presented by position on the RT gene; the nucleotide mutation causing the AA exchange is presented by the position on HBV genome reference gene (GI 1155012). This work was performed using the MegAlign DNASTAR program.

RT Non-synonymous mutations (Nucleotide position) A2	**Amino acid Mutation (AA position)**	**Occurrence in samples**
406:C/T	L93F	1
436:A/G	I103V	1
779:G/T	L217R	3
886:A/G	I253V	3
950:G/A	R274K	1
952:A/G	K275E	1

Five synonymous mutations were found in the Palestinian A2 samples as presented in table 3.13. Y252Y, G258 and K268K were present in all A2 samples. V268V was the only common synonymous mutation between Palestinian A2 and D1 subgenotypes respectively.

Table 3.13: Synonymous mutations in the RT region of Palestinian A2 subgenotypes. The position of nucleotide mutation is shown as well as the unaffected AA position on the RT gene. The nucleotide is presented by the position on HBV genome reference gene (GI 1155012). This work was performed using the MegAlign DNASTAR program.

RT synonymous mutations (nucleotide position) A2	Amino acid (AA position)	Occurrence in samples
885:C/T	Y252Y	3
903:A/G	G258G	3
933:G/A	K268K	3
987:A/C	V286V	2
994:A/C	R289R	2

4. DISCUSSION

HBV is the leading cause of chronic liver disease and currently infects an estimated 240 million people worldwide (Ott et al., 2012). There are no published data regarding the prevalence of active (i. e. acute or more probably chronic) HBV infection in Palestine. Therefore, our institute performed a statistical analysis on HBsAg tests results from the referral hospital in the West Bank (including East Jerusalem) and Gaza Al-Makassed Islamic charity hospital. According to the hospital's policy, all admitted patients are subjected to HBsAg testing. Data for statistical analysis was kindly provided by Mr. Sabri Baraghithi, director of the General Laboratory, Al-Makassed Charity Hospital, Jerusalem. Hereby, 738 patients (2.02 %) out of 36,512 tested between 2007 and April of 2012 were positive for HBsAg. This cohort may not be representative for the entire Palestinian population but gives a first hint on the relatively high prevalence of active HBV infection in Palestine

Genotyping of pathogens circulating in a region is the first step in their molecular characterization. Studies had shown that genotypes of HBV are specific to geographic areas or ethnical groups. The research presented here analyzed two overlapping gene regions of HBV, thereby addressing both the genotype and the drug susceptibility.

4.1 Correlation between HBsAg and HBV DNA

All of the 200 samples subjected to HBV DNA detection were tested positive for HBsAg using qualitative methods. Nevertheless 10% of these samples were tested negative for HBV DNA. In these cases the sensitivity of our HBV DNA assay was probably insufficient. Kuhns et al. (2004) reported that 36 % of HBsAg positive US blood donors had <400 copies HBV DNA/ml and 3% were negative even with the most sensitive PCR assay at a very low detection limit of 1.3 copies/ml.

The index value of the HBsAg immune assay known for 150 of the samples did not correlate positively with the number of the genome copies of HBV DNA revealed. These results were not surprising as the qualitative HBsAg assay reaches its upper range of signals at very low HBsAg concentrations around 100 IU/ml whereas HBsAg carriers have usually >1000 IU/ml (Jaroszewicz et al., 2010; Brunetto et al., 2010). Thus, a quantitative assay of HBsAg levels would require dilution of the samples which was not done here. Furthermore, the correlation between HBsAg and HBV genome copy number is still a subject of debate. Although some assays for quantitative HBsAg detection were shown to correlate well with HBV DNA levels (Werle-Lapostolle et al., 2004; Deguchi et al., 2004; Chen et al., 2004; Chan et a., 2007; Ganji et al., 2011), other studies found this correlation to be weak or absent (Kuhns et al., 2004; Manesis et al., 2010; Thompson et al., 2010; Tuaillon et al., 2012; Park et al., 2012). These discrepancies among studies may be explained by the fact that the correlation between HBsAg and HBV DNA levels is dependent on the phase of the infection (Brunetto et al., 2009; Nguyen et al., 2010; Jaroszewicz et al., 2010; Brunetto et al., 2010). The highest correlation between HBsAg levels and HBV DNA was found in early phases of infection (Van Bommel et al., 2004, Van Bommel et al., 2010; Thompson et al., 2010). This positive correlation disappears in later phases of

infection as the level of viral DNA replication becomes low despite persistence of HBsAg (Janssen et al., 1994; Fan et al., 2001; Brunetto et al., 2009). In contrary to HBsAg, a positive correlation was found between HBeAgpositivity and HBV DNA levels (Van Bommel et al., 2004, Van Bommel et al., 2010; Manesis et al., 2011; Thompson et al., 2010). Our results showed a high HBV viral load in the nine HBeAg positive samples, consistent with these previous studies.

4.2 Amplification of the S/RT gene region

Samples subjected for HBV viral load assay used in this work, were sent to the Virology Laboratory for diagnostic reasons mainly. Initially the PCR amplification of the S/RT region was carried out on any sample reported to be tested positive for HBsAg. However, we soon realized that a well-defined amplification product could not be obtained from samples with a viral load $<10^4$ HBV genome copies/ml despite various optimization efforts. Similar observations were reported in the literature regarding the amplification of HBV genome from samples containing low viral loads. It was shown earlier that DNA polymerases with proof reading activity do not amplify efficiently less than 10^5 copies/ml of template genome (Günther et al., 1998). Therefore, we used the Advantage® 2 Polymerase mix for amplification, as it is an enzyme mix, which allows efficient, accurate amplification in presence of a sufficient proof reading activity. As the intention of this research was to detect mutations, we wanted to make sure that any mutation detected in the PCR amplification is related to the sample and not to the infidelity of the *Taq* polymerase. Nevertheless, with few exceptions the enzyme failed to generate a clear amplification product from samples with viral load $<10^5$ copies/ml. Possibly, the use of a PCR machine with a more rapid temperature change profile like the LightCycler (Roche Diagnostics) might have allowed amplification of smaller HBV DNA levels (Prof. W. Gerlich, University of Giessen, Germany, personal communication).

4.3 HBV Genotypes and subgenotypes occurring in Palestinian samples

HBV genotype D was the most prominent among Palestinian patients. Only 7.5 % of the samples were representing genotype A. The predominance of genotype D is consistent with regional reports from Egypt, Jordan, 1948 area of Palestine, Syria and Lebanon (Khaled et al., 2011; Zekri et al., 2007; Masaadeh et al., 2008; Ben-Ari et al., 2004; Antaki et al., 2010; El Chaar et al., 2012). Genotype D has a worldwide distribution, but it is predominant in some regions, and a minor component in others. Regions of high genotype D prevalence are the Mediterranean and large parts of Asia except East and South East Asia (Norder et al., 2004). The most prominent subgenotype among Palestinians was D1, which was detected in 97% of the D genotype samples. Subgenotype D1 is the most common subgenotype in Turkey, Greece, Iran, Pakistan, Egypt, Lebanon, Israel and others (Cox et al., 2011; Sayan and Dogan, 2012; Fylaktou et al., 2011; Garmiri et al., 2011; Baig et al., 2009; Ragheb et al., 2012; El Chaar et al., 2012; Norder et al., 2004). One single Palestinian sample belonged to D3 subgenotype. Subgenotype D3 is found prominently in Europe (Norder et al., 2004), but some regional studies reported a low prevalence of D3 subgenotype (Cox et al., 2011; Sayan and Dogan, 2012). Surprisingly three Palestinian samples belonged to subgenotype A2. Subgenotype A2 is common in Northern and Central Europe, and in the European offspring of Caucasians living in South Africa and USA (Norder et al., 2004). Genotype A was hardly reported in the region, with the exception of one report from Egypt, where a mix of genotypes D and A were detected in pediatric cancer patients (Zekri et al., 2007). Two of the three A2 samples were from residents of Bethlehem, one from Jerusalem. AQ-31, one of these three A2 samples was submitted to the Virology Laboratory by the patient personally. Personal communication with this patient revealed that she was operated and received blood transfusions. We propose that this blood was not donated from local donors but rather came from abroad within the medical aid for Palestinians. We were unable to allocate further information in this regard or regarding the other two A2 samples.

4.4 Phylogenetic analysis of Palestinian HBV genotypes

The HBV samples representing the predominant subgenotype D1 isolated from Palestinian patients clustered very well together among regional D1 subgenotypes as presented in figure 3.6. These, AQ-08, AQ-36 AQ-33, AQ-22, AQ-13 and AQ-14, are distributed on two subclades including D1 reference sequences, which are from Belgium and Belarus, respectively. This finding does not mean that subgenotype D1

is typical for Belgium or Belarus. It is more likely that the Belgian or White Russian isolates came from immigrants, because in Central-Western Europe population subgenotype A2 is predominant and in Belarus D2. The only D3 Palestinian sample clustered on one branch with a Turkish reference sequence. All Palestinian A2 samples clustered on one branch within the A2 reference sequences.

