Hepatitis B virus genotypes and pre-core/core-promoter mutations in Palestinian patients with chronic HBV and liver complications

By:

Sawsan Sudqi Said

Supervisor:

Dr. Mahmoud A. Srour

This thesis was submitted in partial fulfillment of the requirements for the Master's Degree in Clinical Laboratory Science from the Faculty of Graduate Studies at Birzeit University –Palestine

June, 2008

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تحديد الأنماط الجينية و التغيرات الوراثية في الجين ما قبل الرئيس و موقع التحكم في الجين الرئيس في فيروس التهاب الكبد الوبائي (ب) في المرضى الفلسطينيين المصابين بالتهاب الكبد الوبائي (ب) المزمن ومضاعفات المرض على الكبد

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This thesis was successfully defended and approved on Tuesday 17-06-2008

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2. Dr. Mohammad Farraj (Internal Examiner)	……..
3. Dr. As'ad Ramlawi (External Examiner)	……..

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Dedication

To my parents who taught me the value of education and pushed me to achieve more and more. Thank you for all the unconditional love, guidance, and support that you have always given me, helping me to succeed and instilling in me the confidence that made me capable of doing anything I put my mind to. Thank you for everything. I love you!

تحديد الأنماط الجينية و التغيرات الوراثية في الجين ما قبل الرئيس و موقع التحكم في الجين الرئيس في فيروس التهاب الكبد الوبائي (ب) في المرضى الفلسطينيين المصابين بالتهاب الكبد الوبائي (ب) المزمن ومضاعفات المرض على الكبد

إعداد: سوسن صدقي سعيد

إشراف: د. محمود عبد الرحمن سرور

ملخص

هناك ثماني أنماط جينية لفيروس التهاب الكبد الوبائي (ب), والتي تمتاز بتوزيع جغرافي مميز لكل منها. و يبدو أن هذه الأنماط الجينية تؤثر على التطور السريري للمرض. في حين أن التغيرات الو راثية في الجين ما قبل الرئيس (PC) وفي موقع التحكم الأساسي في الجين الرئيس (BCP) قَدْ تتنبأُ بتطويرَ السرطانِ الكبدي.

في هذه الدراسة، تم دراسة 150 عينة تم جمعها في منطقة الضفة الغربية. و قد صنف المرضى إلى ثلاث مجموعات, (1) التهاب الكبد المزمن النشط (ع = 118), (2) تشمع الكبد (ع = 27) و (3) سرطان الكبد (ع = 5). ولدراسة الأنماط الجينية لفيروس التهاب الكبد الوبائي (ب) تم استخدام تقنية تفاعل البلمرة المتسلسل ((PCR المتعدد والمتداخل والمحددة للجينS لتحديد سبعة أنماط جينية, .بينما النمط الجيني الثامن (G) فقد تمت دراسته في موقع الجين الرئيس (core) والذي يحتوي على صيغة مميزة لهذا النمط ناتجة عن إدخال 36 نيوكليوتيدة (bp) في الجينِ الرئيس. تم التحقق من النتائج السابقة بتحليل تسلسل الأحماض النووية لهذه الأنماط و كذلك بتحليل التطور النوعي. في حين استخدمت تقنية((RFLP لدراسة الطفرات أو التغيرات الو راثية في الجين ما قبل الرئيس (PC) وفي موقع التحكم الأساس في الجين الرئيس (BCP).

و قد أظهرت الدراسة أن النمطين الجينيين D) 25,3 ٪) و A) 74, ٪) (هما الأكثر انتشارا في منطقة الضفة الغربية / فلسطين, إضافة إلى وجود انتشارا كبيرا و ملحوظا للانماط المختلطة و خاصة النمطين D+ A 35,3٪)). أما النمطين B و C فقد وجدت كجزء من عدوى من أنماط مختلطة ولكن ليس كنمط وحيد. و أظهرت العدوى المختلطة انتشارا أكبر بين المرضى الذين يعانون من تشمع الكبد (19 من 27) مقارنة بالمرضى الذينلا يعانون من تشمع الكبد (77 من 123). و قد أظهرت الدراسة أيضا أن نسبة عالية من مرضى

التهاب الكبد الوبائي لديهم سلبية ل HBeAg. ولا سيما المرضى المصابين بعدوى مختلطة نتيجة النمطين (D+A (٪71, 1.

وفيما يتعلق بالطفرات BCP و precoreو codon 15 varaints , فقد وجدت الطفرة (BCP) بنسبة أعلى من الطفرات الجينية الأخرى بين المرضى الفلسطينين, وتم إيجاد علاقة ذات دلالة إحصائية ($p<0.03$) ما بين طبيعة الأنماط الجينية للالتهاب الكبد الوبائي (ب) وطفرة في لأساسا موقع التحكم الجين الرئيس (BCP).لفيروس الكبد (ب), ومن ناحية أخرى أظهرت هذه الدراسة عدم وجود علاقة إحصائية ما بين الطفرة (PC) والأنماط الجينية. ولكنها أظهرت شيوعا أكثر في المرضى الفلسطينين الذين يعانون من تشمع الكبد الوبائي.

وفي النتيجة, فقد بينت هذه الدراسة إن النمط الجيني Dهو الأكثر انتشارا إضافة إلى وجود ارتفاع ملحوظ في انتشار الأنماط المختلطة ولا سيما النمطين D+A بين المرضى الفلسطينيين. و تشير هذه النتائج أيضا إلى إن الإصابة بالأنماط المختلطة قد يرتبط بمضاعفات سريرية للالتهاب الكبد الوبائي . كما أن وجود طفرة (PC) يعتبر كمؤشر ايجابي للدلالة عن تشمع الكبد وسوء الحالة السريرية, في حين أن وجود الطفرة (BCP) يعتبر كمؤشر سلبي لتطور سرطان الكبد.

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Abstract

There are eight genotypes (A-H) of hepatitis B virus (HBV). The HBV genotypes show distinct geographic distribution and have been shown to influence the clinical course of infection. Genetic mutations in the Pre-core C (PC) and basal Core Promoter (BCP) may predict the development of hepatocellular carcinoma.

In this study, we have genotyped 150 HBV clinical isolates from West Bank region, Palestine. Patients were categorized into three groups, (i) chronic active hepatitis (n=118), (ii) liver cirrhosis (n=27) and (iii) hepatocellular carcinoma (n=5). Genotyping was performed by multiplex nested-PCR using genotype-specific primers complementary to pre-S1/S gene for A-F genotypes. While genotype G was genotyped by nested PCR using primers specific for a 36_bp insertion in the core gene. Genotyping was verified by DNA sequencing and phylogenetic analysis. PCR-RFLP analysis was used to investigate PC / BCP mutations.

The most prevalent genotypes were genotype D (25.3 %) and A (4.7 %) in the West Bank region/Palestine. Remarkably a high prevalence of mixed HBV genotypes-infections mainly due to A+D (35.3 %) were observed. Genotypes B and C were observed only as part of a mixed infection but not as a single genotype. Infection with mixed genotypes was more predominant among patients with liver cirrhosis (19/27)

than patients without cirrhosis (77/123). The prevalence of negative HBeAg (68 %) was higher than positive HBeAg among our study population. However, mixed genotypes particularly A+D (71.1 %), tend to predominate among negative HBeAg patients compared to positive HBeAg patients.

Concerning the BCP mutation and precore region/ codon 15 variants, the BCP mutation was the predominant mutation among HBV isolates analysed in this study. A statistically significant correlation was observed between the HBV genotypes and BCP mutation ($p<0.03$). Analysis of the correlation between the HBV genotypes and PC mutation ($p<0.16$) and codon 15 variants ($p<0.16$), showed no significant correlation ($p<0.16$). But PC mutation tends to occur more predominantly among cirrhosis patients than CAH patients.

This study showed that genotype D as the most prevalent one, and a markedly high prevalence of mixed A+D genotypes' infections among Palestinian patients with chronic HBV. These findings indicate that mixed genotypes' infection is associated with a higher risk for development of liver complications or bad clinical outcome (bad prognosis). The PC mutation is a positive predictive marker for liver cirrhosis and the presence of BCP mutation (G1896A) is a negative predictor for HCC.

ACKNOWLEDGMENT

I would like to acknowledge Dr. Tamer Essawi, Head of Clinical Laboratory Science Program, for providing support and helpful discussions during preparation of this study as well as in facilitating the cooperation with the Central Public Health Laboratory at Ramallah necessary for conducting this study.

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The words are unable to express my thanks and gratitude for Dr. As'ad Ramalwi, for understanding the significance of this study to palestinian hepatitis patients, infinite and moral support for this research. Dr. Ramalwi has also kindly provided me access to all the facilities necessary for conducting this research, providing fund from UNFPA, which overall allowed me to successfully accomplish my research goals in a comfortable work environment at the Central Public Heath Laboratory, Ministry of Health at Ramallah.

Special thanks for all physicians and clinical staff in preventive medicine department-MOH for their kind cooperation in data collection and filling the study questionnaire.

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I would like to thank my colleagues at the Molecular Biology division at the Central Public Heath Laboratory- MOH, Mr. Issa Eshtia and Miss Hiba Abu Zayyad as well as Mr. Shadi Rifa'i at the Clinical Laboratory Science program- Birzeit University, for their kind cooperation and for the excellent work environment during this work.

I would like to thank all my friends for whom I shared the hard moments. Warm and special thanks also go to my parents for their support and effort to achieve this success.

I Introduction

Hepatitis B virus (HBV) infection is a global public health problem. Of the two billion persons worldwide who come in contact with HBV, 400 million fail to resolve acute viral infection and become chronic carriers. Seventy five percent of these live in Asian countries (Zuckerman & Zuckerman, 2000; Seeger, *et al*., 2007). Infection with HBV can lead to severe liver complications such as cirrhosis and hepatocellular carcinoma (HCC) (Lin & Kao, 2008).

Human HBV, a prototype strain of the family *Hepadnaviridae*, is a circular, partially double-stranded DNA virus of approximately 3200 nucleotides and contains four open reading frames. HBV strains isolated worldwide have been classified into nine different serological subtypes, (ayw1, ayw2, ayw3, ayw4, ayr, adw2, adw4, adrq+ and adrq) (Stuyver, 2000; Kao, 2002) and eight different genotypes (A-H) (Schaefer, 2007; Seeger, *et al*., 2007) reflecting the genetic variability of HBV. The HBV genotypes have distinct geographical distribution and some genotypes correlate with the severity of liver disease (Sugiyama, *et al*., 2007; Yuan *et al.,* 2007).

HBV mutants have recently been identified in patients with acute, fulminant or chronic infections. Mutations in the basic core promoter (BCP) of HBV and pre-core region were previously reported in association with severe chronic hepatitis (Yuan, *et al*., 2007). The prevalence of the pre-core mutation (PC) is variable, being high in the Mediterranean region and in the Far East and rare in the USA and northern Europe (Hadziyannis, 2001). There is a clear association between HBV genotypes and PC/BCP mutations. The genotyping of HBV can predict the risk of adverse outcomes (fulminant

disease, cirrhosis, and HCC) and can influence decision-making in management of HBV patients (Kidd-Ljunggren, 2002; Kato, *et al*., 2005).

I.1 HBV genome

HBV has a partially double-stranded DNA genome that is covalently linked to the viral reverse transcriptase (RT) at the 5' end of the full length negative strand (Delius, 1983). The complete genome is 3020-3320 nucleotides long (for the full length negative strand) or 1700-2800 nucleotides long (for the short length positive strand), which is incomplete and lacks from 15-50 % of its nucleotide complement. The double stranded DNA is held in relaxed circular configuration by overlapping of several hundred nucleotides at the 5' extremities of the negative and positive strands, forming a direct repeat sequence of complementary base pairs (Shafritz & Lieberman, 1984). The double stranded genome has a nick at a unique site on full length negative strand opposite at a position 242 nucleotides downstream from the 5' end of the positive strand (Fig 1) (kidd-Ljunggren, 2002).

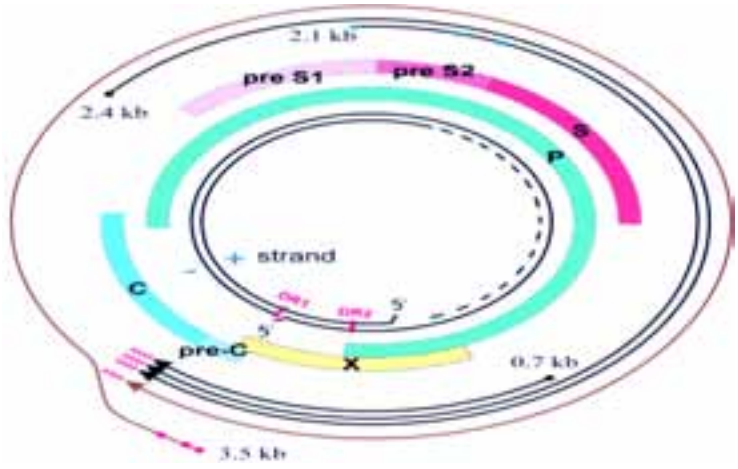

Figure 1**. HBV genome map.** Four main classes of transcripts (arrows) the longest of which corresponds to pregenomic RNA (brown). Variations in 5´end positions for the 2.1 and 3.5 kb size classes are shown with blue and red dots, respectively. Colored boxes represent protein-coding regions. DR1 and DR2 are 10-12 bp direct repeat sequences which play a role in priming the synthesis of the minus- and plus-strand DNA (Kidd-Ljunggren, 2002).

I.2 Structure of HBV virion

HBV genome contains four Open Reading Frames (ORFs) encoding: the envelope (Pre S1, Pre S2, S), core (core, pre core), polymerase and X protein. The S-gene (nt 2848 to nt 833; type ayw3) codes a 226 aa protein, named major antigen (S protein or HBs Ag). The preS2 gene codes a 55 aa protein, which together with the previous major antigen form the medium antigen (M protein). The preS1 gene codes a 108 aa protein which together with the previous medium antigen form the large antigen (L protein). The C-gene (nt 1814 to 2450) codes the core protein (HBcAg) which forms the viral nucleocapsid. The pre-core gene (nts 1816-2457) codes for the pre-core protein or the e-antigen (HBeAg). Both the HBcAg and HBeAg are important diagnostic tools and can be used to determine the status of ongoing HBV infections. The smallest viral X-gene (nt 1374 to 1836) encodes the HBx or X protein which is required for infection *in vivo* and probably has various gene-regulating functions. The polymerase gene (nt 2307 to 1621) codes the viral DNA polymerase (Fig 1) (Kreutz, *et al*., 2002).

HBsAg is found in several forms in serum, 22-nm spherical aggregates and filamentous or tubular structures of 22-nm in diameter and up to 1000 nm long. Both of these structures are composed specifically of the viral envelope protein (HBsAg) and do not contain nucleic acids. A third structure, present in lesser amounts in serum, is comprised of an outer envelope and inner core (or nucleocapsid) and contains both DNA and protein. The latter structure, the so-called Dane particle, is 42 nm in diameter, has the morphologic appearance of a virus, and represents the infectious hepatitis B virion (Shafritz & Lieberman, 1984).

I.2.1 HBV surface proteins

The HBV surface or S-coding region is divided into three parts by two internal initiation codons: the pre-S1, pre-S2, and S regions. The production of the three forms of surface protein (L, M and S) is controlled by two tandem promoters. The upstream pre-S1 promoter controls the transcription of a 2.4-kb transcript that encodes L protein only. The downstream S promoter, 240 bp away, specifies transcripts with heterogeneous 5' termini, with the largest transcript encoding the M protein and the remaining transcript encoding the S protein (Huang, *et al*., 2006).

I.2.2 HBV pre-core and core proteins

The pre-core/core region encodes hepatitis B e antigen (HBeAg) and core antigen (HBcAg). These two proteins are also derived by alternative initiation of translation at two in-frame AUG codons. The internal AUG encodes the 21-kD C protein, the structural polypeptide of the viral capsid, whereas the upstream AUG directs production of the 24-kD PC protein. The PC region encodes a signal sequence, which directs the chain into the secretory pathway. As the chains traverse the Golgi complex, cleavage by cellular proteases generates HBeAg, a 16-kD fragment that is secreted into the blood. The HBeAg is not required for viral assembly or viral replication but it might exert a role in the regulation of the immune response against HBV, particularly against the core protein (Ganem & Prince, 2004; Bruss, 2007).

I.2.3 HBV DNA polymerase and X proteins

The pol coding region is specific for the viral DNA polymerase or reverse transcriptase (RT), a multifunctional enzyme involved in DNA synthesis and RNA encapsidation.

The RT has three functional domains and a hinge region, known as the spacer. The three domains are: the N-terminal terminal protein domain (TP), the RT domain and the C-terminal RNase H domain. The X open reading frame encodes the viral X protein (HBx), which modulates the host-cell signal transduction and can directly and indirectly affect host and viral gene expression. X-protein activity is absolutely required for the *in vivo* replication and spread of the virus (Ganem & Prince, 2004; Beck & Nassal, 2007).

I.3 HBV replication cycle

Hepatocytes are the primary site of viral replication. In addition, lymphocytes are a second reservoir for virus persistence. During initiation of infection the viral particles contain predominately relaxed-circular DNA (rcDNA) with a complete minus strand and a partially synthesized plus strand as well as a small amount of double-stranded linear DNA (DSL) genomes. Following transport of the viral genome into the host cell nucleus the rcDNA genome with RT attached to the 5'end of the minus strand and a short RNA attached to the 5' end of the plus strand, is converted to the covalently closed circular DNA form, called CCC DNA. During this process, both the RT and the RNA are removed. The CCC DNA serves as the transcriptional template for host RNA polymerase II. This enzyme generates a series of genomic and subgenomic transcripts (Seeger & Mason, 2000).

The replication cycle of HBV include attachment, endocytosis and fusion of the virus particle to hepatocytes, delivery of the released nucleocapsid to the nucleus, release and repair of the partially double stranded DNA genome by cellular enzymes, transcription and transport of pre-genomic and subgenomic viral RNAs, translation of the pre-genomic (core and polymerase) and subgenomic (surface proteins and X-protein)

RNAs, encapsidation of the pre-genomic RNA and the polymerase into RNA-containing nucleocapsids, reverse transcription into DNA containing particles that are either re-transported into the nucleus or enveloped and secreted as virions (Fig 2). Once partially double-stranded DNA has been produced, nucleocapsids can undergo a maturation event that facilitates their acquisition of an outer envelope via budding into the ER (Seeger & Mason 2000*;* Ganem & Prince, 2004).

