Detection and Molecular Characterization of Hepatitis B virus (X gene) in Sudanese Patients with Chronic Hepatitis B

الكشف والتوصيف الجزيئي لفيروس التهاب الكبد الوبائي ب (جين اكس) في المرضى السودانيين المصابين بالالتهاب الكبدي المزمن (ب)

A thesis submitted in fulfillment of the requirements for the degree of Ph.D. in Medical Laboratory Sciences (Medical Microbiology)

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2018
DEDICATION

This research work is dedicated to the soul of my father, dear brother and sister.

My family and all those who supported me.

With love and appreciation
Acknowledgments:

I would like to thank the following

Kordofan University for giving me a chance to continue with my studies

My supervisor Professors Shamsoun Khamis Kafi for his generous guidance and instruction with every aspect of my PhD study. His broad perspective, rich experience, clear questions and insightful suggestions mean a lot to me.

The Department of Medical Microbiology, Sudan University of Science and Technology for accepting me to work in their Laboratory

Lastly, the University of Khartoum, Institute of Endemic Diseases for helping me to complete my research practical with them.
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Abstract:

**Background:** HBx gene mutations may have a role in the progression of liver disease from chronic infection to liver cirrhosis and/or hepatocellular carcinoma. These mutations affect the biological functions of HBx protein, which may influence the development of liver diseases. The aim of this study was to detect and characterize Hepatitis B virus X gene in Sudanese patients with chronic Hepatitis B, describe the variability of this gene among patients and find out the prevalent genotypes and their association with HBV infection outcome.

**Methods:** A total of 185 patients positive for HBV infection (88 asymptomatic carriers, 39 chronic hepatitis, 38 liver cirrhosis and 20 with hepatocellular carcinoma) were recruited to participate in this study. DNA was extracted from serum samples using chelex method. HBx DNA of nineteen patients was successfully amplified using nested PCR. HBx gene positive products were sequenced and genotyped. Nucleotide and amino acid variability were determined.

**Results:** HBx30, HBx127, HBx130 and HBx131 were the most detected mutations in HCC and LC patients. Double mutations K130M/V131I and F30L/V mutation were associated with high risk of LC and HCC development. Our study found that the most prevalent genotype was genotype D (47.4%), followed by E (42.1%) and
A (10.5%). Patients with genotype E had ALT elevation exceeding those with genotype D and A. HBx30, HBx130 and HBx131 mutations were associated with liver disease progression from chronic hepatitis to cirrhosis and hepatocellular carcinoma.

**Conclusion:** In conclusion, HBx30, HBx130 and HBx131 mutations may be useful markers for predicting the clinical course of patients with chronic hepatitis B.
المستخلص

خلفية: الطفرات الجينية الموجودة في فيروس التهاب الكبد الوبائي (ب) الجين اكس ربما يكون لها دورا في تقدم التهاب الكبد من الحالة المزمنة الى حالات تليف الكبد أو سرطان الكبد. هذه الطفرات تؤثر على الوظيفة البيولوجية لبروتين اكس مما قد يؤدي الى تطور الحالة المرضية للكبد. كان الغرض من هذه الدراسة هو الكشف والتوصيف الجزيئي لهذا الجين وايضا ايجاد التغيرات في هذا الجين في وسط المرضى السودانيين المصابين بالتهاب الكبد الوبائي إضافة الى ايجاد الانماط الجينية المنتشرة في السودان واحتمال علاقة اي نمط جيني مع نوع المرض الناتج من اصابة الكبد بهذا الفيروس.

الوسائل العملية: ضمت هذه الدراسة عدد 185 مريضا مصابين بفيروس التهاب الكبد الوبائي (ب) تشخيصهم كالاتي (88) مريضا ينتمون الى مجموعة المرضى الحاملين للمرض من غير اعراض او علامات, (39) مريضا ينتمون الى مرضى الالتهاب الكبدي المزمن, (38) مريضا ينتمون الى مجموعة المرضى المصابين بالتليف الكبدي و (20) مريضا مصابون بسرطان الكبد. تم استخلاص الحمض النووي (دنا) من عينات المصل للمرضى. تم الكشف جين اكس من عدد 53 مريضا بنجاح.