Detailed phylogenetic analysis of the Palestinian D1 sequences revealed that the divergence between the samples was between 0 and 2.8%. AQ-21 presented with the highest divergence among the D1 samples; AQ-20 presented with the lowest divergence. The bootstrap value on the main branches of figure 3.7 supports the degree of similarity among the Palestinian D1 samples and the reference D1 sequences. With the exception of AQ-20, all Palestinian samples clustered together on one clade, along with the reference samples from Iran, Turkey, Syria and India. AQ-20 clustered on the second main branch with reference sequences from Egypt, Sudan and Syria. The three Palestinian A2 subgenotypes were also similar to each other than to the reference A2 sequences, and clustered on one subclade together. The only Palestinian D3 sample formed one branch, while the reference sequences another.

In case of the two mother /child pairs AQ-24/AQ-25 and AQ-39/AQ-40, the HBV DNA sequences were identical, a clear evidence for HBV transmission from mother to the child, either in utero or perinatal. It is noteworthy to mention here that the mother/child serum samples were collected on different days and the DNA extraction was performed on different days too.

4.5 Mutation analysis of the S gene

Each sample was subjected to sequencing using forward and reverse primer. Following verification and correction of each sample's forward and reverse sequences, both sequences were aligned for mutation detection. Only mutations found in both, forward and reverse, sequences were considered valid. Seventeen non-synonymous mutations (Table 3.3) were found all over the 36 Palestinian D1 subgenotypes. Six mutations were found in the "a" determinant, three upstream and eight downstream in the S gene. Single mutations were found in one, two or three

samples. As these were rare they were considered to be selected in the individuals after infection. Mutations present in more than 10% of samples with subgenotype D1 were considered frequent and more likely to present a local polymorphism than an individual selection process in a patient.

Mutation I86F, found in one patient, was reported earlier from chronic HBV carriers with D1 subgenotype from Isfahan, Iran (Norouzi et al., 2012). The mutation was found in one patient, along with other mutations in the same patient. According to the authors, who reported different mutations in the immune epitopes of the S protein in 19 patients, this is a result of virus-host interaction with a prolonged infection period. Although the mutation is located in the highly conserved transmembrane helix II of HBsAg and not surface exposed, it appears possible that it has a sterical effect on the B cell epitopes downstream.

Mutation I92T, found in one single patient, was reported earlier in two different studies on Korean and Chinese patients (Kim et al., 2010; Zhang et al., 2011). Zhang et al. detected the mutation in a patient with subgenotype C1 (Zhang et al., 2011). I92T was not studied concerning its influence on viral or S gene function in any of these studies (Kim et al., 2010; Zhang et al., 2011). This mutation is also within the hydrophobic transmembrane helix II of the small HBs protein. Independent occurrence in different regions of the world and in different genotypes suggests a strong selective pressure in some carriers on that site of HBsAg, the nature of which is unknown.

One mutation upstream in the surface exposed HBsAg loop, I110L, detected in two different samples, was caused by two different nucleotide substitutions. Weinberg et al. reported I110L in HBV chronic carrier with genotype A from Germany (Weinberger et al., 2000), without elucidation of a possible role.

In regard to the six mutations found in the “a” determinant, the amino acid exchange Y134F found in two patients was known to be related to HBsAg subtypes adw or ayw (Gerin et al., 1983). F134 is typical for samples which are classified today as A2 (HBsAg adw) whereas Y134 is wildtype for D (HBsAg ayw). This mutation was described in reports from different countries (Ghany et al., 1998; Weinberger et al., 2000; Soussan et al., 2001). S143 is typical for genotype D and T143 for A2. The exchange S143T in genotype D was reported previously (Weinberger et al., 2000).

The other four mutations found in the "a" determinant were known escape mutants; T126S, T127S, D144E and G145R. Each of T126S and P127S were found in two different samples; AQ-1 and AQ-21. Two D1 samples (AQ-27 and AQ-38) were presented with the two other escape mutations; D144E and G145R simultaneously. One patient (AQ-15) was presented with G145R only. Altogether five Palestinian D1 samples were presented with escape mutations, which accounted for 12.5% of the total sequenced samples. G145R is the best known escape mutation (Wallace and Carman, 1997; Kidd-Ljunggren et a., 2002; Osiowy et al., 2006). D144E is another common escape mutation (Wallace and Carman, 1997). T126S and T127S are also known escape mutants (Svicher et al., 2001).

Mutations in the "a" determinant of the S gene are partially responsible for occult HBV infection, a potential risk to blood safety (Carman et al., 1995; Kreutz, 2002; Kay and Zoulim, 2007). This is the case, when HBsAg is seemingly absent in the presence of HBV DNA in serum, which was, however, not the case in our sample. Previous HBsAg assays often failed to detect HBsAg with mutations in the HBsAg loop but last generation assays and the assay used for our study detect most HBsAg escape mutants if they are present in sufficient concentration.

Different studies demonstrated that HBV viruses carrying vaccine escape mutations including the common G145R and D144E reduced binding affinity of anti-HBs antibodies to the S protein, including vaccine generated antibodies, a potential danger for infection despite vaccination (Swenson et al., 1983; Linnemann and Askey, 1984; Wands et al., 1986; Coursaget et al., 1987; Carman et al., 1990; Waters et al., 1992; Wallace and Carman, 1997; Karthigesu et al., 1994; Carman et al., 1995, 1996; Oon et al., 1995; Hsu et al., 1999; Kim et al., 2003). All escape mutants were found in patients, who were above 36 years, which means they were not subjected to immunization. It is probably that an unrecognized antibody response of the patients against their own HBsAg had exerted some selective pressure in favor of classical escape mutants and mutations to an amino acid associated with another genotype like Y134F and S143T. It was reported earlier that S gene mutations accumulate in chronic hepatitis B, particularly after development of hepatitis or loss of HBeAg (Hannoun et al., 2000). These facts were predicted to lead to a global dominance of vaccine escape mutants (Wilson et al., 2000).

Downstream the HBsAg loop, mutation T189I was previously reported not to affect detection of HBsAg (Olinger et al., 2007). However, Kazim et al. (2005) reported that the combination of T189I with V190 and I208T was correlated with resistance to Lamivudine therapy. Mutation Y200F was reported earlier without being connected to functional impact on the S gene (Kim et al., 2010; Liang et al., 2011). A novel mutation Y206L was found in two patients and caused by two different nucleotide substitutions. An Y206H mutation was found among many other mutations in an Iranian blood donor with occult HBV infection who transmitted HBV to the recipient (Saniewski, PhD thesis, 2009, University of Giessen, Germany). Mutation S207N was reported in three different studies, from Germany (Weinberger et al., 2000) and two Iranian studies (Hamkar et al., 2010; Norouzi et al., 2010) without proposing any role in the S gene. Another three mutations; S210R, L213I and S213F, found in 3 different patients belong to yet uncharacterized S gene mutants (Weinberger et al., 2000). Further investigations are needed to address the phenotype/outcome denoted by these mutations. Mutations, which were not characterized yet and not related to the genotypes, may unfortunately have a negative impact on immunoassay based diagnostics of HBV (Coleman et al., 1999; Ireland et al., 2000; Ly et al., 2006).

Beside the non-synonymous mutations causing the amino acid exchanges mentioned above, seven different synonymous mutations (Table 3.4) were found in the 36 D1 subgenotypes, three of which were referred to as polymorphism as they occurred in more than 10% of the samples. The ratio of non-synonymous (17) to synonymous non-polymorphic mutations (4) is an indicator for the evolutionary relevance of a set of mutations (Hanada et al. 2007). Ratios below 1 suggest that these mutations are genetically neutral as suggested by Gojobori et al. (1990) for HBV and other viruses. A ratio of 4.25 for the S genes indicates a strong selection effect on HBV strains circulating in the studied Palestinian patients. A further evidence for this selection may be the amino acid exchanges I110L and Y206L; each occurred twice and was caused each time by different nucleotide substitution (Gojobori et al., 1990).

Regarding the only Palestinian D3 sample (Table 3.5), six non-synonymous mutations were detected, one of which (Y206T) was novel. Mutations Y200F, S204R, S207N were reported earlier without being attributed to functional impact on the S gene (Weinberger et al., 2000; Hamkar et al., 2010; Norouzi et al., 2010; Kim et al., 2010;

Liang et al., 2011). S204R was also found in the HBV quasi-species of an occult infected blood donor among several other mutations (Christophe, PhD thesis, 2008, University of Saarland, Germany). Along with other S gene mutations; P203Q was described earlier to evoke false negative or discrepant results in some HBsAg detection assays (Geretti et al., 2010). In contrast, mutation T125M increased the mean value of HBsAg detection in commercial assays (Araujo et al., 2009).

L209V was the only mutation found in the S gene of all three Palestinian A2 samples. L209V was reported earlier in transplant recipients, who received HBIG (Ghany et al., 1998) and in vaccinated individuals (Mathet et al., 2006). In the second report, authors proposed that antibodies produced due to vaccination may not be effective in neutralizing HBV mutants including the L209V in genotype E (Mathet et al., 2006).