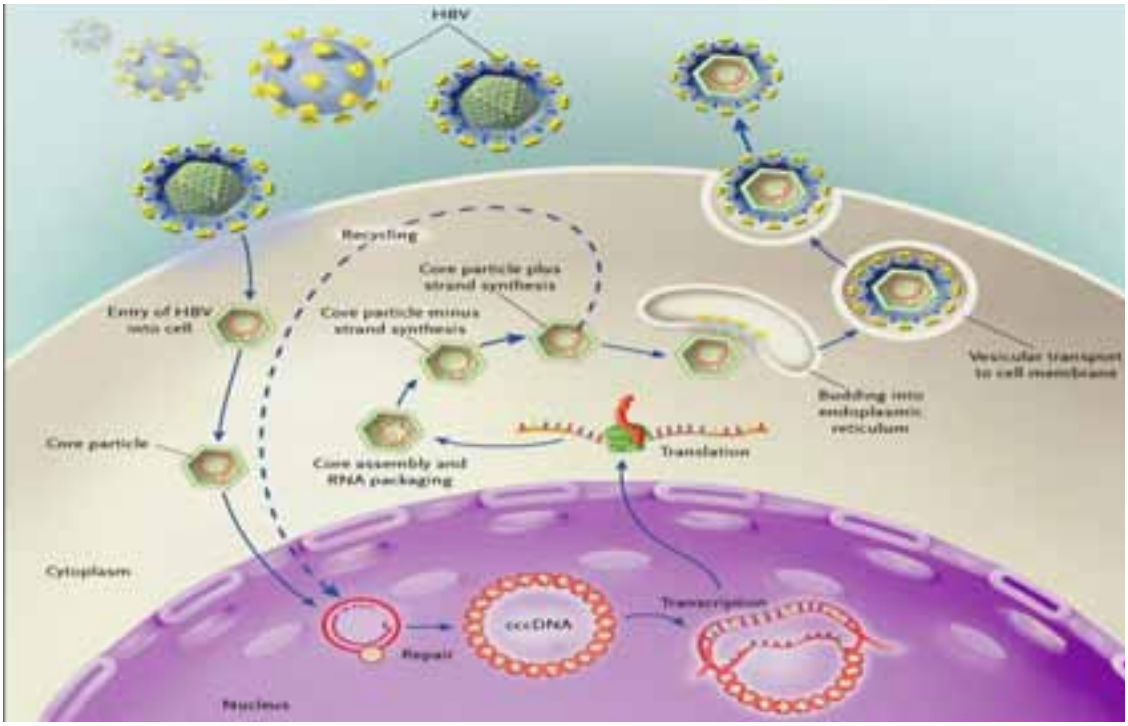

Figure2**. The Replication Cycle of HBV**. HBV virion bind to surface receptors and are internalized. Viral core particles migrate to the hepatocyte nucleus, where their genomes are repaired to form a covalently closed circular DNA (cccDNA) that is the template for viral messenger RNA (mRNA) transcription (Ganem & Prince, 2004).

I.4 Pathogenesis of HBV infection

The clinical spectrum of HBV infection ranges from subclinical to acute symptomatic hepatitis or rarely fulminant hepatitis during the acute phase. Chronic infection is defined as the persistent presence of HBsAg in the serum of an individual for 6 months or longer. Chronic infection occurs in 90 % of infants infected at birth, 30 % of

children infected at an age of 1 - 5 years, 6 % of persons infected after 5 years of age (Robinson, 1995; Mhoney & Kane, 1999). Only 3 to 5 % of adults remain chronically infected and the remainders have acute infections resulting in viral clearance (Lee, 1997). Humans with persistent HBV infection have high levels of viral replication in more than 95 % of the hepatocytes in the liver, and up to 1×10^{10}/ml viral particles and 5×10^{12}/ml non-infectious surface antigen particles in serum (Huang, 2006).

I.4.1 Primary infection

In primary infection, HBsAg becomes detectable in the blood after an incubation period of 4 to 10 weeks, followed shortly by antibodies against the HBV core antigen (anti-HBc antibodies), which early in infection are mainly of the IgM isotype. Viremia is well established by the time HBsAg is detected, and virus titers in acute infection are very high, frequently 10^9 to 10^{10} virions per ml. Circulating HBeAg becomes detectable in most cases. Notably, in most acute hepatitis cases, the infection can be cleared from virtually all hepatocytes without massive hepatic destruction, with clearance of the infection, the viral antigens HBsAg and HBeAg disappear from the circulation, and free anti-HBs antibodies become detectable. However, low levels of HBV DNA in the blood may persist for many years, if not for life. It is not known whether this DNA contains the entire HBV genome, or even whether it is contained in virions (Ganem & Prince, 2004).

I.4.2 Chronic infection

In persistent HBV infection HBsAg remains in the blood and virus production continues, often for life. However, levels of viremia in chronic infection are generally

substantially lower than those during primary infection (Ganem & Prince, 2004). The prognosis for many chronic carriers of HBV who were infected with HBV in the adulthood is poor. The natural history of HBV persistence suggests that there is an ongoing immune attack on infected cells in the liver, an attack that is usually inadequate to eradicate infection altogether, but that does reduce the number of infected cells and thereby lowers the circulating viral load. About 10 to 25 % of patients will die of either liver cancer or cirrhosis (tissue scarring). While smaller number of patients particularly those infected as adults, will die or require liver transplantation due to rapidly progressing liver disease leading to hepatic failure **(**Seeger & Mason**,** 2000**).**

The widely held view that circulating viral DNA disappears when anti-HBe antibodies appear is incorrect. The latter idea reflects the fact that, for many years, HBV DNA was measured by relatively insensitive hybridization methods with a detection limit of 10^5 to 10^6 virions per ml. However, with the implementation of the polymerase-chain-reaction (PCR) method for detection and quantitation of HBV DNA, it's now known that at least 70 to 85 % of people with anti-HBe antibodies have detectable viral DNA in the circulation, typically in the range of 10^3 to 10^5 virions per ml, and sometimes higher. The HBeAg negative carriers are a heterogeneous group. Most such carriers have low levels of viral DNA, relatively normal levels of alanine aminotransferase, and a good prognosis (Ganem & Prince, 2004).

I.4.3 Hepatocellular Carcinoma

Chronically infected subjects have a risk of hepatocellular carcinoma that is 100 times as high as that for non-carriers. Within the HBsAg positive group, HBeAg positive carriers have the highest risk of hepatocellular carcinoma. Even, carriers with anti-HBe antibodies have a substantial risk of cancer. Screening for HCC, with monitoring of α-

fetoprotein levels and six-monthly radiographic examination should be considered for patients at increased risk of developing HCC. However the screening test of liver cancer is imperfect, i.e. α-fetoprotein has an excellent negative predictive value, but its positive predictive value ranges from 9 to 30 percent (Ganem & Prince, 2004**;** Buster & Janssen, 2006).

I.5 Mode of transmission

The most important mode of HBV transmissions is perinatal from mother to her baby. The other common modes of HBV transmissions include sexual contact; sharing toothbrushes or razors, sharing unsterilized needles among drug addicts, body or ear-piercing, tattooing, and acupuncture. Furthermore HBV is able to remain on any surface it comes into contact with for about a week without losing infectivity (Robinson, 1995; Hollinger & Liang, 2001). Unlike the HIV virus, HBV transmission can also occur in settings of continuous close personal contact, such as among family members living together **(**Aitken & Jeffrie**,** 2001).

I.6 Diagnosis of HBV infection

Thirty years ago, the diagnosis of chronic hepatitis B (CHB) was thought to require the presence of hepatitis B e-antigen (HBeAg), as a reliable and sensitive marker of hepatitis B virus (HBV) replication. Individuals positive for hepatitis B surface antigen (HBsAg) but negative for HBeAg were considered to have a non-replicative HBV infection, and if their liver enzymes were normal or nearly normal they were referred to as asymptomatic or healthy HBsAg or HBV carriers. Figure 3 shows typical patterns of serologic and molecular markers in both acute self-limited and chronic HBV infection (Ganem & Prince, 2004).

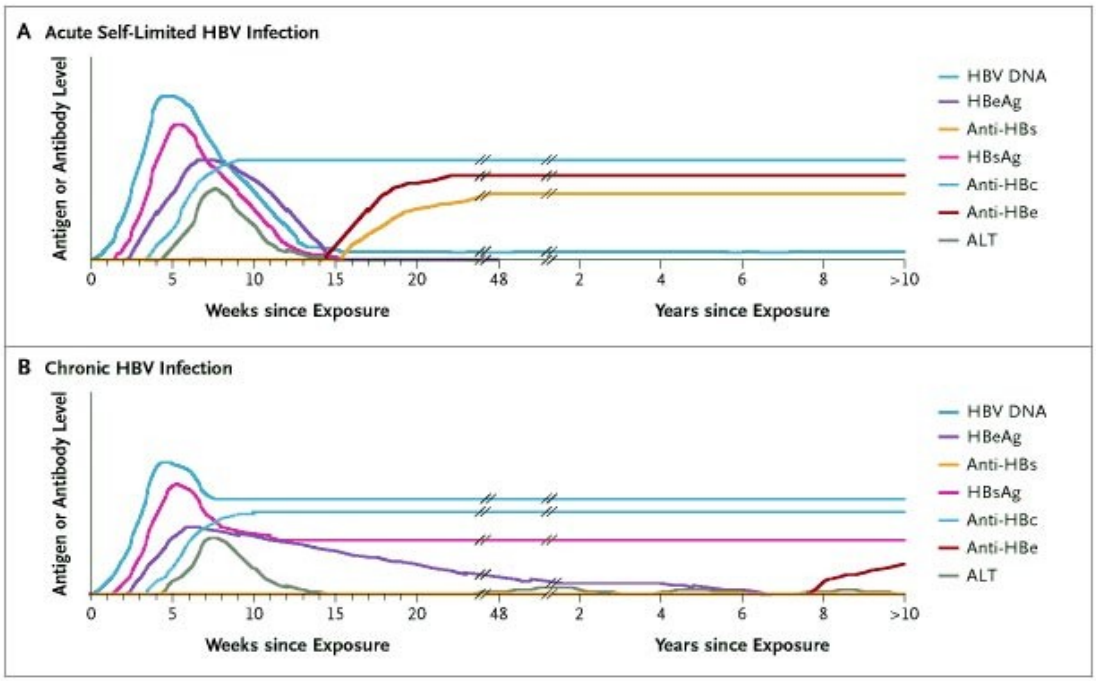

Figure 3**. Patterns of serologic and molecular markers in HBV infection**. Typical levels of alanine aminotransferase (ALT), HBV DNA, hepatitis B s and e antigens (HBsAg and HBeAg), and anti-HBc, anti-HBe, and anti-HBs antibodies are shown in acute and chronic HBV infection. From (Ganem & Prince, 2004).

In the early 1980s it became apparent that HBV could replicate in the absence of HBeAg. Patients from the Mediterranean area, although negative for HBeAg and positive for antibodies to HBeAg (anti-HBe), were reported to have CHB with replicating HBV. The term anti-HBe-positive or HBe Ag negative CHB was then proposed and subsequently became widely accepted. In 1989 the molecular basis of this form of CHB was discovered with the identification of HBV mutations preventing HBeAg formation from an otherwise normally replicating HBV (Jalali1& Alavian, 2006)**.**

Recently, diagnosis of HBV based on a combination of biochemical, serological, virological and DNA tests and histological features have been used to diagnose and classify HBV infection. HBV DNA may be detected in serum using methods that employ DNA hybridization with or without signal amplification and the results of these tests may be expressed qualitatively or quantitatively (The EASL Jury, 2003).

I.7 Treatment

Hepatitis B virus (HBV) infections are treated with antiretroviral drugs such as interferon, lamivudine (Buster & Janssen, 2006). Interferon-alpha was the only therapeutic choice for more than 20 years, but the overall response rate is < 30 %. However, in the advent of nucleoside/nucleotide analogues, lamivudine (LMV), adefovir (ADV) and more recently entecavir (ETV) have been approved for the treatment of chronic hepatitis B (Lung Lai, *et al*., 2006). Lamivudine is given orally, inhibits HBV reverse transcriptase activity and suppresses HBV DNA synthesis. It is very clear that prophylactic treatment with this drug when commenced before chemotherapy for solid tumors or haematological malignancies is highly effective in preventing reactivation in HBsAg positive patients. Problems arise with the emergence of resistant mutants in 14–32 % of patients after one year of therapy and up to 70 % after four years (Leemans, *et al.,* 2007). However, to date, there have been no reports on the efficacy of adefovir and entecavir in the prevention or treatment of HBV reactivation. (Lubel, *et al*., 2007).

I.8 Prevalence of HBV

Of the two billion persons worldwide who come in contact with HBV, 400 millions fail to resolve acute viral infection and become chronic carriers (Zuckermann &

Zuckermann, 2000; Seeger, *et al*., 2007). The world can be divided into three areas where the prevalence of chronic HBV is high (> 8%), intermediate (2-8 %), and low (<2 %) (The EASL Jury, 2003). The prevalence rate of HBV in Palestine was found to be 3.4 % in 2000 for all the population above 8 years of age (Ramlawi & Preventive Medicine Team, 2000). This rate can be considered as intermediate prevalence according to the WHO classification.

I.9 Classification of HBV

HBV exhibits genetic variability which gives rise to the well recognized serotypes and genotypes of the virus. In addition, virus variants may arise during replication as a result of nucleotide misincorporation and in the absence of any proof–reading capacity by the viral polymerase (Zeng, *et al*., 2004).

I.9.1 HBV serotypes

The first classification of HBV isolates was done by serotyping. Within the S-gene different alleles or antigenic determinants have been recognized a, d, y, w (four sub determinants w1 to w4 are described), r and q. The “a” determinant is the major immunogenic region (aa 124 to 147) of HBsAg and is part of all HBs-subtypes. The determinants d/y and r/w are mutually exclusive epitopes. This leads to four main subtypes designated as *ayw, ayr, adw*, and *adr* or nine minor subtypes of the small HBsAg (Kao, 2002). The reagents used in many HBsAg diagnostic assays are directed against epitopes in the “a” determinant, the immunodominant region of HBsAg. The ability to detect HBsAg therefore was of obvious importance for the safety of blood supply, while serotyping was useful for epidemiological studies (Kay & Zoulim, 2007).

I.9.2 HBV genotypes

The first sequence of a complete HBV was published in 1979. By the end of the 1980s, enough full length genome sequences had accumulated in the databases to enable classification of HBV strains by genomic sequence rather than by surface protein antigenicity. Okmoto and his coworkers analyzed 18 full length genomes and divided them into four genotypes, named A to D. HBV genotypes were defined based on divergence of nucleotide sequences exceeding 8 % in the entire genome or 4 % in S gene (Fang*, et al.,* 2004, Yuan, *et al*., 2007). Another four genotypes have been identified, E to H. The diversity within the genotypes has lead to the division of some genotypes into subtypes. To date the HBV genotypes are divided into 24 sub-genotypes that differ by at least 4 % from each other (Glebe, 2007). These genotypes have unique insertions/deletions that may have the potential to alter their replication ability (Sheldon*, et al*., 2006). Each genotype has canonical size, ranging from 3,182 nt for genotype D to 3,248 nt for genotype G. It is interesting to note that genotype F and perhaps genotype H, both lie on the same branch of phylogenetic tree that includes animal HBV, which may suggest that these human genotypes arose from zoonotic infection (Kay & Zoulim, 2007).

I.10 Geographical distribution of HBV genotypes

The evolution of HBV is strikingly highlighted by geographical distribution of the genotypes. Epidemiological studies suggest that these genotypes are common in different parts of the world with various frequencies as follows: genotype A in Western Europe and North America, genotype B in North and Southeastern Asia, genotype C in Asia and Pacific, genotype D in Southern Europe (Mediterranean countries) and Middle East, genotype E in Africa, and genotype F in South America and Alaska,

genotype G in some European countries and North America, and genotype H has been recently reported from Central America (Lai**,** 2003; Palumbo, *et al*., 2007).

However, the distribution of HBV genotypes has not been determined among Palestinian patients with CHB**.** Previous studies that were carried out in the Mediterranean countries such as Turkey, Israel, Egypt and Italy found genotype D as the predominant genotype in patients with CHB (Kao, *et al*., 2000; Saudy, *et al*., 2003; Sunbul & Leblebicioglu, 2005).

A recent study by Zerki *et al*. (2007) indicated the predominance of genotypes D (37.1 %) and B (25.7 %) and a relatively high prevalence of mixed-genotype infections in 15.7 % of 70 Egyptian patients with chronic acute hepatitis. In the former report, the mixed genotypes-infections were mainly caused by A/D (7.1 %), but other combinations of genotypes were also detected such as C/D, B/D and B/C. Another study by Ayed *et al.* (2007) showed the predominance of genotype D among chronic Tunisian patients of hepatitis B virus and that infections caused by mixed genotypes accounted for 12.2 % of all chronic HBV infections.

I.11 HBV genotypes and correlation with clinical outcomes

Several research attempts have been reported to find a correlation between a particular genotype and severe liver disease, but the results were controversial. Preliminary studies in Asia suggest that genotype C is associated more frequently with severe liver disease and HCC than genotype B. Genotype B appears to be associated with seroconversion from HBeAg to anti-HBe at a younger age than genotype C. The

genotypes may also affect response to antiviral therapy as genotypes A and B appear to have greater rates of antiviral response to interferon (IFN)-alpha therapy than D and C. Thus, genotyping of HBV can predict the risk of adverse outcomes (fulminant disease, cirrhosis, and HCC) or can influence decision-making in management of the HBV patients (Kao, *et al*., 2000; Kato, *et al*., 2005).

I.12 HBV mutants and their clinical significance

HBV is a DNA virus and its replication requires an active viral reverse transcriptase /polymerase enzyme. The reverse transcriptase (for both HBV and human immunodeficiency virus) is believed to lack proof-reading function that is common to other polymerases. Indeed, HBV exhibits a mutation rate more than 10-fold higher than other DNA viruses (Jalali, & Alavian, 2006).