النتائج: كانت اكثر الطفرات التي تم اكتشافها في المرضى المصابين بتليف الكبد وسرطان الكبد هي في المواضع 30, 127, 130 و 131 في هذا الجين. الطفرات في المواضع 30,130 و 131 كانت مرتبطة بصورة واضحة مع مرضى التليف الكبدي وسرطان الكبد. وجدنا في هذه الدراسة ان أكثر نمط جيني سائد في السودان هو النمط الجيني (د) بنسبة 47.4%، يتبعه النمط الجيني (ه) بنسبة 42.1% وأخيرا النمط الجيني (أ) بنسبة 10.5%. ايضا وجدنا ان مستويات انزيم ناقل للألانين في النمط الجيني (د) مرتفعة أكثر من التي
الخلاصة: نخلص إلى أن الطفرات في المواضع 30، 130 و 131 مرتبطة بتطور مرض الكبد من الحالة المزمنة إلى حالات التليف الكبدي وسرطان الكبد ويمكن الاستدلال بها لتوقع الحالة المرضية التي يمكن يؤدي إليها مريض الالتهاب الكبدي المزمن.
Chapter (1)
Introduction & Literature review
1-1 Introduction:
Hepatitis B (HBV) is a virus that attacks the liver and can cause both acute and chronic disease (Seeger and Mason., 2000; Feitelson.,1999). An estimated 257 million people are living with hepatitis B infection and overwhelming epidemiological evidence indicates that they are at high risk for the development of liver diseases such as liver cirrhosis and hepatocellular carcinoma (HCC) (https://afro.who.int/health-topics/hepatitis).

HBV belongs to the hepadnaviridae family. The viruses included in this family are small with similar genome organization and have an identical DNA with a sequence homology of about 40% to 70% (Schaefer, 2007). Hepadnaviruses are hepatotrophic and their infection is species-specific or limited to closely related species causing variable degree of pathogenesis. (Guo et al.,2005).

Sudan is an African country with high HBV prevalence of greater than 8% HBsAg-positivity, ranging from 6.8% in central Sudan to 15.7% in southern Sudan. HBV infection occurs in early childhood in southern Sudan, with the infection increasing with age in northern Sudan (Mudawi, 2008; Mudawi, 2007).

Chronic hepatitis B may progress to liver cirrhosis and hepatocellular carcinoma (HCC) which is one of the most frequent malignant tumor in the world (Lupberger and Hildt, 2007). However, the pathogenesis of these HBV-related diseases has not
been fully clarified. The disease progression may depend on complex and multistep mechanisms and the association between HBV mutations and HCC has not been fully investigated. The compact nature of the genome and the ORFs overlapping lead to a “constrained evolution” of the virus (Key and Zoulim, 2007). Genetic variability of the X gene includes genotypic specific variations and mutations emerging during chronic infection (Song et al., 2006). Thus, a mutation on the X gene may not only induce aminoacid changes in HBV-X, but also can affect other genes and modify HBV expression (Boumert et al., 1998).