4.6 Mutations analysis of the RT region

Twenty three mutations, seven and five were detected in the RT region of the Palestinian D1, D3 and A2 isolates respectively. None of these mutations was reported to confer resistance to any of the known antiviral drugs against HBV. Out of the 40 cases presented here, only AQ-31 was reported to have received anti-viral therapy.

Polymorphism mutations Y135S, N248H, F122I, H124Y and R274K were the most common Palestinian mutations (presented in >10% of the samples). Mutations H94I, N279T (D1), detected in one patient each, and Y135H (D3) were not reported yet. Mutation L91I found in one D1 patient was attributed to lamivudine and adefovir resistance, if it occurred in a context of other mutations (Karatayli et al., 2011), which was not the case here. Mutations R110G, T222S in D1 and K275E in D3 were observed in naïve patients treated with entecavir, with no influence on the therapy (Colonno et al., 2006). S219P and D263E were reported mutations detected during lamivudine therapy (Delaney et al., 2001; Quiros-Roldan et al., 2008). Both L115V (D1) and S213T (D3) were reported substitutions in patients with virological breakthrough, but were not accredited for antiviral resistance in these patients (Santantonio et al., 2009). Substitutions H124Y, found in 11 Palestinian D1 isolates, C256 found in 2 D1 and the D3 isolates and I253 found in all three A2 isolates were

detected in German isolates, without being addressed for antiviral resistance (Geipel, 2011, PhD thesis, University of Giessen, Germany). Y135S was the most common Palestinian mutation found in the RT gene of 35 D1 isolates. It was reported in one patient receiving adefovir therapy (Borroto-Esoda et al.,) but according to our findings it has obviously nothing to do with adefovir resistance. Borroto-Esoda et al. (2007) also reported mutations F122I, the third prevalent mutation found in Palestinian D1 patients, Y137T and L145M. Substitution Y135F was known to be a consequence of mutation T127S in the S gene (Svicher et al., 2011; indeed Y135F was detected in the RT gene of AQ-21, which was presented with the T127S escape mutation in the S gene. RT mutation R153Q and R153K are associated with G145R and D144E in the S gene respectively (Costantini et al., 2011). R153Q reduces the replication efficiency of the viral polymerase (Locarnini, 2003; Coleman, 2006). In contrast, R153K was proposed to enhance viral polymerase fitness (Costantini et al., 2011). Although F122Y was attributed to natural polymorphic selection, it may evoke antiviral resistance in strains with primary mutations (Pollicino et al., 2009). Pollicino et al. (2009) also reported substitution P237T. N248H was the second common Palestinian mutation in D1 subgenotypes. N248H and I266K were detected earlier in patients under nucleotide antiviral therapy; R274K (found in Palestinian D1 and A2 genotypes) was detected in naïve patients (Margeridon-Thermet et al., 2009). I163V detected in the D3 isolate can only then contribute to drug resistance if combined with other mutations (Arrese et al., 2011). Mutation V214 was detected in Tunisian blood donors (submitted sequence, GenBank: FJ904404.1). Finally mutation M309K found in two Palestinian D1 isolates was reported earlier (Olotu, 2010, PhD thesis, University of Hamburg, Germany).

As previously described in the introduction, the S gene is overlapping the RT gene region, which means that mutations in the RT region selected after drug treatment may also lead to mutations in the S region (Sheldon and Soriano, 2008) and vice versa as described for some mutations above. Our work however, did not reveal any drug resistance in treatment-naïve Palestinian patients, not even in the only patient receiving lamivudine. This patient submitted two samples for viral load and genotyping as she was the only patient to have received lamivudine treatment. According to the clinical data AQ-31, she had received lamivudine for 2 years. The treatment was put on hold for unknown reason, in this period; she submitted one

serum sample to the Virology laboratory, which was presented with 10^{10} copies/ml. Half a year later, after another cycle of lamivudine treatment, she submitted another serum sample. This time the viral titer was reduced to 10^{8} copies/ml suggesting premature stop of treatment or noncompliance. Sequences performed on both samples from AQ-31 did not reveal any additional drug resistance mutations yet.

Eleven synonymous mutations were found in the RT region of D1 subgenotypes. Mutation L168L was the only polymorphism, found in 10% of the samples. The ratio of non-synonymous (18) to synonymous (10) mutations without polymorphism is 1.8 which is significantly less than that in the S region.

4.7 HBV infection in the vaccinated generation

There were four HBV children in this study representing 10% of the samples, infected with HBV subgenotype D1. All children were vaccinated according to their vaccination records. Personal communication with the mother (AQ-24) of case AQ-25 revealed that she was not aware of her HBV infection; consequently, the child did not receive HBIG passive immunization. Furthermore, the third vaccine dose was not notified in the vaccination book of the child probably because it was hospitalized during that time period. One mother (AQ-39) was aware of her HBV infection; she also assured us that the child (AQ-40) did indeed receive HBIG. Both children AQ-25 and AQ-40 had a long history of hospitalization due to severe diseases. Parents of AQ-33 were not aware of their child's infection. The infection in this case was detected only due to the hospital's policy of testing HBsAg for every patient booked for operation/admission. Physicians of AQ-37 and AQ-32 assured us that these children were vaccinated, the father of AQ-37 was tested positive for HBsAg and had 1000 HBV genome copies/ml. No further information was available for AQ-32 as the serum was sent to the Virology laboratory for viral load testing due to positive HBsAg.

4.8 Conclusions and recommendations

Identifying specific genotypes of pathogens in the different geographic areas with high prevalence may lead to further steps in optimizing vaccines, diagnostic tools and control measures according to the predominant local strains. Furthermore, genetic profiles of pathogenic genes targeted by drugs may be crucial for the efficiency of treatment. The study presented here is the first comprehensive research addressing genotypes and mutation analysis of HBV virus S gen and polymerase in Palestine and very unique in the Arab world. Considering the fact that that HBV carries studied here were treatment-naïve patients, one would think to expect limited mutations. But the nature of the HBV virus, its small genome and error-prone polymerase, immune selection, unspecific selection and evolutionary relevance are all in favor of mutations of HBV genome. We hope that the scope of this study will be wider and we can analyze treated patients and also HBV carriers represented with low viral loads to establish a reliable data base for health institutions and decision makers.

During this study we were studied the polices and drug choices of the Palestinian Ministry of Health. We found out that pregnant women are not screened for HBV infection. This has a sad health consequence on the babies and their lives. Children born to HBV positive mother (based on the HBsAg test value) should become passive immunization (HBIG) before the first dose of the anti HBV vaccine within the first 24h of their lives (see 1.8). 30% of HBsAg positive mothers and 70% of HBeAg mothers transfer their HBV to their fetus (see 1.8). Screening mothers worldwide led to significant reduction in the infection of their children. According to our best knowledge, in Palestine, only private physicians and clinics ask for the HBsAg test and if positive recommend the parents to buy the HBIG on their own cost. The cost for HBIG may be high, but compared with the costs of treating the onset of the disease, it is economically very convenient.

Finally the only anti HBV drug supported by the Palestinian Ministry of health, lamivudine, is not anymore recommended for naïve patients due to its ability to induce high percentage of mutations during treatment (see 1.9.2 and 1.10.4). So taking the complications related to drug resistance, the more expensive drug Entecavir can also be of a big benefit to the diseased person and to the economy of the country.

5. REFERENCES:

1. Akhter S, Talukder MQ, Bhuiyan N, Chowdhury TA, Islam MN, et al. (1992) Hepatitis B virus infection in pregnant mothers and its transmission to infants. Indian journal of pediatrics 59: 411-415.
2. Alter MJ (2003) Epidemiology and prevention of hepatitis B. Seminars in liver disease 23: 39-46.
3. Angus P, Vaughan R, Xiong S, Yang H, Delaney W, et al. (2003) Resistance to adefovir dipivoxil therapy associated with the selection of a novel mutation in the HBV polymerase. Gastroenterology 125: 292-297.
4. Antaki N, Haffar S, Ali Deeb S, Assaad F, Abou Harb R, et al. (2010) High prevalence of HBV genotype D in Syria and the clinical characteristics of hepatitis B e antigen-negative chronic hepatitis B. Epidemiology and infection 138: 40-44.
5. Araujo NM, Vianna CO, Moraes MT, Gomes SA (2009) Expression of Hepatitis B virus surface antigen (HBsAg) from genotypes A, D and F and influence of amino acid variations related or not to genotypes on HBsAg detection. The Brazilian journal of infectious diseases : an official publication of the Brazilian Society of Infectious Diseases 13: 266-271.
6. Arrese E, Basaras M, Blanco S, Ruiz P, Cisterna R (2011) Evolution of hepatitis B virus during long-term therapy in patients with chronic hepatitis B. Annals of hepatology 10: 434-440.
7. Ascherio A, Zhang SM, Hernan MA, Olek MJ, Coplan PM, et al. (2001) Hepatitis B vaccination and the risk of multiple sclerosis. The New England journal of medicine 344: 327-332.
8. Baig S, Siddiqui A, Chakravarty R, Moatter T (2009) Hepatitis B virus subgenotypes D1 and D3 are prevalent in Pakistan. BMC research notes 2: 1.
9. Bartholomeusz A, Locarnini S, Ayres A, Thompson G, Sozzi V, et al. (2004) Molecular modelling of hepatitis B virus polymerase and adefovir resistance identifies three clusters of mutations. Hepatology 40: 246A.
10. Bartholomeusz A, Locarnini SA (2006) Antiviral drug resistance: clinical consequences and molecular aspects. Seminars in liver disease 26: 162-170.
11. Bayer ME, Blumberg BS, Werner B (1968) Particles associated with Australia antigen in the sera of patients with leukaemia, Down's Syndrome and hepatitis. Nature 218: 1057-1059.
12. Beasley RP (1988) Hepatitis B virus. The major etiology of hepatocellular carcinoma. Cancer 61: 1942-1956.
13. Beasley RP, Hwang LY, Lin CC, Chien CS (1981) Hepatocellular carcinoma and hepatitis B virus. A prospective study of 22 707 men in Taiwan. Lancet 2: 1129-1133.
14. Ben-Ari Z, Mor E, Bar-Nathan N, Shaharabani E, Shapira Z, et al. (2003) Combination hepatitis B immune globulin and lamivudine versus