Naturally occurring mutations have been identified in all viral genes and regulatory elements. Most notably mutations in the genes coding for the structural envelope and nucleocapsid proteins, the HBeAg and HBsAg, may result in infection or viral persistence despite the presence of antibodies against HBsAg (anti-HBs). Mutations in the gene encoding the pre –core/core protein (pre-core stop codon mutant) result in loss of HBeAg (HBeAg minus mutant) and seroconversion to antibodies (anti-HBe) with persistence of HBV replication (Yuan, 2007; Huang, 2006). Mutations in the core gene may lead among others to an immune escape due to a T cell receptor antagonism. While mutations in gene coding for polymerase /reverse transcriptase can be associated with viral persistence or resistance to therapy by nucleotide analogues (Keeffe, *et al*., 2006).

I.13 Mutations in the pre-core region

The major HBV variants have been described with mutations in the pre-core region (PC) that prevent HBeAg synthesis despite continuing production of infectious-virions. The PC mutation is a G to A substitution at nt 1896 (A1896G), which creates a premature stop codon in the PC region. This mutation is also located at the highly conserved encapsidation (e) signal, which forms a secondary structure (stem-loop) in the pg RNA that is essential for initiation of encapsidation within the viral replication cycle. The G1896 forms a base pairing with nucleotide 1858 at the base of the stem loop. In HBV genotypes B, D, E, G and some C strains the nucleotide 1858 is a thymidine; therefore the stop codon stabilizes the e-structure (Sheldon*, et al*., 2006; Abbas, *et al*., 2006). Instead, the A1896 mutation is restricted to HBV isolates that have T instead of C at nt 1858, and is most commonly found in association with HBV genotypes which are more frequent in patients infected with B, D and E genotypes, and rare in patients infected with other genotypes (Fig 4).

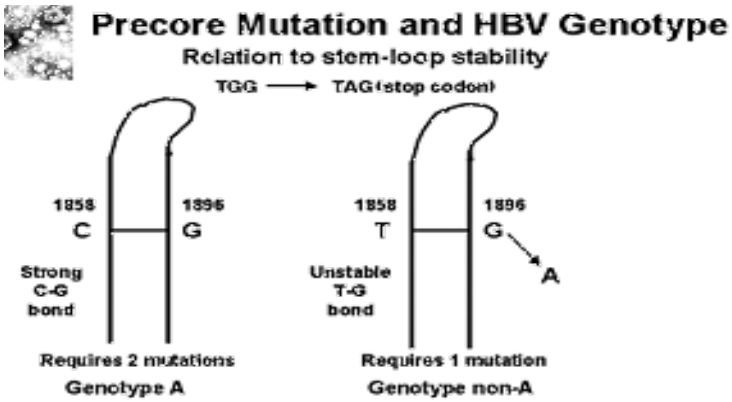

Figure 4: **Pre -core mutation and HBV genotype.** From (Jalali & Alavian, 2006).

However, in the event of impaired base pairing when C-1858 tries to pair with A-1896, there is decreased encapsidation of pg RNA and decreased replication. The prevalence

of the A1896 mutant varies in different geographical regions, according to the distribution of HBV genotypes with T1858, and is not found in HBV genotypes with C1858. Therefore, T1858 predominates in all none A genotypes. (Jalali & Alavian, 2006).

I.14 Mutations in the core promoter region

The most common basal core promoter (BCP) mutations involve two nucleotide substitutions: A to T at nt 1762 and G to A at nt 1764. The BCP mutation has been predominantly found in Japan and Hong-Kong and was shown to down regulate the transcription of the precore mRNA and therefore decrease the expression of HBeAg. Patients with BCP mutants compared with those with wild-type have also been shown to have a higher risk for development of HCC. It has been demonstrated further that HBV genotype C is frequently associated with BCP mutations. Furthermore, the BCP mutations may induce not only amino acid change in X protein but also an alteration of HBV gene expression. The alternation of X protein might play a role in hepatocarcinogenesis, because its coding sequence overlaps regions of crucial importance for viral replication such as enhancer II and the BCP. Additionally, BCP mutations may give rise to HCC through probably other unknown pathways that are not well understood yet (Yuen, *et al*., 2004).

It is important to elucidate the interplay between HBV genotypes and BCP mutations since both are associated with the development of HCC. There is also a possible association between HBV genotypes and pre-core and core promoter mutations and earlier studies have suggested that genotyping of HBV can predict the risk of adverse

outcomes (fulminant disease, cirrhosis, and HCC) or can influence decision-making in management (Kao*, et al*., 2000; Kato, *et al*., 2005).

Currently, HBV genotypes and mutants can be determined by several methods, including direct DNA sequencing, restriction fragment length polymorphysim (RFLP), line probe assay, PCR using genotype-specific primers, colorimetric point mutation assay, and enzyme –linked immunosorbent assay for genotype specific epitopes. Direct sequencing is the most accurate and permits detection of the common as well as uncommon mutations but is also the most expensive and tedious (Schaefer, 2005 Is this 2005 or 2007?). HBV genotyping and detection of pre core/ core mutations should be simple, accurate and specific to facilitate clinical application.

I.15 Objectives of the study

Chronic hepatitis is an important medical problem in Palestine. With the implementation of vaccination against HBV in the national vaccination program for newborns since 1995, the incidence rate of hepatitis B carriership has markedly decreased from 67.5 per 100,000 in 2000 to 53 in 2004 with an annual average incidence rate of 56.1 per 100,000 in the last five years. However, Palestine is considered as moderately endemic area for HBV carriers, (Ministry of Health, 2005).

However, the distribution of HBV genotypes among Palestinian patients with CHB has not been determined**.** Furthermore, the distribution of HBV mutants within the pre-core and core promoter regions among chronic Palestinian patients has not been determined yet.

Therefore, the objectives of this study are:

1. To determine the prevalence of HBV genotypes (A to H) among Palestinian patients with chronic infection.

2. To asses the correlation between HBV genotypes and liver complications such as cirrhosis and hepatocellular carcinoma**.**

3. To investigate the clinical significance of mixed HBV genotype infections.

4. To detect the major pre-core and core promoter mutations among chronic HBV Palestinian patients and their correlation with HBV genotypes as well as with the clinical outcomes.

IV.I Discussion

HBV infection is a global health problem and is a continuously increasing burden in developing countries. Recently, Hepatitis B genotyping has received more attention and research throughout the world. Hepatitis B virus has been classified into eight genotypes based on intergroup divergence of 8 % or more in the complete nucleotide sequence (Schaefer, 2007; Purdy, *et al*., 2008). The distribution of HBV genotypes may vary with time and with population immigration. The clinical significance of HBV genotypes on the clinical outcomes of HBV infection has been partially clarified. Several studies reported that HBV genotypes may differ in their serologic reactivity, pathogenicity, virulence, their association with HBV core antigen, HBeAg seroconversion, activity of liver disease, and response to therapy (Kao, *et al*., 2002; Idrees, *et al*., 2004; Tong, *et al*., 2007; Lin & Kao, 2008, Balsano & Alisi, 2008).

Previous reports have studied the correlation between HBV genotypes and mutations in the precore and core promoter regions that abolish or decrease the production of HBe Ag. Although, the findings were not conclusive in some studies, other studies have found a correlation between the core promoter and precore mutations and the clinical outcomes of the HBV infection (Kuang, *et al*., 2005). The occurrence of the precore mutation seems to be a strategy of the virus to evade the host immune response (Rezende, *et al*., 2005). Thus permitting the persistence of viral replication and maintenance of hepatic necro-inflammatory activity. However, the HBeAg negative state was predominant among Asian population and the frequency of precore mutations was significantly higher among HBeAg negative compared to HBeAg positive patients (Premchandra, *et al*., 2003).

In this study, the prevalence of HBV genotypes and precore/core mutations were studied in 150 chronic HBV patients. Among our study population, genotype D (25.3 %) was the predominant HBV genotype in chronic HBV palestinian patients. While genotype A was observed in a small proportion of patients, 4.7 %. Genotypes B and C were observed only as part of a mixed infection but not as a single genotype. The latter results agree with the prevalence of HBV genotypes in the Mediterranean and Middle East region. Remarkably, a high prevalence of mixed infections was observed in this study (64.7 %) and was attributed mainly to D+A co-infections (35.3 %).

Recent HBV genotyping reports originating from Mediterranean and Middle East countries such as Egypt (Saudy, *et al*., 2003; Zekri, et al., 2007), Saudi Arabia (Abdo, *et al*., 2006), Tunisia (Ayed, *et al.,* 2007), Morocco (Sbai, *et a*l., 2007) and Turkey have demonstrated that HBV genotype D is the most common genotype circulating in these populations (Serin *et al*., 2005). Additionally, a high frequency of HBV genotype D (96%) and a low frequency of genotype A (4%) were reported in "Israel" (Ben-Ari, *et al*., 2004). Although the latter reports have demonstrated that in addition to the common genotype D, other genotypes are also present, albeit at a lower and variable frequencies. Indeed, the molecular heterogeneity of HBV genotypes is being increasingly reported worldwide (Onganer, *et al*., 2006). Table 17 summarizes the distribution of different HBV genotypes and mixed genotypes (see further discussion below) in Mediterranean countries as well as other Asian countries for comparison.

Table 17. Prevalence of HBV genotypes and mixed genotypes in different Mediterranean and Asian countries.

Country	Study Population	Prevalence of HBV genotypes	References
Egypt	Chronic carrier HBV	Predominance D	Saudy , *et al*., 2003
Egypt	Pediatric cancer patients with acute and chronic active HBV infection	D (37.1 %), B (25.7 %) Mixed (15.7 %; mainly A+D)	Zekri, *et al*., 2007
Saudi Arabia	Chronic HBV patients (CAH, cirrhosis, HCC)	D (81.4 %), A (1.4 %) C (1.4 %) , E (5.7 %), Mixed (10 %; includes ADG, DE, DF, ADFG)	(Abdo, *et al*., 2006)
Tunisia	Chronic carrier HBV	D (84.75 %), B (0.6 %), A (0.6 %) C (1.82 %), Mixed (12.2 %)	Ayed, *et al.,* 2007.
Morocco	Chronic HBV	D (97.5 %), A (2.5 %)	Sbai, *et al*., 2007
Pakistan	Chronic HBV carriers	C (41.07 %), A (21 %), B (17.86 %) D (8.03 %), Mixed (7.14 %)	Idrees, *et al*., 2004
China	HBV carrier patients with HBV related-liver disease	B (48.0 %), C (40.8 %), A (0.8 %) D (0.8%), Mixed (9.6 %; all are B+C)	Zumbika, *et a*l., 2005
Malaysia	Chronic HBV carriers	B (56.9 %), C (31.4 %) Mixed (7.8 %)	Lim, *et al*., 2006

Since the beginning of HBV genotyping, it was noted that HBV genotypes show a distinct geographical distribution. In this regard, earlier reports of HBV genotyping have shown that a single genotype-infection is usually the major genotype circulating in the respective geographic region with other mixed genotype-infections (usually 2) constituting a minor proportion of HBV infections (Lindh, *et al*., 1996; Lim, *et al*., 2006; Tong, *et al*., 2006; Mello, *et al*., 2007; Sbai, *et al*., 2007). The presence of different patterns of HBV genotypes in many countries have been partly attributed to the well-known waves of immigration that have occurred over time in the respective country (Palumbo, *et al.,* 2007). The geographical distribution of HBV genotypes in earlier studies is summarized in Table 17.

Remarkably, a high prevalence of mixed infections was observed in this study (64.7 %) and was attributed mainly to D+A co-infections (35.3 %). However, other combinations were also detected and included D+C (0.7 %), A+C+D (10 %), A+B+C (0.7 %), B+C+D (1.3 %) and A+B+C+D (16.7 %). Similar finding was reported by Hannoun *et al*. (2002) who found a mixed genotype infection in 67 % among his study population of 30 HBeAg-positive patients treated with Interferon. However, the findings of a high frequency of mixed genotypes in this study and by Hannoun *et al*. (2002) are in contrast with most earlier reports who found mixed genotypes for example in 0.0 % (Tong, *et al*., 2006; Sbai, *et al*., 2007), 0.8 % (Kao, *et al*., 2001), 1.6 % (Zeng, *et al*., 2004), 10.9 % (Kato, *et al*., 2003), 15.7 % (Zekri, *et al*., 2007), 17.3 % (Olinger, *et al*., 2006) and 17.5 % of the respective study HBV isolates (Chen, *et al*., 2004) (see Table 17 for geographic distribution of mixed genotypes).

Little is known about the nature and origin of mixed genotypes (Coinfection). But based on the higher frequency of mixed infections, it was suggested that chronic carrier HBV patients often become superinfected with additional strains or that *de novo* infection with genotype mixtures is common (Hannoun, *et al*., 2002). The presence of mixed genotypes also may depend on the routes of acquired HBV infection which are usually not known for most patients (Hannoun, *et al*., 2002). Coinfection may be acquired first by injection drug use in subjects, who then introduced it into HBV carrier population by other routes. The latter explanation is plausible, because it is difficult for a superinfecting genotype to establish itself against a genotype that is already present throughout the liver (Hannoun, *et al*., 2002). Also our results indicated that the mode of HBV infection is unknown in the majority of patients (58.8 %), but 28.1 % of patients have acquired infection via a household member (Table 8). These results indicate that

despite the well known routes of HBV transmission, most patients are still not aware of these routes or more specifically of the event that transmitted the infection.

The coexistence of different patterns of HBV genotypes and mixed genotypes probably reflects the distribution of Palestinian people (mostly refugees) in different countries and their mixing through return or social relations. In this study some patients have admitted that before return in late 1990s to Palestine they have been living for long periods mainly in Tunisia, Kuwait, and Lebanon (data not shown).

The method for genotyping influences the detection of mixed genotypes (Kato, *et al*., 2003). Common HBV genotyping methods include direct DNA sequencing (Serin, *et al*., 2005) or RFLP analysis (Zeng, *et al*., 2004) of preS1/S2/S region, RFLP analysis of core region (Hannoun, *et al*., 2002), PCR with genotype-specific primers (Naito, *et al*., 2001) and genotype specific probes assay (GSPA) (Kato, *et al*., 2003). The PCR-RFLP method showed a prevalence of coinfection of 0.8% in a highly endemic region (Kao, *et al*., 2001), while a genotype specific probes assay (GSPA) showed a prevalence of coinfection of 10.9 % from countries where HBV is not highly endemic (Kato, *et al*., 2003). In a recent report from Turkey, all of the 50 HBV chronic patients genotyped by DNA sequencing of the preS1 through S region, showed genotype D and none showed mixed genotypes (Serin *et al*., 2005). On the other hand, using PCR with genotype-specific primers for genotyping of 164 Egyptian patients (Zekri, *et al*., 2007) and 70 Tunisian patients (Ayed, *et al*., 2007) with chronic HBV infection, showed a prevalence of mixed genotypes of 15.7 % and 12.2 %, respectively. The DNA sequencing probably detects the predominant genotype, while the RFLP analysis fails to detect any quasi genotype constituting less than 10 % of the genotype mixture. While

HBV genotyping using RFLP-PCR failed in 18 samples out of 158 samples, which were successfully genotyped by a nested PCR using genotype-specific primers, which reflects the fact that nested PCR much more sensitive than PCR-RFLP (Leblebicioglu, *et al*., 2004). However, technically the PCR with genotype-specific primers (this study) and genotype-specific probe assay (Kato, *et al*., 2003) are highly sensitive and capable of detecting quasi genotypes, provided the method is properly optimized. In this study, when some samples with mixed genotypes (D+A) were further analyzed by DNA sequencing, only the predominant genotype D was observed. While when genotype-specific PCR amplicons were analyzed by DNA sequencing, the results were concordant with the PCR results.

Also several explanations are provided for the source of mixed genotypes, conclusive experimental evidences are not available yet. However, the detection of several recombinant HBV arising from different combinations of genotypes, may suggest that they arise as a result of mixed genotype infection (Schaefer, 2007). Additionally, Schaefer (2007) has raised the possibility that mixed genotypes may arise as a consequence of fast adaptation of HBV to a certain genetic and immunologic environment in different human populations.

The detection of coinfection by different HBV genotypes has an important implication for prognosis and treatment of CHB. The presence of mixed genotypes has been found to be associated with a more severe liver disease compared with infections with a single HBV genotype (Ayed, *et al*., 2007).

The prevalence of negative HBeAg cases among CHB patients seems to vary geographically and in correlation with the geographical distribution of HBV genotypes. In Italy, 41 % of patients with CHB during the period of 1975-1985 were HBeAg negative but in the last decade this has increased to 90 % (Jalali & Alavian, 2006). There is a worldwide increase in the prevalence of negative HBeAg cases. In this study the distribution of negative HBeAg cases (68 %) was higher than positive HBeAg (31.5 %) cases. Analysis of the correlation between HBV genotypes and the status of HBeAg showed a predominance of mixed genotypes (mainly A+D) among negative HBeAg patients (71.1 %) compared to positive HBeAg patients.

CHB infection with negative HBeAg is frequently associated with precore and core promoter mutants (Yuan, *et al*., 2007). The predominant precore mutation involves a G-to-A change at nucleotide (nt) 1896 (G1896A), which creates a premature stop codon and abolishes the synthesis of HBeAg. Another common precore variant includes the C1858/T1858, where this variant nucleotide pairs with nucleotide at 1896 to form the stem-loop structure that acts as a binding site for viral polymerase on the HBV pgRNA. Genotype A contains invariably the C1858 variant, which favors the presence of G at position 1896 (wild type). The latter observation explains the lower frequency of precore mutation at position 1896 in Northern Europe, where HBV genotype A predominates (Laperche, *et al*., 2006). On the other hand the non-A HBV genotypes (B, C, D and E) usually have the T1858 variant which pairs with A at nucleotide 1896 (Zumbika, *et al*., 2005). Thus precore mutations at position 1896 is frequently observed in the Mediterranean region where HBV genotype D predominates (Leblebicioglu, *et al*., 2004).