1.2 Literature Review:

1.2.1 HBV Historical Background:

Before the viruses causing hepatitis were isolated, transmission was differentiated on the basis of epidemiological observations. 'Serum hepatitis' (HBV) was distinguished clinically from 'infectious hepatitis' (HAV) in the 1930's, indicating that at least two different infectious agents were responsible for hepatitis. Type A hepatitis was considered predominantly transmitted via the fecal-oral route while type B hepatitis was believed to be primarily transmitted parenterally. (Kidd-Ljunggren et al., 2002). In order to categorize infectious and serum hepatitis the terms, hepatitis A and hepatitis B, were introduced by MacCallum in 1947. These terms were eventually adopted by the World Health Organization Committee on viral hepatitis (World Health Organization., 1973). HBV was
discovered in Australia through the identification of Australian Antigen in 1963, when searching for polymorphic serum proteins, Blumberg discovered a previously unknown protein in the blood of an Australian aborigine (Blumberg et al., 1965; Blumberg et al., 1967). The subsequent development of acute hepatitis in a laboratory technician provided the essential link to the clinical illness. This protein was denoted as the Australia (Au) antigen. It became apparent that this protein was related to type B hepatitis. For his achievements, Dr. Baruch Blumberg won Nobel Prize in Physiology in 1976. The work of other investigators, notably Prince, established that the Au antigen (now known as the hepatitis B surface antigen) was only found in the serum of type B hepatitis infected patients (Prince, 1968). In 1970, Dane found 42nm virus-like particles, ‘Dane particles’ in the serum of patients suffering from type B hepatitis (Dane et al., 1970). These particles were discovered when serum from Australia antigen-positive patients was studied by electron microscopy. A vast number of spheres and filaments of 22 nm in diameter were seen but also were present larger particles of 42 nm with a central nucleocapsid and an outer coat. These Dane particles were subsequently shown to constitute the complete virion whereas; the smaller filaments and spheres were found to be excess Australia antigen or HBsAg. HBV was initially characterized into different antigenic subtypes and then later into nucleotide divergence-based genotypes. Kaplan confirmed the viral nature of these particles by detecting an
endogenous DNA-dependent DNA polymerase within its core (Kaplan et al., 1973). Discovery of this polymerase allowed Robinson to detect and characterize the HBV genome (Robinson, Greenman., 1974).

1.2.2 Classification of HBV:
The hepadnaviridae is subdivided into mammalian and avian hepadnaviruses. HBV is belong to the mammalian Hepadnaviridae family and genus Orthohepadnavirus. It is a preferentially hepatotropic agent, and was originally called serum hepatitis. (Heathcote, 2007). The members of Hepadnaviridae are the woodchuck hepatitis virus, which was isolated from eastern woodchucks (Valsakamis A, 2007), ground squirrel hepatitis virus obtained from Beechy ground squirrels (Wright, 2006), tree squirrel hepatitis and duck hepatitis B virus (DHBV) isolated from Pekin ducks (Pungpapong, 2007). They all share unique genomic and structural features including similar virion size and ultra-structure, with an envelope surrounding a spherical nucleocapsid that contains a similar viral DNA genome in terms of size, structure and organization. HBV has unusual features, similar to retroviruses. Hepadnaviruses have moderate narrow host range, which is usually limited to each species (Hoofman, Thio , 2007; Lin , Kao , 2008).

1.2.3 Epidemiology of HBV:
HBV infection is a major global public health problem, warranting high priority efforts in prevention and control. The global prevalence of chronic HBV infection
varies from high (≥8%) in Africa, Asia and Western Pacific, to intermediate (2%-7%) in Southern and Eastern Europe and low (<2%) in Western Europe, North America and Australia (Figure1) (Mphahlele, Moloto, 2002). Over two billion of the world’s populations have been exposed to HBV and an estimated 257 million are chronically infected, with a rate of 10 million new carriers each year. Approximately 17% of the carriers die from the consequences of their infection, with an overall annual mortality from HBV infection of about 1 million (Schaefer, 2007; Alexander, Kowdley, 2006).