hepatitis B immune globulin monotherapy in preventing recurrent hepatitis B virus infection in liver transplant recipients. Transplantation proceedings 35: 609-611.
15. Block TM, Mehta AS, Fimmel CJ, Jordan R (2003) Molecular viral oncology of hepatocellular carcinoma. Oncogene 22: 5093-5107.
16. Blumberg BS (1977) Australia antigen and the biology of hepatitis B. Science 197: 17-25.
17. Borroto-Esoda K, Miller MD, Arterburn S (2006) Metaanalysis across adefovir clinical trials demonstrates the absence of novel adefovir-associated mutations and confirms the role of the rtA181V and rtA236T mutations in HBV polymerase with virologic failure. Hepatology 44: 552A.
18. Borroto-Esoda K, Miller MD, Arterburn S (2007) Pooled analysis of amino acid changes in the HBV polymerase in patients from four major adefovir dipivoxil clinical trials. Journal of hepatology 47: 492-498.
19. Bridges EG (2006) Telbivudine preclinical safety studies suggest minimal risk of chronic toxicity ,reproductive toxicity or carcinogenicity. J Hepatol 44: S147.
20. Brown SE, Howard CR, Zuckerman AJ, Steward MW (1984) Affinity of antibody responses in man to hepatitis B vaccine determined with synthetic peptides. Lancet 2: 184-187.
21. Brunelle MN, Jacquard AC, Pichoud C, Durantel D, Carrouee-Durantel S, et al. (2005) Susceptibility to antivirals of a human HBV strain with mutations conferring resistance to both lamivudine and adefovir. Hepatology 41: 1391-1398.
22. Brunetto MR, Colombatto P, Bonino F (2009) Bio-mathematical models of viral dynamics to tailor antiviral therapy in chronic viral hepatitis. World journal of gastroenterology : WJG 15: 531-537.
23. Brunetto MR, Moriconi F, Bonino F, Lau GK, Farci P, et al. (2009) Hepatitis B virus surface antigen levels: a guide to sustained response to peginterferon alfa-2a in HBeAg-negative chronic hepatitis B. Hepatology 49: 1141-1150.
24. Brunetto MR, Oliveri F, Colombatto P, Moriconi F, Ciccorossi P, et al. (2010) Hepatitis B surface antigen serum levels help to distinguish active from inactive hepatitis B virus genotype D carriers. Gastroenterology 139: 483-490.
25. Bruss V, Ganem D (1991) The role of envelope proteins in hepatitis B virus assembly. Proceedings of the National Academy of Sciences of the United States of America 88: 1059-1063.
26. Cacciola I, Pollicino T, Squadrito G, Cerenzia G, Villari D, et al. (2000) Quantification of intrahepatic hepatitis B virus (HBV) DNA in patients with chronic HBV infection. Hepatology 31: 507-512.
27. Carman WF, Korula J, Wallace L, MacPhee R, Mimms L, et al. (1995) Fulminant reactivation of hepatitis B due to envelope protein mutant that escaped detection by monoclonal HBsAg ELISA. Lancet 345: 1406-1407.
28. Carman WF, Zanetti AR, Karayiannis P, Waters J, Manzillo G, et al. (1990) Vaccine-induced escape mutant of hepatitis B virus. Lancet 336: 325-329.
29. Chan HL, Wong VW, Tse AM, Tse CH, Chim AM, et al. (2007) Serum hepatitis B surface antigen quantitation can reflect hepatitis B virus in the liver and predict treatment response. Clinical gastroenterology and

hepatology : the official clinical practice journal of the American Gastroenterological Association 5: 1462-1468.
30. Chang SF, Netter HJ, Bruns M, Schneider R, Frolich K, et al. (1999) A new avian hepadnavirus infecting snow geese (Anser caerulescens) produces a significant fraction of virions containing single-stranded DNA. Virology 262: 39-54.
31. Chen CJ, Yang HI, Su J, Jen CL, You SL, et al. (2006) Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. JAMA : the journal of the American Medical Association 295: 65-73.
32. Chen HS, Kaneko S, Girones R, Anderson RW, Hornbuckle WE, et al. (1993) The woodchuck hepatitis virus X gene is important for establishment of virus infection in woodchucks. Journal of virology 67: 1218-1226.
33. Chen MT, Billaud JN, Sallberg M, Guidotti LG, Chisari FV, et al. (2004) A function of the hepatitis B virus precore protein is to regulate the immune response to the core antigen. Proceedings of the National Academy of Sciences of the United States of America 101: 14913-14918
34. Christophe M. Olinger (2008) The worldwide distribution and evolution of the hepatitis B virus: recombinations, new genotypes and subtypes. Saarland University.Germany.
35. Chu CJ, Hussain M, Lok AS (2002) Quantitative serum HBV DNA levels during different stages of chronic hepatitis B infection. Hepatology 36: 1408-1.415
36. Coleman PF (2006) Detecting hepatitis B surface antigen mutants. Emerging infectious diseases 12: 198-203.
37. Coleman PF, Chen YC, Mushahwar IK (1999) Immunoassay detection of hepatitis B surface antigen mutants. Journal of medical virology 59-19 : .24
38. Colonno RJ, Rose R, Baldick CJ, Levine S, Pokornowski K, et al. (2006) Entecavir resistance is rare in nucleoside naive patients with hepatitis B. Hepatology 44: 1656-1665.
39. Colonno RJ, Rose RE, Pokornowski K, Baldick CJ, Klesczewski K, et al. (2006) Assessment at three years shows high barrier to resistance is maintained in entecavir treated nucleoside naive patients while resistance emergence increases over time in lamivudine refractory patients. Hepatology 44: 229A.
40. Confavreux C, Suissa S, Saddier P, Bourdes V, Vukusic S (2001) Vaccinations and the risk of relapse in multiple sclerosis. Vaccines in Multiple Sclerosis Study Group. The New England journal of medicine 344: 319-326.
41. Costantini A, Marinelli K, Biagioni G, Monachetti A ,Ferreri ML, et al. (2011) Molecular analysis of hepatitis B virus (HBV) in an HIV co-infected patient with reactivation of occult HBV infection following discontinuation of lamivudine-including antiretroviral therapy. BMC infectious diseases 11: 310.
42. Cougot D, Neuveut C, Buendia MA (2005) HBV induced carcinogenesis. Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology 34 Suppl 1: S75-78