The dual basal core promoter (T1762 and A1764) mutations have been reported to reduce HBeAg production by approximately 50 to 70 %. However, it is controversial whether the T1762 and A1764 mutations lead to the seroconversion of HBeAg to the negative phenotype (Chen, *et al*., 2005).

In this study, it was observed that the basal core promoter mutation occurred at a higher percentage among HBV isolates (single mutant 17/135 or as a co-infection with Wt 58/135) compared to the other precore mutation. Although no statistically significant correlation was found between the BCP mutation and HBeAg status, the percentage of BCP mutation (present in 55.5% of samples) was more predominant among negative HBeAg patients compared with positive HBeAg patients. The latter results are consistent with the findings reported by a recent Tunisian's study that also showed the predominance of BCP mutation among HBeAg-negative patients compared to the HBeAg–positive patients (65.4 % vs. 18.2%) (Ayed, *et al*., 2007).

Six out of seven (85.7%) cases with HBV genotype A detected in this study, contained the BCP mutation while only one case of genotype A (14.3 %) contained the wild type sequence at this position. A higher frequency of BCP mutation was also found among mixed genotypes (55.7%) compared to single genotype infections. Additionally, a statistically significant correlation was observed between the BCP mutation and HBV genotypes ($p<0.032$) or serum HBV DNA levels ($p<0.000$).

Regarding the PC mutation, we have found a significant correlation between the PC mutation (G1896A) and the patients' age ($p<0.001$) as well as the serum HBV DNA level ($p<0.001$). No significant correlation was observed between the T1858/C1858

variant and the patients' age, sex, HBV genotype, HBeAg status and serum HBV DNA level. Additionally, a significant correlation was observed between the serum HBV DNA levels and positive HBeAg cases compared to negative HBeAg ($p < 0.002$).

The clinical significance of different HBV genotypes has become increasingly recognized in patients with different clinical outcomes, different genotypes may be associated with different rates of progression from acute to chronic HBV infection. A recent study demonstrated that infection with HBV genotype A is associated with acute hepatitis whereas infection with the most prevalent genotypes B and C lead to chronic infection among Japanese patients (Baig, *et al*., 2007). Genotypes B and C are the most prevalent variants in Taiwan, and it has been reported that genotype C is associated with a more severe liver disease compared to genotype B (Chen, *et al*., 2005). However, a recent report by Zumbika, *et al*. (2005) from China indicated a high prevalence of mixed genotypes (B+C) infection among patients with severe chronic hepatitis and in liver cirrhosis patients (25 vs. 17.7 %).

In this study, it has been observed that older patients are at a higher risk of developing liver cirrhosis than younger patients and that CAH is more predominant among younger patients. Also infection with mixed genotypes was more predominant (23/32) among patients without CAH (including HCC & liver cirrhosis patients) than those with CAH (73/118). While infection with mixed genotypes was more predominant among patients with liver cirrhosis (19/27) than patients without cirrhosis (77/123). Taken together, these findings indicate that mixed genotype infection is associated with a higher risk for development of liver complications or bad clinical outcome (bad prognosis).

The serum HBV DNA level showed a significant correlation with the clinical status of HBV infection in patients with CAH compared to those without CAH ($p<0.009$). Also patients with cirrhosis have a significantly higher levels of serum HBV DNA than those without cirrhosis ($p<0.038$). These findings reflect the fact that patients with severe liver complications have higher levels of viral replication.

A significant correlation was also found between the PC mutation (G1896A) and presence of CAH ($p<0.$ 0.013) or liver cirrhosis ($p<0.001$). Also the PC mutation is found more among liver-cirrhosis patients (11/24) than CAH patients (17/103). Analysis of the correlation between the BCP mutation and codon 15 variants, showed no significant correlation with CAH. However, codon 15 variants show more prevalence of C1858 variant among cirrhosis patients (7/19, 36.25 %) compared to patients without cirrhosis (18/98, 18.4 %). These findings show that PC mutation (G1896A) is a risk factor for development of severe liver complications. While the correlation between BCP and codon 15 variant needs further investigation and probably by a case-controlled prospective study.

Chronically HBV carriers have a 100 times increased risk of hepatocellular carcinoma compared to non-carriers (Ganem & Prince, 2004). HBsAg positively increases the risk of developing HCC by 10-fold and HBeAg positively by 60-fold. Whereas, a detectable HBV DNA level has a 4-fold increased risk of HCC. Generally, all categories of liver diseases showed male dominance; where it is well documented in HCC case. The HCC incidence is three to six times higher in males than in females, suggesting a tumorigenic effect of androgens (Baig, *et al*., 2007).

Of all the 150 study samples, only five patients have had HCC at the time of sample collection, patients in this study who developed HCC were solely males. A significant correlation between patients' age (mean age in years 54.00 ± 4.85) and development of HCC was observed ($p < 0.005$). These findings are in concordant with previous studies that have also found that older age (> 45 years) is an important determinant of HCC development (Pan & Zhang, 2005).

Four out of five HCC patients (80 %) have mixed genotypes compared to 63 % of HBV patients without HCC, and three out of four HCC patients (HBeAg values are available for only 4 of the 5 HCC patients) were negative for HBeAg. However, no significant correlation has been observed between the HCC and mixed genotypes, HBeAg status as well as PC mutation and codon 15 variants. However, the results indicate a high prevalence of mixed genotypes and HBeAg negative status among HCC patients. The lack of significant correlation can be attributed partially to the low sample size in case of HCC group. Moreover, we have observed a significant correlation between wild type BCP (G1896) and HCC ($p<0.023$), i.e., the presence of BCP mutation is a negative predictor of HCC.

Furthermore, HBV is suggested to be directly carcinogenic because of the integration of HBV DNA into the host cellular DNA that may cause instability of host chromosomes in liver cells, interrupt cellular tumor suppressor genes, or cause alterations in the expression of cellular growth factors and/or apoptosis regulating genes (Michielsen , *et al*., 2005). It seems that the integration of the HBV genome regardless of genotype into host chromosome plays an important role in the

development of HCC, especially in non-cirrhotic patients with HBV infection, which may explain the lack of correlation between the HBV genotypes and development of HCC (Wang, *et al*., 2000; Abdo, *et al*., 2006).

In conclusion, HBV genotype D was found to be the most common genotype in West Bank region/Palestine and a high prevalence of mixed HBV genotypes mainly due to A+D (35.3 %) was also found. Genotypes B and C were observed only as part of a mixed infection but not as a single genotype. These findings indicate that mixed genotypes' infection is associated with a higher risk for development of liver complications or bad clinical outcome (bad prognosis). The distribution of negative HBeAg (68 %) was higher than positive HBeAg among our study population. The basal core promoter mutation occurred at a higher percentage among HBV isolates. A statistically significant correlation between the HBV genotypes and BCP mutation was observed. The wild type BCP (G1896) was significantly correlated with HCC and thus the presence of BCP mutation (G1896A) is a negative predictor for HCC. Additionally, the PC mutation is more predominant among cirrhosis patients than CAH Patients. Thus our results indicate that the PC mutation is a positive predictive marker for liver cirrhosis and indirectly for HCC; since it is already known that 15-40 % of patients with liver cirrhosis will develop HCC. Further studies are needed to investigate the correlation between the mixed genotypes and clinical outcome as well as the type of treatment, probably via a case-controlled prospective study including a larger number of patients.

III Results

III.1 Patients

One hundred and fifty HBV patients were enrolled in this study. Patients were selected from all the governorates of West Bank, and the number of patients was representative for the population count for each governorate (Table 6). Selection of patients was performed randomly from chronic HBV patients presenting at one of the Public Health centers in the respective governorate between July 2004 and August 2006. Based on the clinical status, patients were divided into three groups: (i) chronic active hepatitis (n=118), (ii) liver cirrhosis (n=27) and (iii) hepatocellular carcinoma (n=5). The mean age of the patients was 35.92 ± 14.75 years and the study population included males (n=112) and females (n=38). The number of female patients, presenting at the Public Health centers collaborating with this study at the time of sample collection, was lower than male patients for unknown reasons.

Table 6. Geographical distribution of HBV patients included in this study.

Place of birth	No. of patients (%)
Ramallah	35 (23.3 %)
Hebron	41 (27.3 %)
Alquds	3 (2.0 %)
Nablus	23 (15.3 %)
Tulkarm	7 (4.7 %)
Jenin	9 (6.0 %)
Salfiet	2 (1.3 %)
Bethlehem	23 (15.3 %)
Tunisia	2 (1.3 %)
Lebanon	1 (0.7 %)
Kuwait	1 (0.7 %)
Sample origin not identified	3 (2.0 %)
Total	150 (100.0 %)

III.2 Distribution of HBV genotypes

For HBV genotyping, the multiplex nested PCR was used. The multiplex- nested PCR used specific primers for determination of the eight HBV genotypes (A through H). All samples used in this study were HBsAg positive and HBV DNA positive by PCR. However, following genotyping of the study samples, 142 samples were assigned to a specific genotype(s) while 8 samples were untypable (Table 7). Genotypes of HBV samples were determined by identifying the genotype- specific DNA bands on agarose gels. The three different second–rounds PCR amplicons (mix A, mix B and mix G) from each sample were separately electophoresed on a 2 % agarose gel. Mix A allows for the specific detection of genotypes A, B and C. The mix B allows for the specific detection of genotypes D, E and F, and mix G for specific detection of genotype G. The sizes of PCR amplicons were estimated according to the migration pattern of 50-bp or 100-bp DNA ladder.

Figures 6 through 11 show representative agarose gels for mix A, B and G as well as PCR amplicons of all detected HBV genotypes in this study. The size of PCR amplicons representing the different HBV genotypes were as follows; 68 bp, 281 bp, 122 bp and 119 bp for genotypes A (Figs 6-8), B (Figs 7-8), C (Figs 7-8) and D (Figs 9-10), respectively, either as a single genotype or mixed genotypes (Table 7).

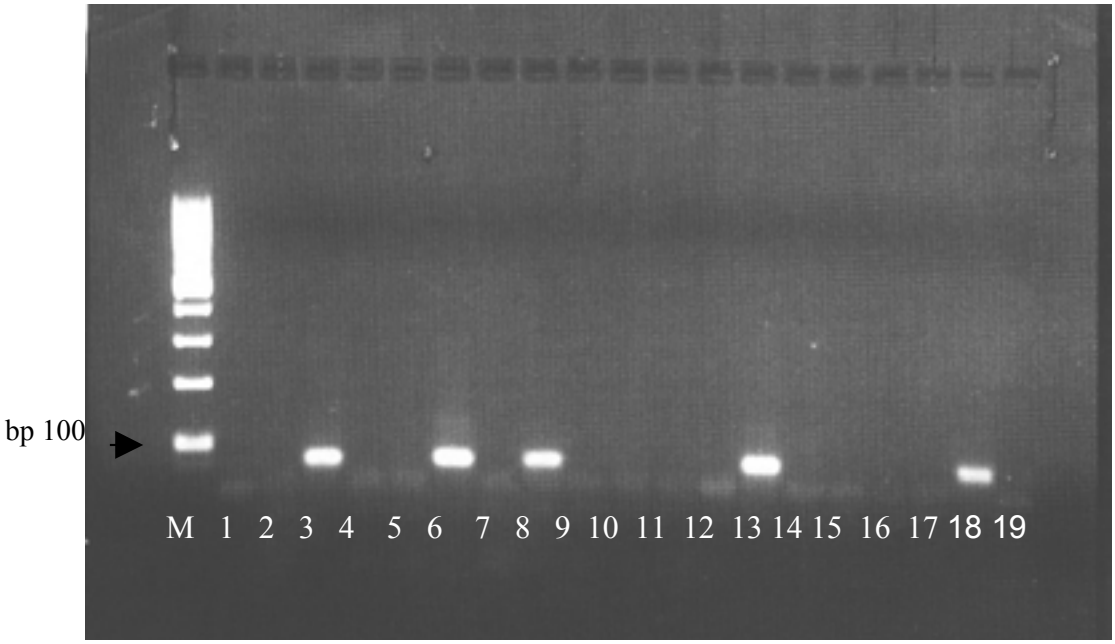

Figure 6. Agarose gel electrophoresis pattern of "Mix A" PCR amplicons showing genotype A. Lane M: 100-bp Ladder; lane 1: Negative control; lanes 3, 6, 8, 13, 18: are positive for genotype A (68bp); and the remaining lanes were negative for Mix A, i.e., no genotype A, B or C was detected.

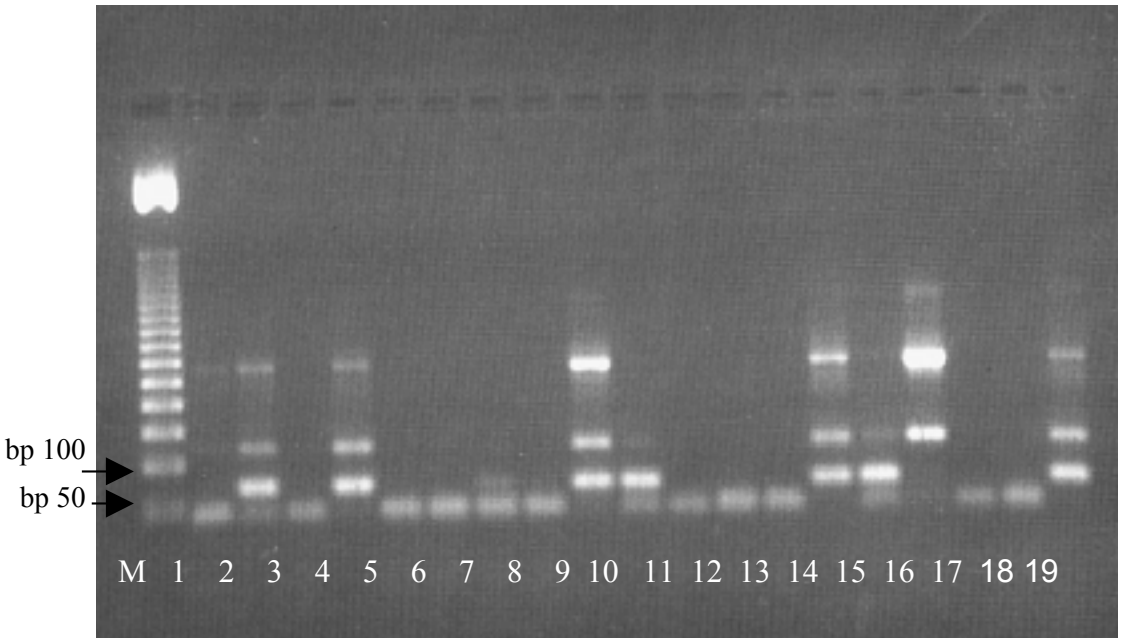

Figure 7. Agarose gel electrophoresis pattern of "Mix A" PCR amplicons showing genotype A, B and C. Lane M: 50-bp Ladder; lanes 2, 4, 9, 14, 19: showed mixed genotypes which include A (68 bp), B (122 bp) and C (281bp); lanes 10, 15: positive for genotypes A and C; Lane 16: positive for genotypes B and C; and the remaining lanes were negative for Mix A, i.e., no genotype A, B or C was detected. The Negative control lane was included in the same run and is not shown here because it was run on a second gel.

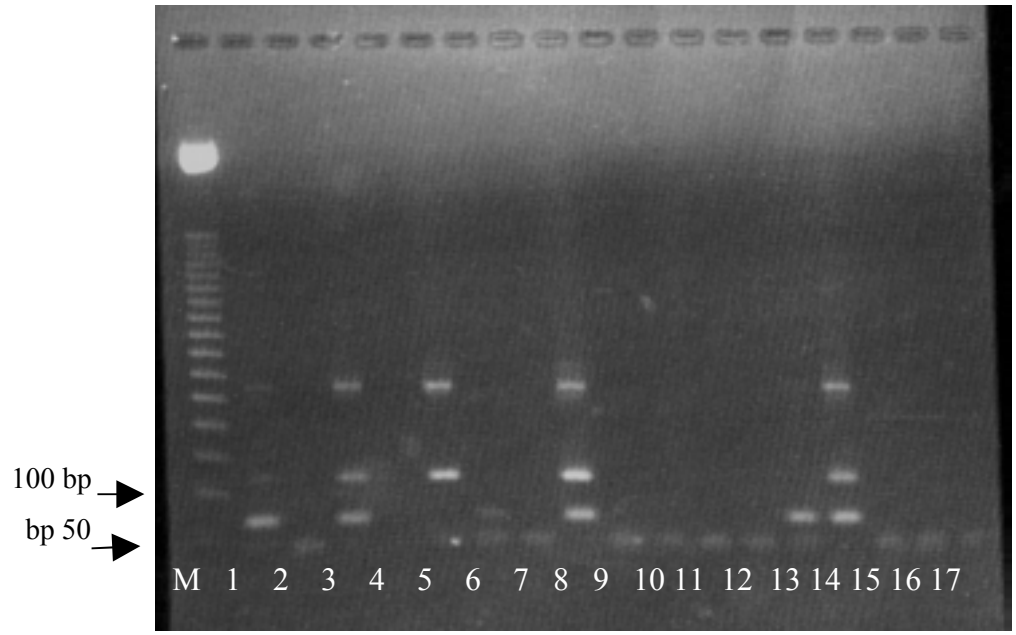

Figure 8. Agarose gel electrophoresis pattern of "Mix A" PCR amplicons showing genotype A, B and C. Lane M: 50-bp Ladder; lanes 1, 3, 8, 14: showed mixed genotypes which include A (68 bp), B (122 bp) and C (281bp); lanes 6, 13: positive for genotypes A ; Lane 5: positive for genotypes B and C; and the remaining lanes were negative for Mix A, i.e., no genotype A, B or C was detected. The Negative control lane was included in the same run and is not shown here because it was run on a second gel.