1.2.3.1 Epidemiology of HBV in Africa:

About 100 million persons in the World Health Organization (WHO) African Region have chronic hepatitis B virus (HBV) infection, and all countries in the region have an intermediate (2%–7%) or high (≥ 8%) population prevalence of chronic HBV infection (WHO, 2016). Chronically infected individuals have a 15%–25% estimated lifetime risk of developing liver cancer or cirrhosis, dependent upon age at infection (WHO, 2009). Africa is on the whole considered to have a high HBV endemicity. HBV infection is hyperendemic > 8% in some sub-Saharan countries such as Nigeria, Namibia, Gabon, Cameroon, Burkina Faso. Other countries like Kenya, Zambia, The Ivory Coast, Liberia, Sierra Leone and Senegal are considered areas of intermediate endemicity (2%-8%), while Egypt, Tunisia, Algeria and Morocco, located in the north of the continent, show a low
endemicity level (< 2%) (Kramvis&Kew, 2007). Prevalences of chronic carriage of hepatitis B virus in South African blacks are 5-16% in rural males, 8-9% in urban males, 4-12% in rural females, and 2.7-4% in urban females. The overall male to female ratio is 2.6:1.0. There are now three to four million South African blacks who are chronically infected with this virus. In rural black populations chronic hepatitis B virus infection is acquired very early in life, predominantly as a result of horizontal transmission of the virus, and by the age of 5 years’ carrier rates approach those seen in adulthood. (Michael Kew, 2008).

WHO recommends that all infants receive hepatitis B vaccine at birth, preferably within 24 hours, this dose acts as a safety net, reducing the risk of getting the disease from moms or family members who may not know they are infected with hepatitis B. Followed by two or three additional doses with a minimum interval of four weeks (WHO, 2009). In November 2014, the WHO African Regional Committee endorsed a resolution for a hepatitis B control goal to reduce chronic HBV infection prevalence to < 2% in children less than 5 years of age in all Members States by 2020 (WHO, 2017). Children acquire HBV infection most frequently by parenteral horizontal transmission (Ugwujaet al.,2010) from parents or siblings, as clearly demonstrated by phylogenetic analysis in Gambian families where HBV transmission occurred in at least two-thirds of the families investigated (Dumpis., 2001). Unsafe sharing in the daily practices of toiletries and sharpening,
cutting, scraping or scratching objects accounts for such a high horizontal transmission. In addition, cultural practices like scarification and tattooing and promiscuous sexual activity greatly increase the risk of HBV infection (Jombo et al., 2005; McCarthy et al., 1994)

1.2.3.2 Prevalence of HBV in Sudan:

In 2012, the Northern and Nahr Alnile states had the lowest prevalence of HBV (0.1%). In 2013 no cases were detected in the Northern Province, probably because of the small size of the population tested. The incidence in Nahr Alnile, in the same year, was 1.7%. In 2014, HBV prevalence in the Northern state was 0.5%, and was 0.9% in Nahr Alnile. The highest prevalence of HBV in 2012 was in South Kordofan, which was 15.7%. In 2013, the White Nile state had the highest percent of 12.3. And in 2014, Al-Gadarif showed the highest prevalence of 8.8%. The prevalence in Khartoum, in 2012-2014 was 3.6%, 4.7%, and 2.7% (Abdo et al., 2015). A study conducted, in 2008-2011 on patients undergoing surgery at Alshab Hospital, one of the major hospitals in Khartoum, revealed a higher percentage of 4.9% (Osman et al., 2013). In a more recent study in 2012 conducted among 843 health care workers in public teaching hospitals in Khartoum showed that Anti-HB core was found to be 57%, HBsAg was 6%, HBeAg was 9%, and Anti-HBsAg was 37% (Elmukashfi et al., 2012). The HBV prevalence in Al-Gezira state (Central Sudan) in 2012-2014, was 4.4%, 5.49%, and 5.2%
respectively. These percentages are lower than those observed in a study conducted in 2000, in Um Zukra village in Al-Gezira state, the percent of HBV was found to be 6.9%. (Mudawi et al., 2007) An earlier study in 1992, revealed much higher percents, it studied the prevalence of hepatitis B surface antigen in blood donors and laboratory technical staff in Al-Gezira state, and was found to be 17.3%, and 12.1% respectively (McCar thy et al., 1989). A study of the epidemiology of HBV in Al-Gezira State in 1989 reported a high HBsAg rate of 18.7% and anti-HBc of 63.9%. (Hyams et al., 1989). The prevalence of HBV in South Darfor (West Sudan) in 2012-2014 was 2.5%, 6% and 4.5% respectively. An earlier study in 2007 conducted in blood donors in Nyala (West Sudan) revealed a percent of 6.25%(Elsheikh et al., 2007). The prevalence of HBV in Kassla (East Sudan) in 2012-2014 was 3.6%, 5.7% and 4.2 % respectively. A much higher percentage of 8.2% was found in a study conducted in 2011 on healthy visitors at Kassala Hospital (Abdallah et al., 2011).
1.2.4 HBV Virion and genome structure