43. Coursaget P, Gharbi Y, Khrouf N, Depril N, Boukhris N, et al. (1994) Familial clustering of hepatitis B virus infections and prevention of perinatal transmission by immunization with a reduced number of doses in an area of intermediate endemicity (Tunisia). Vaccine 12: 275-278.
44. Cox LE, Arslan O, Allain JP (2011) Characterization of hepatitis B virus in Turkish blood donors, and the prevalence of the SP1 splice variant. Journal of medical virology 83: 1321-1325
45. Cornberg M, Protzer U, Petersen J, Wedemeyer H, Berg T, Jilg W, Erhardt A,
Wirth S, Sarrazin C, Dollinger MM, Schirmacher P, Dathe K, Kopp IB, Zeuzem S,
Gerlich WH, Manns MP; AWMF. (2011) Prophylaxis, diagnosis and therapy of
hepatitis B virus infection - the German guideline. Z Gastroenterol. 49(7):871-930.
46. Damme P (2001) Hepatitis B: vaccination programmes in Europe--an update. Vaccine 19: 2375-2379.
47. Dane DS, Cameron CH, Briggs M (1970) Virus-like particles in serum of patients with Australia-antigen-associated hepatitis. Lancet 1: 695-698.
48. De Clercq E, Ferir G, Kaptein S, Neyts J (2010) Antiviral treatment of chronic hepatitis B virus (HBV) infections. Viruses 2: 1279-1305.
49. Deguchi M, Yamashita N, Kagita M, Asari S, Iwatani Y, et al. (2004) Quantitation of hepatitis B surface antigen by an automated chemiluminescent microparticle immunoassay. Journal of virological methods 115: 217-222.
50. Delaney WEt, Locarnini S, Shaw T (2001) Resistance of hepatitis B virus to antiviral drugs: current aspects and directions for future investigation. Antiviral chemistry & chemotherapy 12: 1-35.
51. Delaney WEt, Ray AS, Yang H, Qi X, Xiong S, et al. (2006) Intracellular metabolism and in vitro activity of tenofovir against hepatitis B virus. Antimicrobial agents and chemotherapy 50: 2471-2477.
52. El Chaar M, El Jisr T, Allain JP (2012) Hepatitis B virus DNA splicing in Lebanese blood donors and genotype A to E strains: implications for hepatitis B virus DNA quantification and infectivity. Journal of clinical microbiology 50.3167-3159 :
53. Enders GH, Ganem D, Varmus H (1985) Mapping the major transcripts of ground squirrel hepatitis virus: the presumptive template for reverse transcriptase is terminally redundant. Cell 42: 297-308.
54. Fan YF, Lu CC, Chen WC, Yao WJ, Wang HC ,et al. (2001) Prevalence and significance of hepatitis B virus (HBV) pre-S mutants in serum and liver at different replicative stages of chronic HBV infection. Hepatology 33: 277-286.
55. Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA (2005) Virus Taxonomy: Eighth Report of the International Committee of Taxonomy of Viruses. Hepadnaviridae. San Diego,Calif: Elsevier Academic Press. pp. 373-384
56. Findlay GM, MacCallum FO (1937) An interference phenomenon in relation to yellow fever and other viruses. The Journal of Pathology and Bacteriology 44: 405–424.

57. Fox AJ, Mason D, Panna HA, Para M (1942) Observations on occurrence of icterus in Brazil following vaccination against yellow fever. american journal Hygene 36: 63-116.
58. Franco A, Paroli M, Testa U, Benvenuto R, Peschle C, et al. (1992) Transferrin receptor mediates uptake and presentation of hepatitis B envelope antigen by T lymphocytes. The Journal of experimental medicine 175: 1195-1205.
59. Fujii H, Moriyama K, Sakamoto N, Kondo T, Yasuda K, et al. (1992) Gly145 to Arg substitution in HBs antigen of immune escape mutant of hepatitis B virus. Biochemical and biophysical research communications .1157-1152 :184
60. Fylaktou A, Papaventsis D, Daoudaki M, Moskophidis M, Reiberger T, et al. (2011) Molecular epidemiology of chronic hepatitis B virus infection in Greece. Journal of medical virology 83: 245-252.
61. Garmiri P, Rezvan H, Abolghasemi H ,Allain JP (2011) Full genome characterization of hepatitis B virus strains from blood donors in Iran. Journal of medical virology 83: 948-952.
62. Gavilanes F, Gonzalez-Ros JM, Peterson DL (1982) Structure of hepatitis B surface antigen. Characterization of the lipid components and their association with the viral proteins. The Journal of biological chemistry 257: 7770-7777.
63. Ganji A, Esmaeilzadeh A, Ghafarzadegan K, Helalat H, Rafatpanah H, et al. (2011) Correlation between HBsAg quantitative assay results and HBV DNA levels in chronic HBV. Hepatitis monthly 11: 342-345.
64. Geipel A (2011) Phänotypische Charakterisierung klinisch relevanter Hepatitis-B-Virus-Mutanten: university Gießen.
65. Geretti AM, Patel M, Sarfo FS, Chadwick D, Verheyen J, et al. (2010) Detection of highly prevalent hepatitis B virus coinfection among HIV-seropositive persons in Ghana. Journal of clinical microbiology 48: 3223-3230.
66. Gerin JL, Purcell RH (1983) New approaches to hepatitis B vaccines. Progress in clinical and biological research 143: 369-377.
67. Gerlich WH (2007) The enigma of concurrent hepatitis B surface antigen (HBsAg) and antibodies to HBsAg. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 44: 1170-1172.
68. Ghany M, Liang TJ (2007) Drug targets and molecular mechanisms of drug resistance in chronic hepatitis B. Gastroenterology 132: 1574-1585.
69. Ghany MG, Ayola B, Villamil FG, Gish RG, Rojter S, et al. (1998) Hepatitis B virus S mutants in liver transplant recipients who were reinfected despite hepatitis B immune globulin prophylaxis. Hepatology 27: 213-222.
70. Girones R, Miller RH (1989) Mutation rate of the hepadnavirus genome. Virology 170: 595.597-
71. Gojobori T, Moriyama EN, Kimura M (1990) Molecular clock of viral evolution, and the neutral theory. Proceedings of the National Academy of Sciences of the United States of America 87: 10015-10018.
72. Gough NM (1983) Core and E antigen synthesis in rodent cells transformed with hepatitis B virus DNA is associated with greater than

genome length viral messenger RNAs. Journal of molecular biology 165: 683-699.
73. Guerrero E, Swenson PD, Hu PS, Peterson DL (1990) The antigenic structure of HBsAg :study of the d/y subtype determinant by chemical modification and site directed mutagenesis. Molecular immunology 27: 435-441.
74. Gunson RN, Shouval D, Roggendorf M, Zaaijer H, Nicholas H, et al. (2003) Hepatitis B virus (HBV) and hepatitis C virus (HCV (infections in health care workers (HCWs): guidelines for prevention of transmission of HBV and HCV from HCW to patients. Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology 27: 213-230.
75. Gunther S ,Fischer L, Pult I, Sterneck M, Will H (1999) Naturally occurring variants of hepatitis B virus. Advances in virus research 52: 25-137.
76. Gunther S, Sommer G, Von Breunig F, Iwanska A, Kalinina T, et al. (1998) Amplification of full-length hepatitis B virus genomes from samples from patients with low levels of viremia: frequency and functional consequences of PCR-introduced mutations. Journal of clinical microbiology 36: 531-538.
77. Hannoun C, Horal P, Lindh M (2000) Long-term mutation rates in the hepatitis B virus genome. The Journal of general virology 81: 75-83.
78. Hadziyannis SJ (2006) New developments in the treatment of chronic hepatitis B. Expert opinion on biological therapy 6: 913-921.
79. Hamkar R, Aghakhani A, Soufian S, Banifazl M, Ghavami N, et al. (2010) Surface gene mutations of hepatitis B virus among high-risk patients with occult hepatitis B virus infection. Diagnostic microbiology and infectious disease 66: 285-291.
80. Hanada K, Shiu SH, Li WH (2007) The nonsynonymous/synonymous substitution rate ratio versus the radical/conservative replacement rate ratio in the evolution of mammalian genes. Molecular biology and evolution 24: 2235-2241.
81. Harrison TJ, Hopes EA, Oon CJ, Zanetti AR, Zuckerman AJ (1991) Independent emergence of a vaccine-induced escape mutant of hepatitis B virus. Journal of hepatology 13 Suppl 4: S105-107.
82. Hasegawa K, Huang JK, Wands JR, Obata H, Liang TJ (1991) Association of hepatitis B viral precore mutations with fulminant hepatitis B in Japan. Virology 185: 460-463.
83. Heermann KH, Goldmann U, Schwartz W, Seyffarth T, Baumgarten H, et al. (1984) Large surface proteins of hepatitis B virus containing the pre-s sequence. Journal of virology 52: 396-402.
84. Hino K, Okuda M, Hashimoto O, Ishiko H, Okazaki M, et al. (1995) Glycine-to-arginine substitution at codon 145 of HBsAg in two infants born to hepatitis B e antigen-positive carrier. Digestive diseases and sciences 40: 566-570.
85. Hoofnagle JH (1981) Serologic markers of hepatitis B virus infection. Annual review of medicine 32: 1-11.
86. Hoofnagle JH, Gerety RJ, Barker LF (1973) Antibody to hepatitis-B-virus core in man. Lancet 2: 869.873-