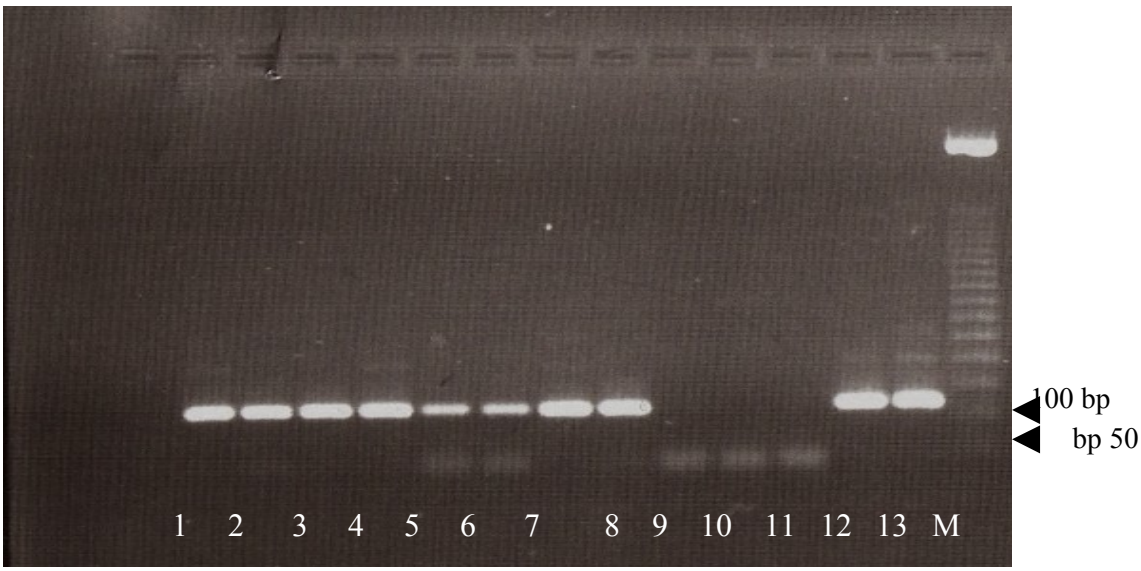

Figure 9. Agarose gel electrophoresis pattern of "Mix B" PCR amplicons showing genotype D. Lane M: 50-bp Ladder; lanes 1, 2, 3, 4, 5, 6, 7, 8, 12, 13: are positive for genotype D (119 bp); and the remaining lanes were negative for Mix D, i.e., no genotype D, E or F was detected. The Negative control lane was included in the same run and is not shown here because it was run on a second gel.

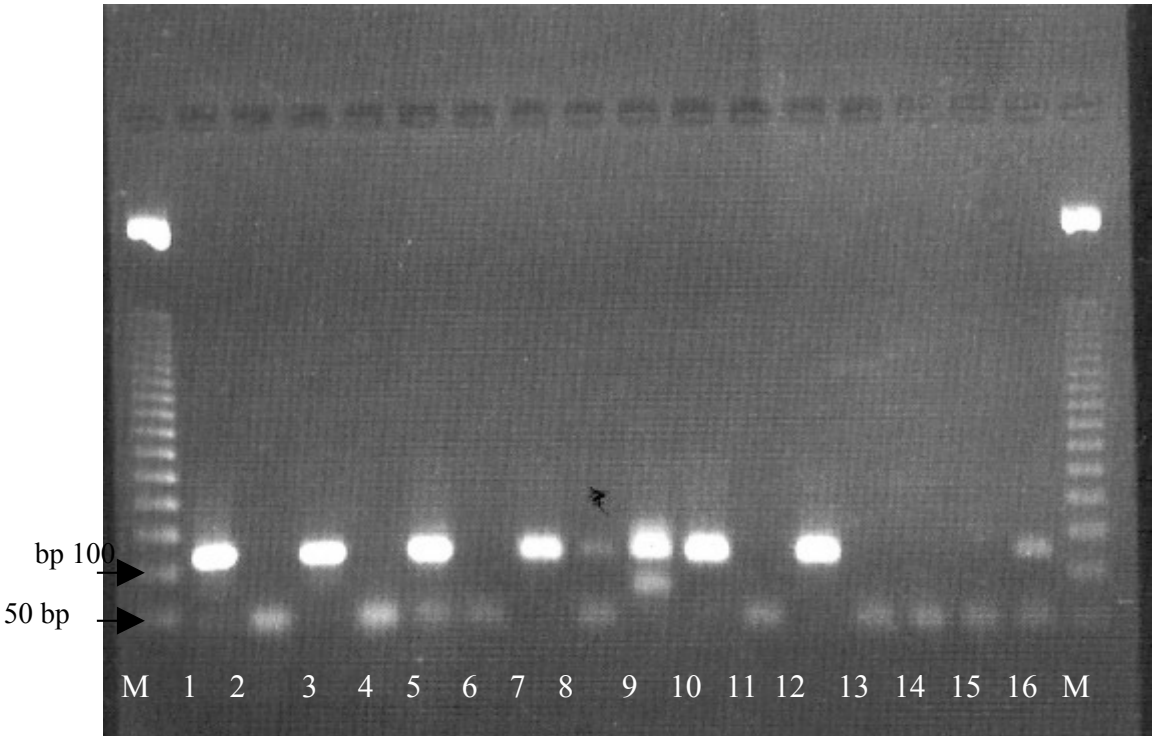

Figure 10. Agarose gel electrophoresis pattern of "Mix B" PCR amplicons showing genotype D. Lane M: 50-bp Ladder; lanes 1, 3, 5, 7, 8, 9, 10, 12, 16: are positive for genotype D (119 bp); lane 6; Negative control; and the remaining lanes were negative for Mix D, i.e., no genotype D, E or F was detected.

All the results for Mix G were negative for HBV genotype G (Fig 11). However, the specificity of Mix G PCR reaction was checked by including a pair of primers that generate an internal control fragment to verify the functionality for the Mix G PCR. As shown in Figure 11, all samples showed the internal control fragment of 406 bp.

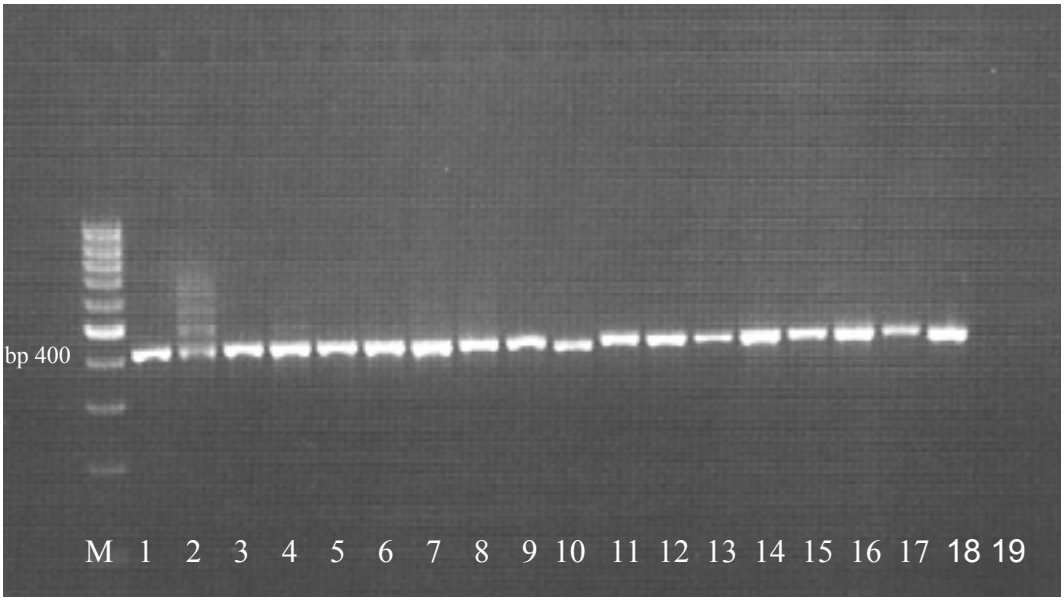

Figure 11. Agarose gel electrophoresis pattern of "Mix G" PCR amplicons. Lane M: 100 bp DNA ladder; lanes 1-18: in all lanes the genotype G-specific fragment was not detected but the fragment corresponding to the internal control (406 bp) was detected in all lanes. Lane 19: Negative control.

This study showed that the majority of palestinian HBV patients were infected with either genotype D (25.3 %) or A (4.7 %) (Table7). However, a high prevalence of mixed genotypes infections was observed and was attributed mainly to D+A infections (35.3 %). Genotypes B and C were observed only as part of a mixed infection but not as a single genotype (Table 7). However, in this study genotypes E, F, G and H were not detected.

Table7. Prevalence of HBV genotypes among palestinian HBV patients.

HBV Genotype	No. of patients (%)	No. of HBeAg(+) patients (%)	No. of HBeAg(-) patients (%)
A	7 (4.7 %)	3 (50.0 %)	3 (50.0 %)
D	38 (25.3 %)	11 (37.9 %)	18 (62.1 %)
A/D	53 (35.3 %)	11 (28.9 %)	27 (71.1 %)
A/C/D	15 (10.0 %)	0 (100.0 %)	11 (0.0 %)
A/B/C/D	25 (16.7 %)	8 (38.1 %)	13 (61.9 %)
A/B/C	1 (0.7 %)	1 (100.0 %)	0 (0.0 %)
B/C/D	2 (1.3 %)	2 (0.0 %)	0 (0.0 %)
D/C	1 (0.7 %)	0 (0.0 %)	1 (1.3 %)
Non –type able	8 (5.3 %)	0 (0.0 %)	5 (100.0 %)
Total	150 (100 %)	36	78

III.2.1 Distribution of HBV genotypes based on HBeAg status

Analysis of the distribution of HBV genotypes according to the clinical status of HBeAg showed that mixed genotypes, particularly A+D (71.1 %), tend to predominate among negative HBeAg patients compared to positive HBeAg patients (Table 7). The genotype D also was more predominant among negative HBeAg patients (62.1 %) and was only second to the mixed genotypes A+D (Table 7).

Table 8 shows that there was no significant correlation between the HBeAg status and patients' age and sex as well as any of the risk factors recorded in this study. However, the results indicate that in more than half of the patients, either positive or negative for HBeAg, the risk factor for transmission or acquisition of HBV infection is unknown (Table 8).

Table 8. General characteristics of chronic HBV patients according to presence or absence of HBeAg in serum.

	HBeAg(+)	HBeAg(-)	*P - value*
No. of patients	36*	77*	
Age	32.97 ± 14.34	35.36 ± 14.18	0.41
Sex M/F	25/11	58/20	0.58
Risk factors			0. 33
Household Member (Non - Sexual)	14 (38.9 %)	18 (23.3 %)	
Sex Partner	0 (0.0 %)	1 (1.3 %)	
Perinatal	1 (2.8 %)	3 (3.9 %)	
Dentist	0 (0.0 %)	4 (5.2 %)	
Blood Borne Disease	0 (0.0 %)	4 (5.2 %)	
Nosocomial	0 (0.0 %)	1 (1.3 %)	
Unknown	21 (58.3 %)	46 (59.7 %)	

*Represent the no. of patients for whom complete data presented in this table is available

III.2.2 DNA sequencing and phylogenetic analysis.

Although the nested multiplex PCR used for genotyping has successfully genotyped the majority of the study samples, the remarkably high incidence of mixed genotypes has prompted us to refer to DNA sequencing to confirm the PCR results. For this purpose, selected samples representing all genotypes detected in this study were subjected for DNA sequencing. For DNA sequencing two types of DNA fragments were used. Firstly, a fragment covering most of the S-region (nts 203 to 704) was used for sequencing. Electronic genotyping using the NCBI HBV genotyping tool (available at www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi; Rozanov, *et al*., 2004) or HBV STAR (available at www.vgb.ucl.ac.uk/starn.shtml; Myers, *et al*., 2006) indicated that genotype D is the predominant genotype circulating in West Bank. Additionally phylogenetic analysis showed that our isolates were closely clustered with genotype D (Gene Bank accession no. X65259 and M32138) (Fig 12).

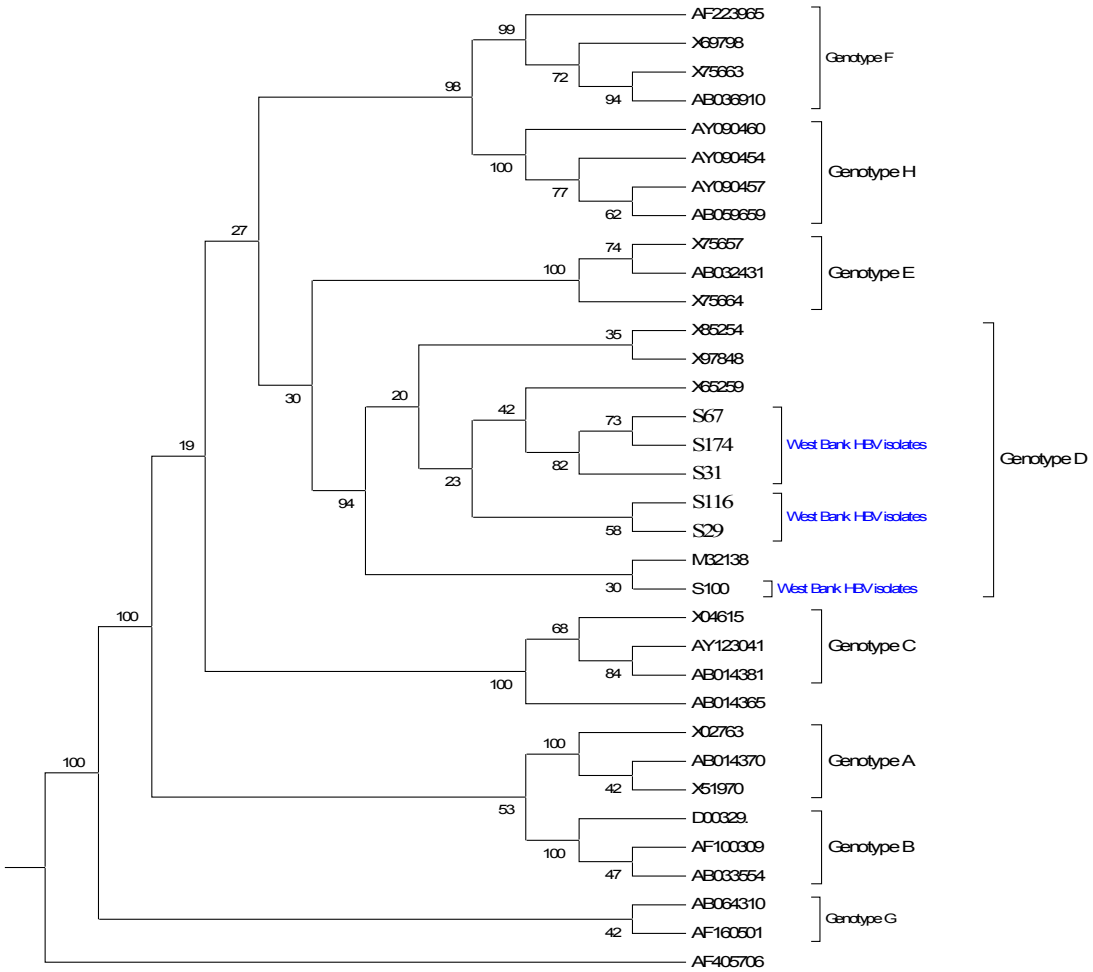

Figure 12. Consensus phylogenetic tree constructed on a 502 bp of the preS1 through S genes. HBV Genotypes and subtypes were retrieved from Gene Bank. The blue color shows HBV isolates from this study which were clustered with D subtypes. Alignments were obtained with clustal 1X (1.81) program. The statistical significance of the phylogenies constructed was estimated with the SEQBOOT program. The 100 data sets obtained were analyzed with the DNADIST program. While the observed nucleotide distance matrix was then processed with the NEIGHBOR program, and the tree was generated with the CONSENSE program. The numbers at the nodes represent the percentage of 100 bootstrap replicates that contained the cluster distal to the node.

Although the results of DNA sequencing analysis confirmed the presence of genotype D, but it did not yield conclusive results to confirm the presence of mixed HBV genotypes among our isolates. Because the DNA fragment used for DNA sequencing, was generated by a primer pair common to all genotypes, which probably amplifies preferably the most predominant genotype, i.e., genotype D. Since agarose gel electrophoresis of the primer-specific nested multiplex PCR showed, for most samples, that genotype D is the predominant one in case of mixed genotypes. Therefore, a

second type of DNA fragments were used, which included the genotype-specific PCR amplicons generated by the genotype-specific primers. For this purpose, two samples representative of each genotype detected in this study were selected for DNA sequencing. Phylogenetic analysis showed a 100 % concordance between the two genotyping assays; the nested multiplex PCR with genotype-specific primers and DNA sequencing (Figs 13-16).

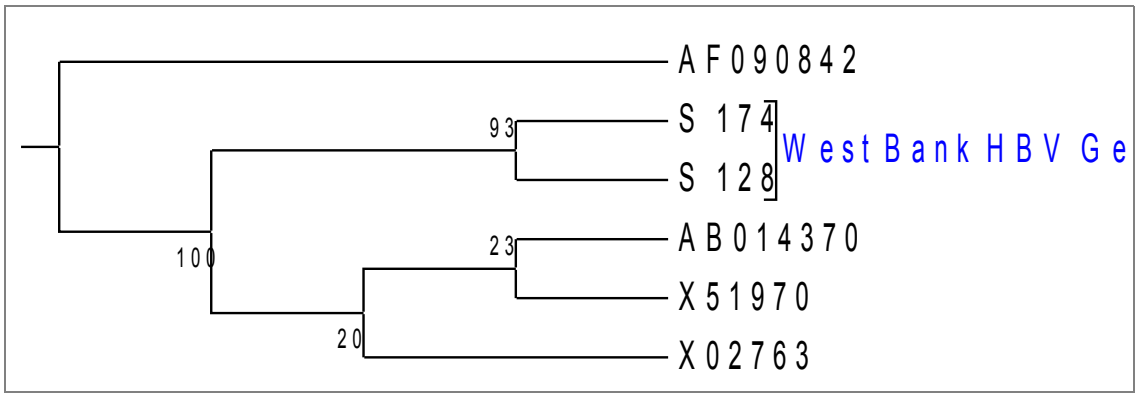

Figure 13. Phylogentic tree of two different genotype A (genotyped by primer-specific PCR) samples as well as HBV prototype and subtypes A retrieved from Gene Bank (accession no. are shown on the tree). The Gene Bank accession no. AB014370 represents the prototype of genotype A.