1.2.4.1 Virion structure:

HBV-infected cells produce three types of viral particles: 40-47-nm double-shelled particles called Dane particles, 22-nm spheres, and small quantities of filaments of 20- nm diameter and variable length (Figure 2). All three have a common antigen on their surface, termed hepatitis B surface antigen. HBs are present in enormous quantity in the serum of infected hosts allowing the physician to use direct antigen detection as a sensitive diagnostic test for HBV infection.
The Dane particle is the infectious virion of HBV. The outer envelope of the HBV virion contains three related glycoproteins (L, M and S); the inner nucleocapsid contains a single capsid protein (C). The viral DNA contains a terminal polymerase protein (P) attached to the negative strand (figure: 3). The major structural protein of the core is the C protein. Within the core are the viral DNA and a polymerase activity known to be involved in viral genomic replication. The 20-nm spheres and filaments lack nucleic acid and hence are noninfectious. Nonetheless, in pure form,
these particles are highly immunogenic and served as the initial form of HBV vaccine before development of recombinant HBs preparations.

**Figure (3):** The HBV virion is 42 nm in size, with the L, M, and S envelope proteins, with their preS1, preS2 and S domains in sub-boxes of size proportional to their polypeptide length. Embedded inside the envelope is the capsid containing the HBV genome, which is shown as a partially double-strand, incomplete circle, and viral polymerase protein (pol) (Diagram adapted from Block *et al.*, 2007)

HBV has a compact genome with partially double stranded DNA of approximately 3200 bases in length (Lau, Wright, 1993). The genome encodes four overlapping open reading frames (ORFs), overlapping each other and covering the entire genome (Figure 4). The ORFs include: surface (S) [(pre- S1/S2/S)], core (C) [pre-core/core], polymerase (P) and HBX-encoding (X) regions (Carman, 1996). The viral polymerase is covalently attached to the 5’ end of the minus strand which is approximately 3200 bases in length. The minus strand is the longest, and
contains ORF that encode for viral proteins and the elements responsible for regulation of HBV gene expression and replication. A plus strand of variable length maintains the circular structure of the cohesive hybridisation that straddles the 5’ and 3’ ends of the minus strands (Gerlich, Robinson, 1980).

**Figure (4):** The circular map of HBV genomic organization, with minus-strand and plus-strand DNA form the partially double-strand, incomplete circle. The four open reading frames (ORFs), denoted by S, P, C and X, overlapped partially. The viral mRNA transcripts are shown in the peripheral, with their polypeptide products indicated (Diagram adapted from Block *et al.*, 2007).
1.2.4.2 The Surface gene of HBV:

The S ORF encodes for the viral surface envelope proteins, and can be structurally and functionally divided into the pre-S1, pre-S2, and S regions. Each begins with its own translation start codon (ATG), but all end with a common translation stop codon (TAA). The three resulting proteins are LHBsAg, MHBsAg and SHBsAg or HBsAg proteins. All three proteins are required for the formation of the HBV envelope (Pungpapong et al., 2007). The HBsAg is coded for by the S-gene and is composed of 226 amino acids. HBsAg is of crucial importance, because it contains major neutralising epitope and is therefore used in commercial hepatitis B vaccines. The presence of antibodies to HBsAg (anti-HBs) alone is sufficient to protect from HBV infection (Carman, 1996).