87. Howard CR, Stirk H, Brown SE, Steward MW (1988) Towards the development of synthetic hepatitis B vaccines. Viral Hepatitis and Liver Disease: 1094-1101
88. Hsu HY, Chang MH, Liaw SH, Ni YH, Chen HL (1999) Changes of hepatitis B surface antigen variants in carrier children before and after universal vaccination in Taiwan. Hepatology 30: 1312-1317.
89. Hunt CM, McGill JM, Allen MI, Condreay LD (2000 (Clinical relevance of hepatitis B viral mutations. Hepatology 31: 1037-1044.
90. Iloeje UH, Yang HI, Su J, Jen CL, You SL, et al. (2006) Predicting cirrhosis risk based on the level of circulating hepatitis B viral load. Gastroenterology 130: 678-686.
91. Ireland JH, O'Donnell B, Basuni AA, Kean JD, Wallace LA, et al. (2000) Reactivity of 13 in vitro expressed hepatitis B surface antigen variants in 7 commercial diagnostic assays. Hepatology 31: 1176-1182.
92. Janssen HL, Kerhof-Los CJ, Heijtink RA, Schalm SW (1994) Measurement of HBsAg to monitor hepatitis B viral replication in patients on alpha-interferon therapy. Antiviral research 23: 251-257.
93. Janssen HL, van Zonneveld M, Senturk H, Zeuzem S, Akarca US, et al. (2005) Pegylated interferon alfa-2b alone or in combination with lamivudine for HBeAg-positive chronic hepatitis B: a randomised trial. Lancet 365: 123-129.
94. Jaroszewicz J, Calle Serrano B, Wursthorn K, Deterding K, Schlue J, et al. (2010) Hepatitis B surface antigen (HBsAg) levels in the natural history of hepatitis B virus (HBV)-infection: a European perspective. Journal of hepatology 52: 514-522.
95. Kann M, Bischof A, Gerlich WH (1997) In vitro model for the nuclear transport of the hepadnavirus genome. Journal of virology 71: 1310-1.316
96. Kaplan PM, Greenman RL, Gerin JL, Purcell RH, Robinson WS (1973) DNA polymerase associated with human hepatitis B antigen. Journal of virology 12: 995-1005.
97. Karatayli E, Karatayli SC, Cinar K, Gokahmetoglu S, Guven K, et al. (2012) Molecular characterization of a novel entecavir mutation pattern isolated from a multi-drug refractory patient with chronic hepatitis B infection. Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology 53: 130-134.
98. Karthigesu VD, Allison LM, Fortuin M, Mendy M, Whittle HC, et al. (1994) A novel hepatitis B virus variant in the sera of immunized children. The Journal of general virology 75 (Pt 2): 443-448.
99. Kay A, Zoulim F (2007) Hepatitis B virus genetic variability and evolution. Virus research 127: 164-176.
100. Ken-ichi O, Mizokami M, Ohno T, Suzuki K, Orito E, et al. (1995) Relationships between serotypes and genotypes of hepatitis B virus : genetic classification of HBV by use of surface genes .Virus Research 39: 25-34.

101. Khaled IA, Mahmoud OM, Saleh AF, Bioumie EE (2011) Prevalence of HBV genotypes among Egyptian hepatitis patients. Molecular biology reports 38: 4353-4357.
102. Kidd-Ljunggren K, Miyakawa Y, Kidd AH (2002) Genetic variability in hepatitis B viruses. The Journal of general virology 83: 1267-1280.

103. Kim JH, Jung YK, Joo MK, Yim HJ, Park JJ, et al. (2010) Hepatitis B viral surface mutations in patients with adefovir resistant chronic hepatitis B with A181T/V polymerase mutations. Journal of Korean medical science 25: 257-264.
104. Koff RS, Galambos JT (1987) Viral Hepatitis; Schiff L, Schiff ER, editors. Philadelphia : JB Lippincott. 457-581 p.
105. Kreutz C (2002) Molecular, immunological and clinical properties of mutated hepatitis B viruses. Journal of cellular and molecular medicine 6: 113-143.
106. Krugman S, Giles JP (1973) Viral hepatitis, type B (MS-2-strain). Further observations on natural history and prevention. The New England journal of medicine 288: 755-760.
107. Kuhns MC, Kleinman SH, McNamara AL, Rawal B, Glynn S, et al. (2004) Lack of correlation between HBsAg and HBV DNA levels in blood donors who test positive for HBsAg and anti-HBc: implications for future HBV screening policy. Transfusion 44: 1332-1339.
108. Ladner SK, Otto MJ, Barker CS, Zaifert K, Wang GH, et al. (1997) Inducible expression of human hepatitis B virus (HBV) in stably transfected hepatoblastoma cells: a novel system for screening potential inhibitors of HBV replication .Antimicrobial agents and chemotherapy 41: 1715-1720.
109. Lai CL, Dienstag J, Schiff E, Leung NW, Atkins M, et al. (2003) Prevalence and clinical correlates of YMDD variants during lamivudine therapy for patients with chronic hepatitis B. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 36: 687-696.
110. Lai CL, Leung N, Teo EK, Tong M, Wong F, et al. (2005) A 1-year trial of telbivudine, lamivudine, and the combination in patients with hepatitis B e antigen-positive chronic hepatitis B. Gastroenterology 129: 528-536.
111. Lai CL, Lim SG, Brown NA, Zhou XJ, Lloyd DM, et al. (2004) A dose-finding study of once-daily oral telbivudine in HBeAg-positive patients with chronic hepatitis B virus infection. Hepatology 40: 719-726.
112. Lampertico P, Vigano M, Manenti E, Iavarone M, Lunghi G, et al. (2005) Adefovir rapidly suppresses hepatitis B in HBeAg-negative patients developing genotypic resistance to lamivudine. Hepatology 42: 1414-1419.
113. Lanford RE, Chavez D, Brasky KM, Burns RB, 3rd, Rico-Hesse R (1998) Isolation of a hepadnavirus from the woolly monkey, a New World primate. Proceedings of the National Academy of Sciences of the United States of America 95: 5757-5761.
114. Lau GK, Piratvisuth T, Luo KX, Marcellin P, Thongsawat S, et al. (2005) Peginterferon Alfa-2a, lamivudine, and the combination for HBeAg-positive chronic hepatitis B. The New England journal of medicine 352: 2682-2695.
115. Lau JY, Wright TL (1993) Molecular virology and pathogenesis of hepatitis B. Lancet 342: 1335-1340.
116. Lavanchy D (2004) Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. Journal of viral hepatitis 11: 97-107.

117. Le Seyec J, Chouteau P, Cannie I, Guguen-Guillouzo C, Gripon P (1999) Infection process of the hepatitis B virus depends on the presence of a defined sequence in the pre-S1 domain. Journal of virology 73: 2052-2057.
118. Leistner CM, Gruen-Bernhard S, Glebe D (2008) Role of glycosaminoglycans for binding and infection of hepatitis B virus. Cellular microbiology 10: 122-133.
119. Li SY, Qin L, Zhang L, Song XB, Zhou Y, et al. (2011) Molecular epidemical characteristics of Lamivudine resistance mutations of HBV in southern China. Medical science monitor : international medical journal of experimental and clinical research 17: PH75-80.

120. Liang M, Ma S, Hu X, Zhou B, Zhang J, et al. (2011) Cellular immune responses in patients with hepatitis B surface antigen seroclearance induced by antiviral therapy. Virology journal 8: 69.
121. Lindh M, Horal P, Dhillon AP, Norkrans G (2000) Hepatitis B virus DNA levels, precore mutations, genotypes and histological activity in chronic hepatitis B. Journal of viral hepatitis 7: 258-267.
122. Linnemann CC, Askey PA (1984) Susceptibility to hepatitis B despite high titre anti-HBs antibody. Lancet 1: 346-347.
123. Locarinin S (2004) Molecular Virology of Hepatitis B Virus. Molecular Virology 24: 03-10.
124. Locarnini S (2003) Hepatitis B viral resistance: mechanisms and diagnosis. Journal of hepatology 39 Suppl 1: S124-132.
125. Lok AS, Akarca U, Greene S (1994) Mutations in the pre-core region of hepatitis B virus serve to enhance the stability of the secondary structure of the pre-genome encapsidation signal .Proceedings of the National Academy of Sciences of the United States of America 91: 4077-4081.
126. Lutwick LI, Robinson WS (1977) DNA synthesized in the hepatitis B Dane particle DNA polymerase reaction. Journal of virology 21: 96-104.
127. Ly TD, Servant-Delmas A, Bagot S, Gonzalo S, Ferey MP, et al. (2006) Sensitivities of four new commercial hepatitis B virus surface antigen (HBsAg) assays in detection of HBsAg mutant forms. Journal of clinical microbiology 44: 2321-2326.
128. Mac CF (1946) Homologous serum hepatitis. Proceedings of the Royal Society of Medicine 39: 655-657
129. Madalinski K, Sylvan SP, Hellstrom U, Mikolajewicz J, Zembrzuska-Sadkowska E, et al. (2001) Antibody responses to preS components after immunization of children with low doses of BioHepB. Vaccine 20: 92-97..
130. Manesis EK, Papatheodoridis GV, Tiniakos DG, Hadziyannis ES, Agelopoulou OP, et al. (2011) Hepatitis B surface antigen: relation to hepatitis B replication parameters in HBeAg-negative chronic hepatitis B. Journal of hepatology 55: 61-68.
131. Margeridon-Thermet S, Shulman NS, Ahmed A, Shahriar R, Liu T, et al. (2009) Ultra-deep pyrosequencing of hepatitis B virus quasispecies from nucleoside and nucleotide reverse-transcriptase inhibitor (NRTI)-treated patients and NRTI-naive patients. The Journal of infectious diseases 199: 1275-1285.