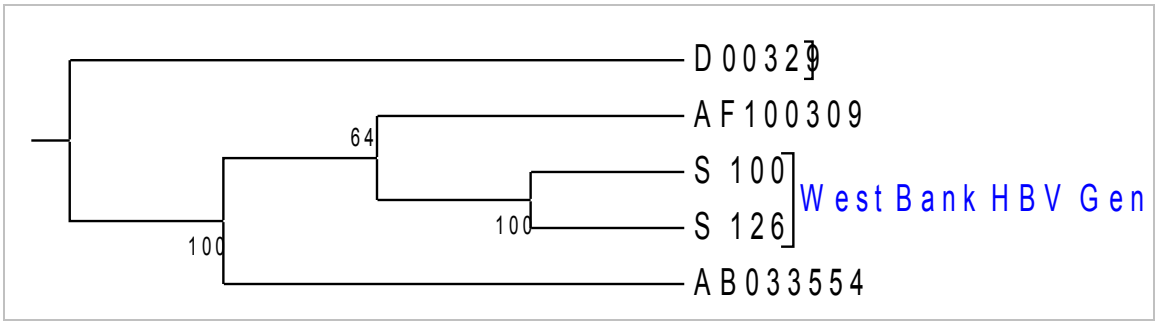

Figure 14. Phylogenetic tree of two different genotype B (genotyped by primer-specific PCR) samples as well as HBV prototype and subtypes B retrieved from Gene Bank (accession no. are shown on the tree). The Gene Bank accession no. AB033554 represents the prototype of genotype B.

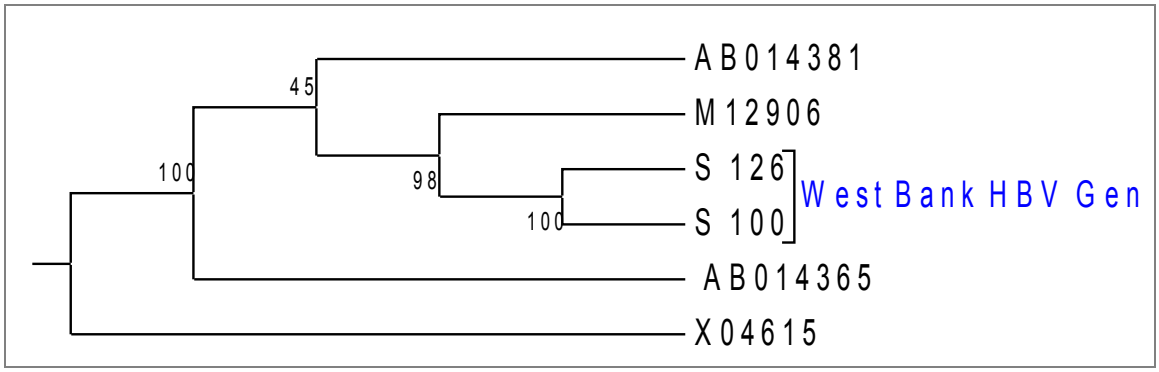

Figure 15. Phylogenetic tree of two different genotype C (genotyped by primer-specific PCR) samples as well as HBV prototype and subtypes C retrieved from Gene Bank (accession no. are shown on the tree). The Gene Bank accession no. AB014365 represents the prototype of genotype C.

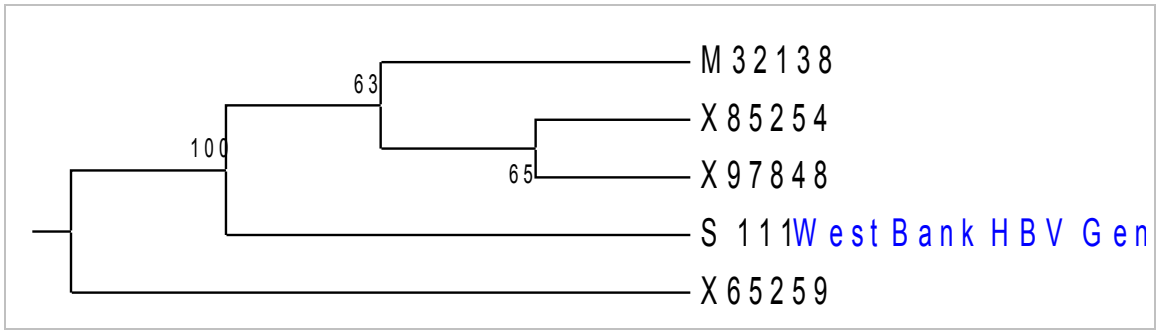

Figure 16. Phylogenetic tree of two different genotype D (genotyped by primer-specific PCR) samples as well as HBV prototype and subtypes D retrieved from Gene Bank (accession no. are shown on the tree). The Gene Bank accession no. X97848 represents the prototype of genotype D.

Table 9 shows the demographic and virologic characteristics of HBV genotypes. There was no significant correlation between the HBV genotype and patients' age, sex and serum DNA level. Analysis of the correlation between the HBeAg status and genotype shows a predominance of mixed genotypes among patients negative for the HBeAg, although this difference was not statistically significant (Table 9).

Table 9. Demographic and virologic characteristics of HBV genotypes.

Characteristic	Genotype A	Genotype D	Mixed	NA*	p-value
No. of Patients	7	38	97	8	
Age yr (mean ± SD)	40.14 ± 11.01	35.92 ± 15.08	35.98 ±15.09	31.50 ± 13.07	0.74
Male /Female	4/3	31/8	70/26	7/1	0.48
HBeAg, *n* (%)	**n=6	**n=29	**n =74	**n =5	0.27
+	3 (50 %)	11 (37.9 %)	22 (29.7 %)	0 (0.0 %)	
-	3 (50 %)	18 (62.1 %)	52 (70.3 %)	5 (100.0 %)	
HBV-DNA level (mean ± SD)	4.72 ± 0.74 log10	4.62 ± 1.66 log10	5.05 ± 1.83 log10	4.44 ± 1.83 log10	0.53

*NA: not-typeable samples.
** Represent the no. of patients for whom HBV genotypes data presented in this table is available

III.3 Determination of pre-core, basal core promoter mutants and Codon 15 variants

The three precore/core mutations were analyzed by a nested RFLP-PCR. All the 150 study samples were subjected for analysis of the latter three mutations, but some samples failed to yield PCR amplicons in one or more of these mutation respective PCRs. Thus, Table 10 shows the frequency of basal core promoter (BCP) mutation, Codon 15 variants, and pre-core stop codon (PC) mutations in 135, 117 and 132 patients, respectively. The basal core promoter mutation was found at a higher percentage among HBV isolates (single mutant 17/135 or as co-infection with mutant and wt 58/135) compared to the other two mutations.

Table 10. Frequency of basal core promoter and precore mutations among palestinian chronic HBV patients.

	Basal Core promoter mutation (A/G 1762/4 T/A)	Codon 15 variants (C1858/T1858)	Pre-core stop codon mutation (G1896A or codon 28 TGG>TAG)
Wild type	60	92*	104
Mutant	17	8**	14
Con- infections (Mutant/ Wild type)	58	17	14
Total	135	117	132

*C1858 variant; **T1858 variant

Basal core promoter mutation:

For analysis of BCP mutation, the nested RFLP-PCR amplicons were digested with *BfuC1*, which in the presence of the mutation generates a mutant-specific fragment of 110 bp (Fig 17). The same PCR amplicon also contains a wild type restriction site in the majority of HBV isolates, and thus serves as an internal control for the *BfuCI* digestion reaction (Fig 17). As shown in Table 11, there was a significant correlation between the BCP mutation and the HBV genotypes. The BCP mutation was more predominant among cases with A and mixed genotypes. Analysis of the correlation between the HBeAg status and the BCP mutation showed a predominance of BCP mutation among negative HBeAg patients compared to wild type, although this correlation was statistically not significant.

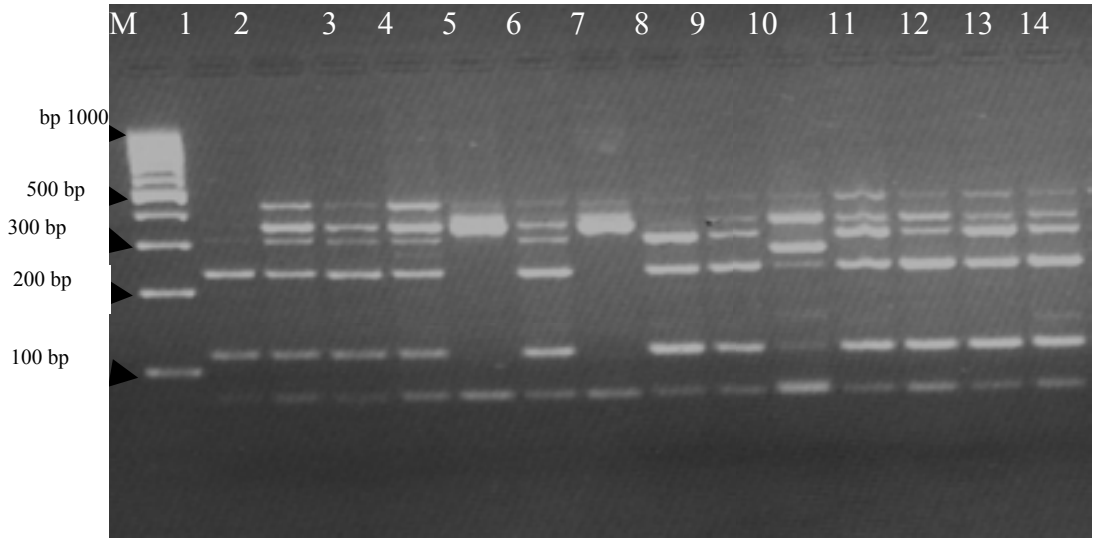

Figure 17. Agarose gel electrophoresis pattern of nested RFLP-PCR amplicons for the BCP mutation (A/G1762/1764T/A). Lane M: 100 bp DNA ladder; lanes; 1, 2, 3, 4, 6, 8, 9, 10, 11, 12, 13, and 14: show the BCP mutant with the mutant-specific fragment of 110 bp; lanes 5 and 7: show the wild type digestion pattern.

Table 11. Demographic and virologic characteristics of basal core promoter mutation (A/G 1762/1764 T/A).

Characteristic	*BCP 1762/1764 Mutant & Co-infection*	*Wild Type*	*P Value*
No. of patients	75/135	60/135	
Age yr (mean ± SD)	36.88 ± 15.01	36.51 ± 16.79	0.91
Male / Female	54/21	49/11	0.19
Genotype, n			0.03
A	6	1	
D	13	19	
Mixed	49	39	
Not type -able	7	1	
HBe Ag, n	*n*=57	*n*=43	0.44
+	17	16	
-	40	27	
HBV- DNA level (mean ± SD)	4.45 ± 1.56 log10	5.60 ± 1.83 log10	0.000

Pre-Core G1896A/ codon 28 stop mutation:

The nested RFLP-PCR specific for the PC mutation was successful in 132 samples. The PCR amplicons were digested with *Bsu36I* and the presence of PC G1896A mutation was identified by the detection of the mutation specific fragments of 164 bp and 30 bp (Fig 18), while the presence of the undigested fragment of 194 bp indicates the presence of wild type genotype. As shown in Table 12, there was a significant correlation between the PC mutation and the patients' age ($p<0.001$) as well as the serum HBV DNA level ($p<0.001$). Analysis of the correlation between the HBV genotypes and PC mutation, showed no significant correlation. However, the number of samples showing the PC mutation was low (28/132), and thus a larger sample size is needed to reach a conclusive result regarding this point.

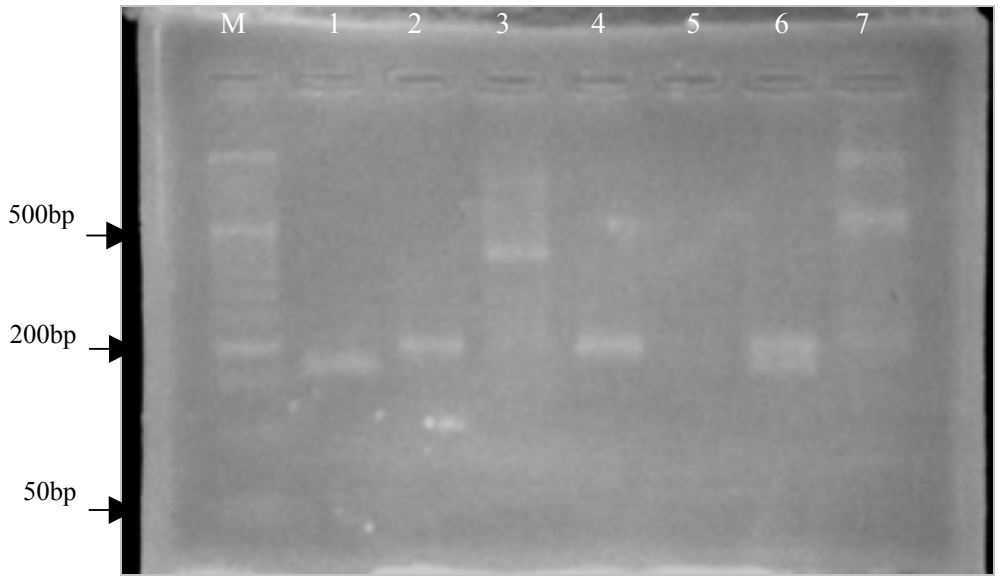

Figure 18. Agarose gel electrophoresis pattern of nested RFLP-PCR amplicons specific for the PC (G1896A) mutation. Lane M: 50 bp DNA Ladder; lane 1: positive for the mutant and show mutant specific fragment of 160 bp; lanes 2, 3, 4, 7: wild type and show the undigested fragment of 194 bp; lane 5: empty; lane 6: show both the mutant and wild type pattern. The 30 bp fragment that accompanies the mutant specific fragment of 160 bp, can not be detected using the above gel.

Table 12. Demographic and virologic characteristics of pre-core stop codon mutation (G1896A or codon 28 TGG>TAG).

characteristic	*Mutant PC1896 (mutant & co-infection)*	*Wild type*	*P-value*
No. of patients	28/132	104/132	
Age yr (mean ± SD)	44.61 ± 15.26	33.60 ± 14.24	0.001
Male / Female	23/5	78/26	0.25
Genotype, n	*n=28*	*n=104*	0.16
A	0	6	
D	11	22	
Mixed	16	69	
Not type -able	1	7	
HBe Ag, n	*n*=17	*n*=82	0.39
+	4	28	
-	13	54	
HBV- DNA level (mean ± SD)	5.91 ± 1.33 log10	4.71 ± 1.77 log10	0.001

Precore region/ Codon 15 variants:

The nested RFLP-PCR specific for codon 15 variants was successful in 117 samples. The amplicons were digested with *EcoNI*, and the detection of undigested fragment of 208 bp indicates the presence of the C1858 variant while the detection of 178 bp and 30 bp indicates the presence of T1858 variant (Fig 19). As shown in Table 13, no significant correlation was observed between the T1858/C1858 variants and the patients' age, sex, HBV genotype, HBeAg status and serum HBV DNA level.

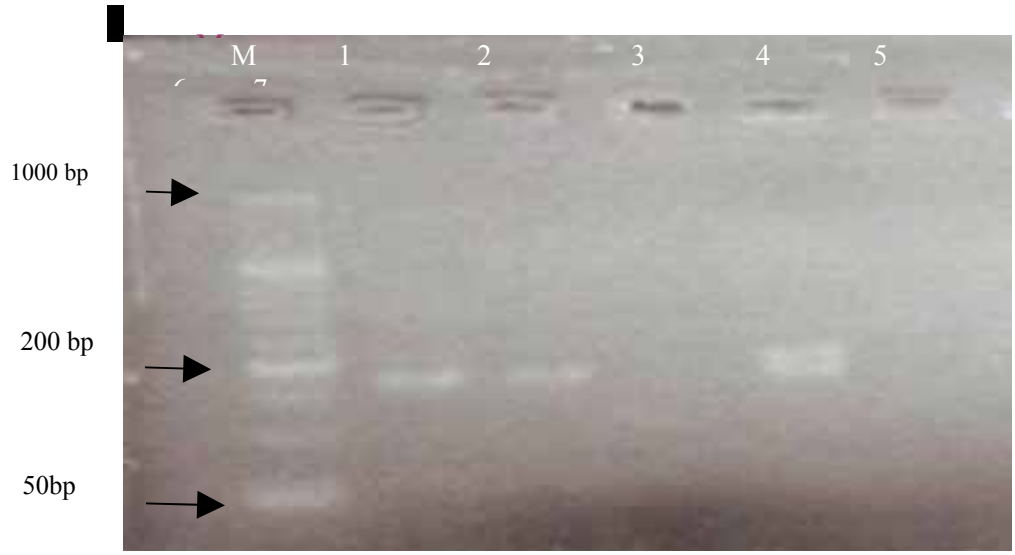

Figure 19. Agarose gel electrophoresis pattern of nested RFLP-PCR amplicons specific for the precore codon 15 variants (T1858/C1858). Lane M: 50 bp DNA ladder; lane 1 and 2: positive for the T1858 variant and show the specific fragment of 178 bp; lanes 4: show both the T1858 (178 bp) and C1858 (208 bp) variants. The 30 bp fragment that accompanies the T1858 variant specific fragment of 178 bp, can not be detected using the above gel.

Table 13. Demographic and virologic characteristics of pre-core codon 15 variants (C1858/T1858).

Characteristic	*Codon 15 Mutant & Co-infection (T1858 variant)*	*Wild Type (C1858 variant)*	*P-Value*
No. of patients	25/117	92/117	
Age yr (mean ± SD)	40.04 ± 12.235	35.51 ± 15.789	0.195
Male / Female	20/5	69/22	0.66
Genotype, n	*n=25*	*n=92*	0.16
A	3	3	
D	7	21	
Mixed	15	61	
Not type -able	0	7	
HBeAg, n	*n=18*	*n=70*	0.156
+	8	19	
-	10	51	
HBV- DNA level (mean ± SD)	4.97 ± 1.85 log10	4.95 ± 1.80 log10	0.970

III.4. Serum HBV DNA level

The presence of detectable levels of serum HBV DNA was determined by a quantitative PCR method on the COBAS-Amplicore (Roche diagnostics) and was

considered as one of the criteria for enrolment into this study. Table 14 summarizes the serum HBV DNA level and their correlation to the patients' sex and HBeAg status. The results show that the DNA level in patients with positive HBeAg was significantly higher than those with negative HBeAg (Table 14; $p<0.002$). However, no significant difference in the HBV DNA levels was observed between male and female patients.

Table 14. Demographic and virologic characteristics of baseline serum HBV DNA levels (mean ± SD; copies/ml).

characteristic	*Mean HBV-DNA levels*	*p-value*
Sex		0 .365
Male	4.95 ± 1.76 log10	
Female	4.641 ± 1.65 log10	
HBe Ag		0 .002
+ (n=34)	5.63 ± 1.64 log10.	
- (n=75)	4.53 ± 1.72log10	

III.5 Correlation between HBV genotypes and clinical outcomes in patients with chronic HBV

Table 15 shows the statistical analysis of factors associated with the clinical outcomes in patients with chronic HBV infection. A significant correlation was observed between the presence or absence of CAH or cirrhosis and patients' age. Where older patients are at a higher risk of developing liver cirrhosis than younger patients and that CAH is more predominant among younger patients. Additionally, no significant correlation was observed between HBV genotypes and presence or absence of CAH or cirrhosis. However, the results indicate that infection with mixed genotypes was more predominant among patients with CAH than those without CAH. While infection with mixed infection was more predominant among patients without liver cirrhosis than cirrhotic patients. Table 15 also shows that patients with CAH have a significantly lower levels of serum HBV DNA compared to those without CAH ($p<0.009$). While

patients with cirrhosis have a significantly higher levels of serum HBV DNA than those without cirrhosis ($p<0.038$). A significant correlation was also found between the PC mutation (G1896A) and presence of CAH ($p<0.013$) or cirrhosis ($p<0.001$). Analysis of the correlation between the BCP mutation and codon 15 variants, showed no significant correlation with CAH or cirrhosis.

Table 15. Statistical analysis of factors associated with clinical outcomes in HBV patients.

	CAH	No CAH*	P- value	Cirrhosis	No Cirrhosis	P-value
No. of patients	118	32		27	123	
Age, yr (mean ± SD)	31.35 ± 12.56	52.39 ± 9.60	0.000	52.08± 10.31	32.36 ± 13.14	0.000
Male/Female	85/33	27/5	0.155	22/5	90/33	0.37
Genotypes	*n*=118	*n*=32	0.38	*n*=27	*n*=123	0.44
A	5	2		2	5	
D	32	7		6	33	
Mixed	73	23		19	77	
Not type -able	8	0		0	8	
HBe Ag, n	*n*=92	*n*=23	0.32	*n*=18	*n*=96	0.35
-	61	17		14	64	
+	31	5		4	32	
HBV- DNA level (mean ± SD)	4.71 ± 1.76 log10	5.74 ± 1.50 log10	0.009	5.60 ± 1.46 log10	4.76 ± 1.78 log10	0.038
BCP1762/1764	n=105	n=30	0.127	n=26	n=109	0.526
W	43	17		13	47	
M	62	13		13	62	
PC (G1896A)	n=103	n=29	0.013	n=24	n=108	0.001
W	86	18		13	91	
M	17	11		11	17	
Codon 15 variants C/T1858	n=93	n=24	0.109	n=19	n=98	0.072
W (C1858)	76	16		12	80	
M (T1858)	17	8		7	18	

No CAH* represent hepatocellular carcinoma & cirrhosis patients.

Furthermore, we have reported the type of antiviral treatment the patients received at the time of sample collection (data not shown). Where the majority of patients with CAH were treated with Interferon (24/118), Epivirin (17/118) or a combination of

Interferon and Lamivudine (24/118). While patients without CAH were mostly treated with Epivirin (16/32), and a lower number of patients in this group received Interferon (1/32) or Interferon and Lamivudine (4/32). But, still a good proportion of patients with (44/118) or without (6/32) CAH received no treatment. Regarding patients with cirrhosis, a lower number of patients compared to non-cirrhotic patients received antiviral treatment at the time of sample collection. Patients with cirrhosis received Epivirin (16/27), Interferon and Lamivudine (3/27) or no treatment (3/27). While patients without cirrhosis received Interferon (25123/), Epivirin (17/123), Interferon and Lamivudine (47/123) or no treatment (25/123). Since, the type of treatment was reported at one point only; analysis of statistical correlation with liver complications is not useful and thus was not performed. We suggest that different groups of chronic HBV patients receiving different antiviral therapy are followed over a specific time period and the type of liver complications studied, which should allows a more accurate investigation of the correlation between the type of treatment and liver complications.

Table 16 summarizes the statistical analysis of factors associated with the development of HBV infection to HCC. Of all the 150 study samples, only five patients have had HCC at the time of sample collection. As shown in Table 16, HCC tend to develop at an older age among chronic HBV patients ($p<0.005$). Also patients with HCC tend to have a higher serum levels of HBV DNA ($p<0.058$). Remarkably, all of the four HCC patients typed for the BCP mutation showed the wild type pattern ($p<0.023$). Moreover, Table 16 shows that four out of five HCC patients have mixed genotypes and that three out of four HCC patients (HBeAg values are available for only 4 of the 5 HCC patients) were negative for HBeAg. The lack of significant correlation between the HCC and HBV genotype or HBeAg may be attributed to the lower number of HCC

cases included in this study. Moreover, no significant correlation was observed between HCC and the patients' gender, PC 1896 mutation and the codon 15 variants.

Table 16. Statistical analysis of factors associated with development of HCC.

	HCC	No HCC	P value
No. of patients	5	145	
Age, yr (mean ± SD)	54.00 ± 4.85	35.27 ± 14.58	0.005
Male/Female	5/0	107/38	0.185
Genotypes	n=5	n=145	0.85
A	0	7	
D	1	38	
Mixed	4	92	
Not type -able	0	8	
HBeAg	n=4	n=110	0.77
-	3	75	
+	1	35	
HBV- DNA level (mean ± SD)	7.23 ± 2.08 log10	4.86 ± 1.74 log10	0.058
BCP 1762/1764	n=4	n=131	0.023
W	4	56	
M	0	75	
PC (G1896A)	n=5	n=127	0.237
W	5	99	
M	0	28	
Codon 15 variants C/T1858	n=5	n=112	0.939
W (C1858)	4	88	
M (T1858)	1	24	

LIST OF ABBREVIATIONS

ADV	Adefovir
AFP	Alpha-Fetoprotein
ALT	Alanine Aminotransferase
ANOVA.	Analysis Of Variance
Anti-HBe	Antibodies to HBeAg,
AGPC	Acid Guanidinium Thiocynate Phenol –Chloroform
AST	Aspartate Aminotransferase
BCP	Basic Core Promoter
CAH	Chronic Active Hepatitis
CHB	Chronic Hepatitis B
DSL	Double-Stranded Linear DNA
ETV	Entecavir
HBcAg	Hepatitis B Core Antigen
HBeAg	Hepatitis B e-Antigen
HBsAg	Hepatitis B s-Antigen
HBV	Hepatitis B Virus
HBx	Hepatitis B X-protein
HCC	Hepatocellular Carcinoma
IFN	Interferon
LC	Liver Cirrhosis
LMV	Lamivudine
NCBI	National Center for Biotechnology Information
ORFs	Open Reading Frames
PC	Pre-Core Mutation
PCR	Polymerase Chain Reaction
RT	Reverse Transcriptase
SPSS	Statistical Package for the Social Sciences
TP	Terminal Protein domain in HBV polymerase

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II Materials and methods

II.1 Materials

All chemicals and reagents used were of Molecular Biology Grade. A list of chemicals and buffers, and solutions used in this study are shown in Tables 1 and 2, respectively.

Table 1. Chemicals and reagents.

Chemical	Source, country
Puregene DNA Purification kit	Gentra, USA
Wizard® SV Gel and PCR Clean System	Promega, USA
Big Dye ®Terminator Cycle Sequencing Kit	Applied Biosystems, USA
Guanidinium thiocynate	Sigma, USA
Sodium citrate	Finkeman
Sarcosyl	Sigma, USA
2- mercaptoethnol	Sigma, USA
Sodium acetate	ICN
Agarose (ultra pure agarose)	Invitrogen, Scotland
Tris base (99 %)	Avocdo, Johnson Matty
Boric acid	Himedia, India
EDTA (99 %)	Avocdo, Johnson Matty
GoTaq ® DNA polymerase (5U/ µL)	Promega, USA
dNTP's Mix (10 mM)	Promega, USA
Ethidium Bromide (10 mg/ml)	Sigma, USA

Table 2. Buffers and solutions.

Buffer	Components
TE	10 mM Tris, pH 8.0, 1 mM EDTA
Solution D	4 M guanidinium thiocynate, 25 mM sodium citrate (pH 7), 0.5% sarcosyl, 0.1 M 2-mercaptoethanol

All PCR primers used were obtained from Invitrogen (UK) and Metabion (Jerusalem) and the sequences of primers used are shown in Table 3.

Table 3. Primer sequences used for HBV genotyping by nested PCR

Primer name	Primer sequence (position, specificity, and polarity)	length
P1	5'-TCA CCA TAT TCT TGG GAA CAA GA-3' (nt 2823-2845, universal, sense)[1]	23
S1-2	5'-CGA ACC ACT GAA CAA ATG GC-3' (nt 685-704, universal, antisense) [1]	20
B2	5'-GGC TCM AGT TCM GGA ACA GT-3' (nt 67-86, types A to E specific, sense) [1]	20
BA1R	5'-CTC GCG GAG ATT GAC GAG ATG T-3' (nt 113-134, type A specific, antisense) [1]	22
BB1R	5'-CAG GTT GGT GAG TGA CTG GAG A-3' (nt 324-345, type B specific, antisense) [1]	22
BC1R	5'-GGT CCT AGG AAT CCT GAT GTT G-3' (nt 165-186, type C specific, anti sense) [1]	22
BD1	5'-GCC AAC AAG GTA GGA GCT-3' (nt 2979-2996, type D specific, sense) [1]	18
BE1	5'-CAC CAG AAA TCC AGA TTG GGA CCA-3' (nt 2955-2978, type E specific, sense) [1]	24
BF1	5'-GYT ACG GTC CAG GGT TAC CA-3' (nt 3032-3051, type F specific, sense) [1]	20
B2R	5'-GGA GGC GGA TYT GCT GGC AA-3' (nt 3078-3097, types D to F specific, anti sense) [1]	20
HBV-2160A	5'-CTG ACT AC T AAT TCC CTG GAT GCT GGG TCT-3' (nt 2131-2160, antisense)[2]	30
HBHKF1	5'-ACG GGG CGC ACCTCT CTT TAC-3' (nt 1519 to 1539, sense)[3]	21
HBV-1606S	5'-GCA TGG AGA CCA CCG TGA AC-3' (nt 1606-1625, sense) [2]	20
HBHKR2	5' AGC CAA AAA GGC CAT ATG GCA3' (1917-1937, antisense)	21
HBV-1653	5'-CAT AAG AGG ACT CTT GGA CT-3' (nt1653-1672, sense) [3]	20
HBV-2094A	5'-CAC CCA GGT AGC TAG AGT CAT CA-3'	23

	(nt 2094-2116, antisense)[4]	
HBV-2039A	5'-GTA TGG TGA GGT GAA CAA TG-3' (nt 2058-2039 , antisense)[3]	20
YS1-S	5'-GCG GGG TTT TTC TTG TTGA-3' (nt 203-221, sense)[5]	19
HBV-1680S	5'ATG TCG ACA ACC GAC CTT GA-3' (nt 1680-1699, sense)[3]	20
HBV-1888AS	5'CCC AAG GCA CAG CTT GGA GGC TCC TAC AGT-3' (nt 1888-1859, antisense) [3]	30
HBV-1865S	5'CAA GCC TCC AAG CTG TGC CTT GGG TGG CCT T3' (nt 1865-1895, sense) [3]	31

The sequence of primers denoted by a superscript number were obtained from the following references: [1](Naito, *et al*., 2001), [2](Kato, *et al*., 2002), [3](Lindh, *et al*., 1996), [4](described in this study), [5](Zeng, *et al*., 2004).

The restriction enzymes used for detection of precore/ basal core promoter mutations were obtained form New Englan Biolabs (UK) (Table 4).

Table 4. Restriction endonucleases used for detecting HBV pre-core and basal core promoter mutations.

Restriction enzyme	Recognition site	Source
EcoNI	5'…CCTNN▾NNNAGG…3' 3'…GGNNN▴NNTCC...5'	New England Biolabs
Bsu36I	5'…CC▾TNAGG…3' 3'…GGANT▴CC…5'	New England Biolabs
BfuCI	5'…▾GATC….3' 3'…..CTAG▴….5'	New England Biolabs

II.2 Methods

II .2.1 Study design and data collection

The archives in governmental medical centers in the different governorates of West Bank between July 2004 and August 2006 were reviewed for identification of patients

with chronic HBV. Patients with chronic HBV included: chronic active HBV, hepatocellular carcinoma and patients with liver cirrhosis. One hundred and fifty patients were selected. The following information for each patient was reported (see the Questionnaire in Appendix 1): patient's gender, mean age (range), and the presence of liver complications such as liver cirrhosis (LC), chronic active hepatitis (CAH) and hepatocellular carcinoma. Selection of patients was in accordance with the distribution of HBV cases in various regions as recorded in the national annual report of HBV between 2004 and 2006, and correlated to the prevalence of HBV in the year 2000 (Fig

5).

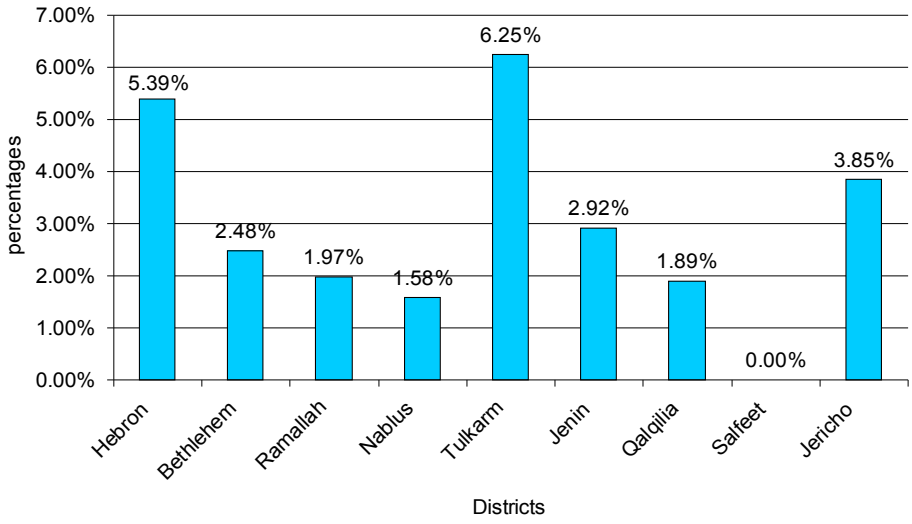

Figure 5. The prevalence of positive HBsAg carriers distributed by districts in West Bank 2000. From (Ramlawi & Preventive Medicine Team, 2000).

II .2.2 Criteria for patients' selection

Patients for this study were selected according to the following criteria: Patients whom age falls between 16 to 65 years old, HBsAg positive at least for a period of 6 months, HBeAg or anti-HBe measured (either positive or negative), HBV- DNA positive

(quantitative) in serum and serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured. Personal and clinical information of selected HBV patients were collected by asking the treating physician to fill the provided HBV questionnaire. Questionnaires were filled only after obtaining an informed consent from the selected patients.

II .2.3 Biochemistry and serologic markers

Liver function tests, ALT and AST were measured for most patients to establish a baseline for patients' examination by using a commercial kit (Diaysis, Biosystem kit) according to the manufacturer's instructions. Serological testing for HBsAg, hepatitis B e-antigen (HBeAg), anti-HBe, anti-HBc IgM, anti-HBc IgG, anti-HBs were done for most patients enrolled in the study using commercial kits (Biokit, Equipar, Organic, Human, and Atlas; respectively) per the manufacturer's instructions.

II. **2.4 Detection of HBV DNA by PCR and viral load determination**

Detection of HBV DNA and viral load determination was performed in serum from 150 patients using a quantitative PCR technique on the COBAS-Amplicore machine (Roche Diagnosis System, USA) and HBV DNA detection kit for COBAS Amplicore (COBAS AMPLICORE HBV MONITOR Test, Roche Diagnosis System, USA), as a diagnostic test to confirm the presence of HBV infection as well as determine the viral DNA load. The test can quantify HBV DNA over the range of 60 to 38,000 International Units (IU)/ml (300 to 200,000 HBV DNA copies /ml). The HBV monitor test uses the primers HBV-104UB and HBV-104D to define a sequence of 104 nucleotides within the highly conserved pre-Core /Core region of the HBV genome.

II .2.5 Sample preparation and storage

Sera were collected from respective patients with HBV infection and stored at -35°C until used.

II. 2.6 Extraction of HBV- DNA from serum

Part of the nucleic acid extraction was carried out by using Gentra DNA extraction kit (Puregene Minneapolis, USA) according to the manufacturer's instructions. Briefly, for extraction of HBV-DNA, 100 µl serum was mixed with 300 µl Cell lysis solution (Tris, EDTA and sodium dodecylsulfate) and 1 µl Rnase was added and incubated at 37ºC for 10 minutes. After that 3 µl of protinase K (20 mg/ml) was added to the lysate, incubated at 55ºC for 1-2 hours and then chilled on ice for 1 minute. The digested lysate was mixed with 200 µl of protein precipitation solution (Ammonium acetate) and centrifuged at 14000 rpm at room temperature for 3 minutes and the supernatant containing the DNA was transferred to a new tube. The DNA was precipitated by addition of 500 µl isopropanol and overnight incubation at -20ºC, followed by centrifugation at 14000 rpm at 4ºC for 15 minutes. The DNA pellet was washed from the isopropanol traces with 700 µl of 70 % ethanol and with further centrifugation at 14000 rpm at 4ºC for 5 minutes. The DNA pellet was air dried at 37ºC for 5-10 minutes. The resulting pellet was resuspended in 25 µl DNA Hydration Solution (10 mM Tris, pH 8, 1 mM EDTA) and was incubated at 37ºC overnight or at 65ºC for one hour and then subjected to nested PCR.