132. Masaadeh HA, Hayajneh WA, Alqudah EA (2008) Hepatitis B virus genotypes and lamivudine resistance mutations in Jordan. World journal of gastroenterology : WJG 14: 7231-7234.
133. Mason WS, Seal G, Summers J (1980) Virus of Pekin ducks with structural and biological relatedness to human hepatitis B virus. Journal of virology 36: 829-836.
134. McMahon G, Ehrlich PH, Moustafa ZA, McCarthy LA, Dottavio D, et al. (1992) Genetic alterations in the gene encoding the major HBsAg: DNA and immunological analysis of recurrent HBsAg derived from monoclonal antibody-treated liver transplant patients. Hepatology 15: 757-766.
135. McNalby AS (1939) Annual Report of the Chief Medical Officer of the Ministry of Health for the Year 1937. The British journal of venereal diseases 15: 59-74.
136. Murakami S, Okochi K (1968) Observation on Australia antigen in Japan. Vox Sang 15: 374–385.
137. Moucari R, Korevaar A, Lada O, Martinot-Peignoux M, Boyer N, et al. (2009) High rates of HBsAg seroconversion in HBeAg-positive chronic hepatitis B patients responding to interferon: a long-term follow-up study. Journal of hepatology 50: 1084-1092.
138. Murray R (1955) Viral hepatitis. Bulletin of the New York Academy of Medicine 31: 341-358.
139. Nguyen T, Thompson AJ, Bowden S, Croagh C, Bell S, et al. (2010) Hepatitis B surface antigen levels during the natural history of chronic hepatitis B: a perspective on Asia. Journal of hepatology 52: 508-513.
140. Norder H, Courouce AM, Coursaget P, Echevarria JM, Lee SD ,et al. (2004) Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. Intervirology 47: 289-309.
141. Norder H, Courouce AM, Magnius LO (1994) Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. Virology 198: 489-503.
142. Norouzi M, Ghorashi S, Abedi F, Nejatizadeh A, Ataei B, et al. (2012) Identification of Hepatitis B Virus Surface Antigen (HBsAg) Genotypes and Variations in Chronic Carriers from Isfahan Province, Iran. Iranian journal of public health 41: 104-111.
143. Nowak MA, Bonhoeffer S, Hill AM, Boehme R, Thomas HC, et al. (1996) Viral dynamics in hepatitis B virus infection. Proceedings of the National Academy of Sciences of the United States of America 93: 4398-4402.
144. Ogata N, Cote PJ, Zanetti AR, Miller RH, Shapiro M, et al. (1999) Licensed recombinant hepatitis B vaccines protect chimpanzees against infection with the prototype surface gene mutant of hepatitis B virus. Hepatology 30: 779-786.
145. Okada K, Kamiyama I, Inomata M, Imai M, Miyakawa Y (1976) e antigen and anti-e in the serum of asymptomatic carrier mothers as indicators of positive and negative transmission of hepatitis B virus to their infants. The New England journal of medicine 294: 746-749.
146. Okamoto H, Imai M, Kametani M, Nakamura T, Mayumi M (1987) Genomic heterogeneity of hepatitis B virus in a 54-year-old woman who

contracted the infection through materno-fetal transmission. The Japanese journal of experimental medicine 57: 231-236.
147. Okamoto H, Tsuda F, Sakugawa H, Sastrosoewignjo RI, Imai M, et al. (1988) Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. The Journal of general virology 69 (Pt 10): 2575-2583.
148. Okamoto H, Yano K, Nozaki Y, Matsui A, Miyazaki H, et al. (1992) Mutations within the S gene of hepatitis B virus transmitted from mothers to babies immunized with hepatitis B immune globulin and vaccine. Pediatric research 32: 264-268.
149. Okochi K, Murakami S (1968) Observations on Australia antigen in Japanese. Vox sanguinis 15: 374-385.
150. Olinger CM, Weber B, Otegbayo JA, Ammerlaan W, van der Taelem-Brule N, et al. (2007) Hepatitis B virus genotype E surface antigen detection with different immunoassays and diagnostic impact of mutations in the preS/S gene. Medical microbiology and immunology 196: 247-252.
151. Olotu CIF (2008) Analyse von virusspezifischen Resistenzfaktoren und phylogenetischer Entwicklung von Hepatitis B Viren unter Therapie mit Adefovir. Hamburg: university Hamburg.
152. Ono SK, Kato N, Shiratori Y, Kato J, Goto T, et al. (2001) The polymerase L528M mutation cooperates with nucleotide binding-site mutations, increasing hepatitis B virus replication and drug resistance. The Journal of clinical investigation 107: 449-455.
153. Oon CJ, Lim GK, Ye Z, Goh KT, Tan KL, et al. (1995) Molecular epidemiology of hepatitis B virus vaccine variants in Singapore. Vaccine 13: 699-702.
154. Osiowy C ,Giles E, Tanaka Y, Mizokami M, Minuk GY (2006) Molecular evolution of hepatitis B virus over 25 years. Journal of virology 80: 10307-10314.
155. Park SH, Jung JK, Lim JS, Tiwari I, Jang KL (2011) Hepatitis B virus X protein overcomes all-trans retinoic acid-induced cellular senescence by downregulating levels of p16 and p21 via DNA methylation. The Journal of general virology 92: 1309-1317.
156. Perrillo R (2009) Benefits and risks of interferon therapy for hepatitis B. Hepatology 49: S103-111.
157. Ott JJ, Stevens GA, Groeger J, Wiersma ST (2012) Global epidemiology of hepatitis B virus infection: new estimates of age-specific HBsAg seroprevalence and endemicity. Vaccine 30: 2212-2219.
158. Peterson DL (1981) Isolation and characterization of the major protein and glycoprotein of hepatitis B surface antigen. The Journal of biological chemistry 256: 6975-6983.
159. Pollicino T, Isgro G, Di Stefano R, Ferraro D, Maimone S, et al. (2009) Variability of reverse transcriptase and overlapping S gene in hepatitis B virus isolates from untreated and lamivudine-resistant chronic hepatitis B patients. Antiviral therapy 14: 649-654.
160. Prince AM (1968) An antigen detected in the blood during the incubation period of serum hepatitis. Proceedings of the National Academy of Sciences of the United States of America 60: 814-821.

161. Qi X, Xiong S, Yang H, Miller MD, Delaney W (2006) In vitro susceptibility of HBV polymerase encoding mutations acquired during adefovir dipivoxil therapy to other Anti-HBV agents. Hepatology 44: 252A.
162. Quiros-Roldan E, Calabresi A, Lapadula G, Tirelli V, Costarelli S, et al. (2008) Evidence of long-term suppression of hepatitis B virus DNA by tenofovir as rescue treatment in patients coinfected by HIV. Antiviral therapy 13: 341-348.
163. Rabe B, Glebe D, Kann M (2006) Lipid-mediated introduction of hepatitis B virus capsids into nonsusceptible cells allows highly efficient replication and facilitates the study of early infection events. Journal of virology 80: 5465-5473.
164. Ragheb M, Elkady A, Tanaka Y, Murakami S, Attia FM, et al. (2012) Multiple intra-familial transmission patterns of hepatitis B virus genotype D in north-eastern Egypt. Journal of medical virology 84: 5.595-87
165. Rall LB, Standring DN, Laub O, Rutter WJ (1983) Transcription of hepatitis B virus by RNA polymerase II. Molecular and cellular biology 3: 1766-1773.
166. Reynaud L, Carleo MA, Talamo M, Borgia G (2009) Tenofovir and its potential in the treatment of hepatitis B virus. Therapeutics and clinical risk management 5: 177-185.
167. Robinson WS, Clayton DA, Greenman RL (1974) DNA of a human hepatitis B virus candidate. Journal of virology 14: 384-391.
168. Robinson WS, Greenman RL (1974) DNA polymerase in the core of the human hepatitis B virus candidate. Journal of virology 13: 1231-1236.
169. Robinson WS, Lutwick LI (1976) The virus of hepatitis, type B (first of two parts). The New England journal of medicine 295: 1168-1175.
170. Saito I, Oya Y ,Yamamoto K, Yuasa T, Shimojo H (1985) Construction of nondefective adenovirus type 5 bearing a 2.8-kilobase hepatitis B virus DNA near the right end of its genome. Journal of virology 54: 711-719.
171. Sandler SG, Courouce AM, Soulier JP (1978) wVariants of the hepatitis B surface antigen in Israeli populations. Vox sanguinis 34: 149-151.
172. Santantonio T, Fasano M, Durantel S, Barraud L, Heichen M, et al. (2009) Adefovir dipivoxil resistance patterns in patients with lamivudine-resistant chronic hepatitis B. Antiviral therapy 14: 557-565.
173. Sayan M, Dogan C (2012) Genotype/subgenotype distribution of hepatitis B virus among hemodialysis patients with chronical hepatitis B. Annals of hepatology 11: 849-854.
174. Schulze A, Gripon P, Urban S (2007) Hepatitis B virus infection initiates with a large surface protein-dependent binding to heparan sulfate proteoglycans. Hepatology 46: 1759-1768.
175. Seeger C, Zoulim, F., Mason, W. S. (2007) Hepadnaviruses. In: Knipe DM, and Howley, P. M. , editor. Fields Virology. fifth ed: Lippincott Williams & Wilkins. pp. 2978-3031.
176. Seeger C, Mason WS (2000) Hepatitis B virus biology. Microbiology and molecular biology reviews : MMBR 64: 51-68
177. . Soussan P, Pol S, Garreau F, Brechot C, Kremsdorf D (2001) Vaccination of chronic hepatitis B virus carriers with preS2/S envelope