The second part of HBV DNA extraction was performed by Acid Guanidinium Thiocynate phenol –chloroform (AGPC) method as described by (Chocmczynski & Sacchi, 1987). Briefly, 200 µL of patient's serum was mixed with 600 µL of

denaturing Solution D (Solution D contains 4 M guanidinium thiocynate, 25 mM sodium citrate, pH 7, 0.5 % sarcosyl, 0.1 M 2-mercaptoethanol)-and 1/10 volume of 3 M sodium acetate (pH 4.2-5.2). The latter mixture was mixed with an equal volume of phenol: chloroform (pH 8) and incubated at room temperature for 20 minutes. The mixture was centrifuged at 13000 rpm at room temperature for 20 minutes. The aqueous phase containing the DNA was transferred to a new tube, mixed with 0.5 volume of chloroform and centrifuged at 13000 rpm at room temperature for 20 minutes. For precipitation of DNA, the aqueous phase was mixed with 900 µL of isopropyl alcohol, incubated overnight at –20ºC, and further processed as described above for the Gentra DNA extraction kit. However, hydration of the DNA pellet was performed by adding 50 µL of sterile free-nuclease water at 37ºC for 1 hour or overnight, and then subjected to nested PCR.

II .2.7 HBV Genotyping

Genotyping of HBV isolates was performed by multiplex- nested PCR using specific primers for determination of the seven HBV genotypes (A through H except G), based on S-region of HBV as described by Naito *et al*., (2001). The specificity of the results was confirmed by employing sequencing and phylogenetic analysis for representative samples.

II. 2.7.1 Determination of HBV genotypes (A-F) by multiplex nested PCR of S region with specific primers

The sequences of PCR primers used in this study were described earlier by Naito, *et al*., (2001), and are shown in Table 3. For the first-round PCR, a 2 µl aliquot of processed serum specimens was subjected to 25 µl polymerase chain reaction (PCR) with the

following amplification mixture: each reaction contained 5 µl of 5X GoTaq buffer containing 1.5 mM $MgCl_2$, 0.2 mM of deoxynucleotide triphosphate mix, 0.3 µM of each of the common primer pair P1 and S1-2, 1U of Taq DNA polymerase (Promega, USA). The common primer pair (P1 and S1-2) hybridizes to a conserved consensus region in the S/preS-gene in all HBV genotypes. Thermal cycling was performed as follows: initial denaturation at 94ºC for 4 minutes**,** followed by 35 cycles, with each cycle consisting of denaturation at 94ºC for 30 seconds, annealing at 52ºC for 30 seconds, extension at 72ºC for 1 minute, followed by a final extension at 72ºC for 5 minutes.

The amplicons (1097 bp) of first-round PCR were subjected to two second – round PCR reactions called Mix A (detects genotypes A, B and C) and Mix B (detects genotypes D, E and F). Mix A used the common universal sense primer (B2) and the genotype-specific primers (BA1R, BB1R and BC1R for genotypes A, B and C, respectively), while mix B used the common antisense primer B2R and the genotype-specific primers (BD1, BE1 and BF1 for genotypes D, E and F, respectively) (Naito, *et al*., 2001; Idrees, *et al*., 2004). However, because genotype H and F show high sequence homology, they were amplified using the same primer pair. Thus discrimination between H and F should be further performed by a specific PCR-RFLP analysis as described earlier (Sunbul & Leblebicioglu; 2005). Since genotype F was not detected in this study, the aforementioned PCR-RFLP analysis for discrimination of genotypes F and H was not performed and thus is not described here.

The second-round PCR was performed by adding 1 µl aliquot of the first-round PCR product (diluted 1:10 with DEPC-dH_2O) to each of two tubes (Mix A and Mix B), each

containing 5 µl of 5X GoTaq buffer containing 1.5 mM MgCl2, 0.25 mM of deoxynucleotide triphosphate mix, 0.3 µM of each mix A or mix B primers, 1U of Taq DNA polymerase in a 25 µL -reaction. Negative PCR control was included in each reaction to ensure the specificity of genotyping results. Thermal cycling was performed for 35 cycles with the following parameters: preheating at 95ºC for 3 minutes, 15 cycles, with each cycle consisting of denaturation at 94ºC for 20 seconds, annealing at 60ºC for 20 seconds, and extension at 72 ºC for 30 seconds, and an additional 20 cycles, with each cycle consisting of denaturation at 94ºC for 20 seconds, annealing at 58ºC for 20 seconds, and extension at 72ºC for 30 second.

II .2.7.2 Determination of HBV genotype G by nested primer-specific PCR

For detection of HBV genotype G, a nested-PCR targeting the core region was performed. The first round PCR amplified a 511-bp fragment in the core region using the outer primers HBV-1606S (the primer HBV-1606S sequence was provided kindly by Professor Chee Kent lim, Monash University, Malaysia) and HBV-2094A (described in this study). The second-round or nested PCR was a multiplex PCR and used 3 primers HBV-1653 /sense, HBV-2039A /antisense and HBHKR2 /antisense. The primer pair HBV-1653 and HBV-2039 amplifies a 406-bp fragment (lindh, *et al*., 1996), which serves as an internal control for the multiplex PCR. While the HBHKR2 primer (Kato, *et al*., 2002) was designed based on genotype G-specific 36-bp insertion in the core region and in combination with the common primer HBV-1653 primer amplifies a 285-bp fragment.

For the first round-PCR, 2 µl of DNA extracts were subjected for PCR essentially as described for first-round PCR of S/preS region in the previous section (II.2.7.1), with

0.3 μM of each of the primer pair HBV-1606S and HBV-2039A. Thermal cycling was performed with the following parameters: preheating at 94ºC for 3 minutes, 40 cycles, each consisting of denaturation at 94ºC for 30 seconds, annealing at 54ºC for 30 seconds and extension at 72ºC for 40 seconds, and a final extension step at 72ºC for 5 minutes. The second-round multiplex PCR of genotype G reaction was run as described for the second-round PCR of S/preS region with 0.3μM of each of the nested primers HBV-2039A/antisense, HBV-1653/sense and HBHKR2/antisense. Thermal cycling included: preheating at 94ºC for 3 minutes, 35 cycles each consisting of denaturation at 94ºC for 1 minute, annealing at 55ºC for 30 seconds and extension at 72ºC for 40 seconds, followed by a final extension at 72ºC for 5 minutes.

For analysis of genotyping PCR products, the respective PCR products were electrophoresed on a 2 % or 3 % agarose gel prepared in 1X TBE buffer and containing ethidium bromide (0.5 μg/ml) and run at 5-8 V/cm. The HBV genotype-specific DNA fragments were determined by comparison with a 50-bp or 100-bp DNA ladder (Promega, USA). The gels were visualized under UV illumination.

II .2.8 Detection of pre-core and core promoter mutants

All HBV samples were subjected for mutation analysis for the three common mutations that occur within the core region: the basal core mutations (A1762/G1764> T1762/1764A), the codon 15 varaints (C1858/T1858) and precore stop muatation at codon 28 (G1896A, TGG>TAG). These three mutations were analysed by nested RFLP-PCR.

For this purpose, the first-round PCR was performed using the primer pair HBV-1606S and HBV-2094A as described for the internal control used in the detection of genotype G (see previous section II.2.7.2).

The validity of restriction enzyme digestion was checked by digesting the two plasmids pMT2-FVIII -BDD (11000 bp) and pEGFP-C1 (4731 bp) with the three enzymes used for analysis of the above mentioned three mutations in the core gene, namely, *BfuCI*, *EcoNI* and *Bsu36I*. Analysis of plasmid digestion products showed that the digestion conditions used in this study are appropriate.

II .2.8.1 Basal core promoter mutations (A1762/G1764>T1762/1764A**) analysis**

Ten µl of the second PCR product (406 bp fragment) of the genotype G/ internal control was digested with 1.2 U of the *BfucI* enzyme (New England Biolabs) at 37°C overnight. The digestion products were analyzed on a 3 % agarose gel. The PCR amplicon contained a wild type restriction site in addition to mutant specific restriction site and thus served as a control for the BfuCI digestion reaction.

II.2.8.2 Codon 15 mutation (C1858/T1858) analysis

The nested second-round PCR for detection of C1858/T1858 variants was performed using the common primer HBV-1680S and the specific primer HBV-1888A. The primer HBV-1888A contains a mismatch at nts -8 to -6 (5'TGA3' > 5'CCT3') to generate an *EcoNI* restriction site in the PCR amplicon. The PCR amplicon (208bp)

was digested with 3 Units of *EcoNI* (New England Biolabs) at 37°C overnight. The digestion products were analyzed on a 3% agarose gel.

II .2.8.3 Pre -core stop codon mutation (TGG>TAG at Codon 28) analysis

The nested second-round PCR for detection of A1896G mutation (codon 28 stop mutation or TGG>TAG) was performed using the common primer HBV-2039A and the specific primer HBV-1865S. The primer HBV-1865S contains a mismatch at nt -3 (T>C) to generate a *Bsu36I* restriction site in the PCR amplicon. The PCR amplicon (194 bp) was digested with 2 Units of *Bsu36I* (New England Biolabs) at 37°C overnight. The digestion products were analyzed on a 3 % agarose gel.

II .2.9 DNA Sequencing and sequence analysis

The validity of primer-specific nested-PCR for HBV genotyping was verified by DNA sequencing of representative samples of each genotype. Additionally, the RFLP-PCR analysis results of precore/core region mutations were verified by DNA sequencing of representative samples.

II. 2.9.1 HBV–DNA purification

The HBV amplicon of different genotypes was cleaned from the residual dNTP's and primers using a commercial DNA gel extraction kit (Wizard® SV Gel and PCR Clean UP System, Promega, USA). Purification was processed from gel slices following electrophoresis, the DNA band was excised from gel in a 1.5 ml microcentrifuge tube, mixed with 10 µL of membrane binding solution (4.5M guanidine isothiocyanate, 0.5M potassium acetate, pH 5) per 10 mg of gel, and incubated at 50-65ºC until the gel slice is completely dissolved. Binding of DNA to SV-Minicolumn was achieved by

centrifugation at 14000 rpm for 1 minute followed by two times washing with membrane washing solution (10 mM potassium acetate, pH 5; 80 % ethanol, 16.7 M EDTA, pH 8). The DNA was eluted with 50 µl of nuclease- free water.

II .2.9.2 HBV–DNA sequencing

Following preparation of the PCR products or sequencing templates, the target DNA fragment was purified using a DNA gel extraction kit as described above (see section II.2.9.1) and the purified products accompanied by the specific primers (see below) were sent for sequencing to a commercial DNA sequencing facility at Bethlehem University (Bethlehem), and Metabion (Jerusalem). DNA sequencing was performed by the dideoxy chain termination method using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and the automated DNA Sequencer 3100 / Genetic Analyzer (Applied Biosystems) per the recommendations of the manufacturer.

For sequencing of the PCR products of the different HBV genotypes, two strategies were used. The first strategy used an amplicon generated by the primers YS1-S (nts 203 to 221) and S1-2 (nts 685 to 704) which amplifies a 502-bp fragment. The latter product has been used in previous studies for HBV genotyping by DNA sequencing (Zeng, *et al*., 2004). The second strategy used the shorter genotype-specific PCR products. The specific primers used and the type of template used for sequencing are shown in Table 5. The amplification of the 502-bp amplicon, was performed using 2 µl of first-round PCR for HBV genotyping (see sections II.2.9.1 & 2), 0.3 µM of each primer YS1-S and S1-2 under the same conditions used for second-round PCR described in section II.2.7.1 While for the second strategy, the genotype-specific PCR

products were prepared as described for the genotyping reaction, purified and used for sequencing.

However, for sequencing the core region covering the three mutations described in section II.2.8, a 406-bp fragment was amplified using the primer pair HBV-1653S and HBV-2039A, as described in section II.2.8.

For DNA sequencing, briefly, the appropriate PCR product (about 100 ng) was mixed with 3.2 pmol of the sequencing primer (shown in table 5) and 8 µl of the BigDye ready reaction mix in a final volume of 20 µl. The thermal cycling of the cycle sequencing step was performed using the following program: preheating at 95°C for 5 minutes and 30 cycles each consisting of denaturation at 95°C for 30 seconds, annealing at 50°C for 10 seconds, and extension at 60°C for 4 minutes. Excess dye terminators were removed from the sequencing mixture by means of ethanol/sodium acetate precipitation. Heat-dried samples were dissolved in 10 µl of nuclease-free distilled water and loaded onto the DNA sequencer according to the manufacturer's instructions.

Table 5. Primers used for DNA sequencing.

Sample name	Primer name & type	PCR product size (bp)
174	BA1R	68
128	BA1R	68
100	BB1R	281
126	BB1R	281
111	BD	119

126	BC1R	122
100	BC1R	122
67	YS1-2	502
116	YS1-2	502
174	YS1-2	502
31	YS1-2	502
116	YS1-2	502
29	YS1-2	502
115	HBV-2039	406
114	HBV-2039	406
129	HBV-2039	406

II. 10 Phylogenetic and statistical analysis

The phylogenetic analysis was made on the amplified products of S gene as indicated in Table 5. The sequencing data were aligned with reference strains retrieved from the Gene Bank database, subtype A: X02763, X51970, AF090842; subtype B: D00329, AF100309; subtype C: X04615, M12906, AB014381; subtype D: X65259, M32138, X85254; subtype E: X75657, AB032431; subtype F: X69798, AB036910, AF223965; subtype G: AF160501, AB064310, AF405706; subtype H: AY090454, AY090457, AY0900460, as well as genomes representing the different prototypes of HBV genotypes (Genotype A: AB014370, Genotype B: AB033554; Genotype C: AB014365; Genotype D: X97848; Genotype E: X75664; Genotype F: X75663; Genotype G: AF160501; Genotype H: AB059659. The Gene bank accession numbers of the HBV prototypes were provided kindly by Professor Hideaki Kato (Nagoya City University, Japan). Sequence alignment was performed using the CLUSTAL 1X (1.81) program, while the GENE doc program version (2.7) was used for exporting the aligned sequences and producing edited sequence to the PHYLIP program.

II.10.1 Phylogenetics

The PHYLIP program was used in a sequential way. The output from the first program was used as an input in the next program. Phylogenetic analysis of S gene sequences was performed using the PHYLIP package, version (3.6). The PHYLIP program SEQBOOT was used to bootstrap data in which 100 data sets were analyzed. First a distance matrix was calculated by Dnadist from the multiple sequence alignment. The matrix was then transformed into a tree by a NEIGHBOR (neighbor joining) to create dendrograms. Consense was used to create consensus trees from multiple trees. Finally, Trees were finalized by tree view program version (1.0).

II.10.2 Statistical analysis

All data analyses were performed using the SPSS package v.15 (SPSS Inc., Chicago, USA). Data are shown as means ± SD. All continuous data were analyzed by One Way ANOVA. While all the independent variables were analyzed by Chi Square and Binary Logistic Regression. A p-value <0.05 was considered statistically significant.

Appendix 1

Hepatitis B Questionnaire

Personal Information:

Patient Name: Date of Birth: _____
Place of Birth: Sex: M F
District: Phone:
Place of residence: 1.city 2.village 3.camp
Occupation: _____
Total monthly income (JD) 1- 0-500 2- 500-1000 3- 1000-2000
4- >2000
Number of persons/ family
Date reported to public health: ___/____/____

Clinical and diagnostic data:

1-Reason for HBV testing: (check all items that apply)

□ Asymptomatic (with risk factors) □ Asymptomatic without risk factors/Prenatal
□ Blood/organ donor screening
□ Symptoms of acute hepatitis □ Evaluation of elevated liver enzymes
□ Follow-up testing for previous marker of viral hepatitis

2 - Was the patient hospitalized for hepatitis? □Yes □No

3- Did the HBV patient develop HBV complications?
□Cirrhosis □HCC □Liver Failure □ Hepatic Encephalopathy
□ Coma

4-If the patient has HBV complications, what confirmatory tests were done to investigate these complications?
□ Alpha- Fetoprotein □ Ultrasound □ Liver Biopsy

5- Did the patient die from hepatitis? □Yes □No
Date of death: ___/___/____

Laboratory diagnostic tests:

CBC result:
WBC_____ RBC_____ Platelets Count_____
Date for first blood sample drawn for hepatitis B testing? __/__/__
Reporting Laboratory:_____

Liver enzyme values:

SGPT (ALT) _____ Test date: ___/___/____ Upper limit normal: _____
SGOT (AST) _____ Test date: ___/___/____ Upper limit normal: _____
Other tests _____

HBV serology results:

Test	Positive	Negative	Borderline	Not Done
HBsAg				
Anti- HBs				
HBe				
Anti_ HB e				
total anti-HBc				
IgM anti-HBc				

PCR result for detection of HBV DNA in serum:

_____

Vaccination history:

1-Has the patient ever received the hepatitis B vaccine? □Yes □No
If yes, how many doses? □ 1 □ 2 □ 3+

2-In what year was the last dose received? _____
3-Was the patient ever tested for antibody to HBsAg (anti-HBs) within 1-2 months after the last dose? □ Yes □ No
4-Was the patient ever given Immune Globulin for HBV? □ Yes □ No
If yes, at what month/year was the last dose received?_____

Mode of transmission:

□ Blood Transfusion □ Perinatal □Sex Partner
□Hemodialysis □Household Member (non-sexual) □ IDU
□Nosocomial.
1-Did the patient experience an accidental stick or puncture with a needle or other objects contaminated with blood? □Yes □No
2-Did the patient receive a tattoo? □Yes □ No
3-Did the patient has any part of his body pierced (other than ear)? □Yes □No
4-Did the patient has a minor surgery (other than oral treatment)? □Yes □ No

Antiviral treatment:

1-Type of treatment _____
2-Date for starting treatment_____
3-Duration of treatment _____

Interviewer name: _____

Interview Date: ___/___/___

Name of health center/clinic:

_____

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