protein is not associated with the emergence of envelope escape mutants. The Journal of general virology 82: 367-371.
178. Sheldon J, Soriano V (2008) Hepatitis B virus escape mutants induced by antiviral therapy. The Journal of antimicrobial chemotherapy 61: 766-768.
179. Sprengel R, Kaleta EF, Will H (1988) Isolation and characterization of a hepatitis B virus endemic in herons. Journal of virology 62: 3832-3839.
180. Standring DN, Bridges EG, Placidi L, al. e (2001) Antiviral beta-L-nucleosides specific for hepatitis B virus infection. Antivir Chem 12: 119-129
181. Stevens CE, Beasley RP, Tsui J, Lee WC (1975) Vertical transmission of hepatitis B antigen in Taiwan. The New England journal of medicine 292: 771-774..
182. Stuyver LJ ,Locarnini SA, Lok A, Richman DD, Carman WF, et al. (2001) Nomenclature for antiviral-resistant human hepatitis B virus mutations in the polymerase region. Hepatology 33: 751-757.
183. Summers J, O'Connell A, Millman I (1975) Genome of hepatitis B virus :restriction enzyme cleavage and structure of DNA extracted from Dane particles. Proceedings of the National Academy of Sciences of the United States of America 72: 4597-4601.
184. Svicher V, Cento V, Salpini R, Mercurio F, Fraune M, et al. (2011) Role of hepatitis B virus genetic barrier in drug-resistance and immune-escape development. Digestive and liver disease : official journal of the Italian Society of Gastroenterology and the Italian Association for the Study of the Liver 43: 975-983.
185. Swenson PD, Escobar MR, Carithers RL, Jr., Sobieski TJ, 3rd (1983) Failure of preexisting antibody against hepatitis B surface antigen to prevent subsequent hepatitis B infection. Journal of clinical microbiology 18: 305-309.
186. Tenney DJ, Levine SM, Rose RE ,Walsh AW, Weinheimer SP, et al. (2004) Clinical emergence of entecavir-resistant hepatitis B virus requires additional substitutions in virus already resistant to Lamivudine. Antimicrobial agents and chemotherapy 48: 3498-3507.
187. Thompson AJ, Nguyen T ,Iser D, Ayres A, Jackson K, et al. (2010) Serum hepatitis B surface antigen and hepatitis B e antigen titers: disease phase influences correlation with viral load and intrahepatic hepatitis B virus markers. Hepatology 51: 1933-1944.
188. Tiollais P, Pourcel C, Dejean A (1985) The hepatitis B virus. Nature 317: 489-495.
189. Touze E, Fourrier A, Rue-Fenouche C, Ronde-Oustau V, Jeantaud I, et al. (2002) Hepatitis B vaccination and first central nervous system demyelinating event: a case-control study. Neuroepidemiology 21: 180-186.
190. Treinin M, Laub O (1987) Identification of a promoter element located upstream from the hepatitis B virus X gene. Molecular and cellular biology 7: 545-548.
191. Tuaillon E, Mondain AM, Nagot N, Ottomani L, Kania D, et al (2012) . Comparison of serum HBsAg quantitation by four immunoassays, and relationships of HBsAg level with HBV replication and HBV genotypes. PloS one 7: e32143.

192. Van Bommel F, de Man RA, Wedemeyer H, Deterding K, Petersen J, et al. (2010) Long-term efficacy of tenofovir monotherapy for hepatitis B virus-monoinfected patients after failure of nucleoside/nucleotide analogues. Hepatology 51: 73-80.
193. Van Bommel F, Wunsche T, Mauss S, Reinke P, Bergk A, et al. (2004) Comparison of adefovir and tenofovir in the treatment of lamivudine-resistant hepatitis B virus infection. Hepatology 40: 1421-1425.
194. Van Bommel F, Zollner B, Sarrazin C, Spengler U, Huppe D, et al. (2006) Tenofovir for patients with lamivudine-resistant hepatitis B virus (HBV) infection and high HBV DNA level during adefovir therapy. Hepatology 44: 318-325.
195. Van Damme P (2001) Hepatitis B: vaccination programmes in Europe--an update. Vaccine 19: 2375-2379.
196. Villeneuve JP, Durantel D, Durantel S, Westland C, Xiong S, et al. (2003 (Selection of a hepatitis B virus strain resistant to adefovir in a liver transplantation patient. Journal of hepatology 39: 1085-1089.
197. Villet S, Pichoud C, Villeneuve JP, Trepo C, Zoulim F (2006) Selection of a multiple drug-resistant hepatitis B virus strain in a liver-transplanted patient. Gastroenterology 131: 1253–1261.
198. Wallace WA, Carman WF (1997) Surface Variation of HBV: scentific and medical relevance. viral Hepatitis Rev 3: 5-16.
199. Wands JR, Fujita YK, Isselbacher KJ, Degott C, Schellekens H, et al. (1986) Identification and transmission of hepatitis B virus-related variants. Proceedings of the National Academy of Sciences of the United States of America 83: 6608-6612.
200. Waters JA, Kennedy M, Voet P, Hauser P, Petre J, et al. (199 (2Loss of the common "A" determinant of hepatitis B surface antigen by a vaccine-induced escape mutant. The Journal of clinical investigation 90: 2543-2547.
201. Weinberger KM, Bauer T, Bohm S, Jilg W (2000) High genetic variability of the group-specific a-determinant of hepatitis B virus surface antigen (HBsAg) and the corresponding fragment of the viral polymerase in chronic virus carriers lacking detectable HBsAg in serum. The Journal of general virology 81: 1165-1174.
202. Werle-Lapostolle B, Bowden S, Locarnini S, Wursthorn K, Petersen J, et al. (2004) Persistence of cccDNA during the natural history of chronic hepatitis B and decline during adefovir dipivoxil therapy. Gastroenterology 126: 1750-1758.
203. WHO (1977) Advances in viral hepatitis. In :Hepatitis WECoV, editor. Geneva
204. . Wilson JN, Nokes DJ, Carman WF (2000) Predictions of the emergence of vaccine-resistant hepatitis B in The Gambia using a mathematical model. Epidemiology and infection 124: 295-307.

205. Yamamoto K, Horikita M, Tsuda F, Itoh K, Akahane Y, et al. (1994) Naturally occurring escape mutants of hepatitis B virus with various mutations in the S gene in carriers seropositive for antibody to hepatitis B surface antigen. Journal of virology 68: 2671-2676.

206. Yang H, Westland CE, Delaney WEt, Heathcote EJ, Ho V, et al. (2002) Resistance surveillance in chronic hepatitis B patients treated with adefovir dipivoxil for up to 60 weeks. Hepatology 36: 464-473.
207. Yao GB (1996) Importance of perinatal versus horizontal transmission of hepatitis B virus infection in China. Gut 38 Suppl 2: S39-42.
208. Ying Ma, Yang Ding, Juan Feng and Xiao Guang Dou (2011) Genotyping the hepatitis B virus with a fragment of the HBV DNA polymerase gene in Shenyang, China 8:315
209. Yu H, Yuan Q, Ge SX, Wang HY, Zhang YL, et al. (2010) Molecular and phylogenetic analyses suggest an additional hepatitis B virus genotype "I". PloS onzje 5: e9297.
210. Yu JW, Sun LJ, Yan BZ, Kang P, Zhao YH (2011) Lamivudine treatment is associated with improved survival in fulminant hepatitis B. Liver international : official journal of the International Association for the Study of the Liver 31: 499-506.
211. Zekri AR, Hafez MM, Mohamed NI, Hassan ZK, El-Sayed MH, et al. (2007) Hepatitis B virus (HBV (genotypes in Egyptian pediatric cancer patients with acute and chronic active HBV infection. Virology journal 4: 74.
212. Zhang Z, Torii N, Hu Z, Jacob J, Liang TJ (2001) X-deficient woodchuck hepatitis virus mutants behave like attenuated viruses and induce protective immunity in vivo. The Journal of clinical investigation 108: 1523-1531.
213. Zoulim F, Seeger C (1994) Reverse transcription in hepatitis B viruses is primed by a tyrosine residue of the polymerase. Journal of virology 68: 6-13
214. Zoulim F, Locarnini S (2009) Hepatitis B virus resistance to nucleos(t)ide analogues. Gastroenterology 137: 1593-1608 e1591-1592..