1.2.4.3 The pre-core/core gene:

The C-ORF encodes either the viral nucleocapsid HBcAg or hepatitis B e antigen (HBeAg), depending on whether translation is initiated from the core or precore regions, respectively. The pre-core/core has two translation start codons (AUG); translation from the first leads to the preprotein that, after loss of the signal sequence and the carboxyterminal nucleophillic sequence, leads to HBeAg which is secreted into the serum, and its function remains largely undefined, although it has been implicated as an immune tolerogen, whose function is to promote persistent infection (Milich, Liang, 2003). Translation from the second AUG
leads to core protein, which is the building block of core particles and, therefore, virions. Both HBeAg and HBcAg translation end at a common translation stop signal (UAG). Both the products share about 160 amino acids. Besides HBcAg and HBeAg being structurally related and sharing some epitopes, they are not immunologically identical. Due to proteolytic processing, HBeAg assumes a different three dimensional conformations. This conformation allows HBeAg to shift the immune response against the virus in a humoral direction (Carman , 1996; Carman \textit{et al.}, 1989; Milich \textit{et al.}, 1998).

\textbf{1.2.4.5 The Polymerase gene of HBV:}

The polymerase gene (Pol) is the largest open reading frame and encodes for the multifunctional polymerase protein. The P ORF encodes 854 amino acid. Its protein is 90kD in size and has RNA- and DNA-dependent polymerase activities, which are responsible for priming and reverse transcription of the pregenomic HBV RNA (Lau, Wright., 1993). The polymerase gene overlaps all six other genes including the core gene that encodes for HBcAg and the precore gene that encodes for the HBeAg, the three envelope genes PreS1, PreS2, and HBsAg, that encodes for the large, middle, and small envelope proteins, respectively, LHBs, MHBs, and HBsAg. It also overlaps the X gene that encodes for the multifunctional X protein. The polymerase gene contains four functional regions: (i) terminal protein used in protein priming of HBV DNA synthesis, (ii) the spacer region, (iii) the reverse
transcriptase (RT) that has RNA and DNA dependent DNA polymerase activities, and the ribonucleaseH (RNase H) that cleaves the RNA in the RNA-DNA hybrids during reverse transcription (Chang et al., 1990; Radziwill et al., 1990; Lanford et al., 1999). The hepatitis B polymerase also requires the presence of the metal ions and the presence of the stem-loop for polymerase/reverse transcription activity to occur (Tavis et al., 1998; Urban, 1998). HBV has a complex genomic replication strategy, which uses protein priming for the synthesis of the minus and plus strands via a number of polymerase strand transfers to generate the partially double-stranded genomic HBV DNA. There are potentially three sites of action for a nucleoside analog in the HBV replication process. The first is the reverse transcription of the HBV pregenomic (pg) RNA into minus strand DNA. The second is the formation of the incomplete positive strand DNA. The third is the conversion of the partially double-stranded DNA (either derived from the intracellular conversion pathway or the initial entrance of the virus into the cell) to the fully complete double-stranded covalently closed circular form (cccDNA). This final conversion is likely to be mediated by cellular enzymes and is therefore not an ideal target for nucleo(ti)side analogs (Mack, 1988).

1.2.4.6 The HBx gene:

The X gene is the smallest of the four ORFs of the HBV genome. It codes for 154-amino acid polypeptide with a molecular weight of 17kD (Pungpapong et
al., 2007). The X gene was the last of the HBV genes to be characterised. The biological functions of the HBxAg have not been fully understood. The main function of the X protein is transcriptional activation and it has been shown to transactivate a number of cellular and viral promoters (Kekule, 1993; Henker, Koshy, 1996; Arbuthnot., 2000). X Protein may play a role in the over-expression of oncogenes and carcinogenesis of hepatic cells by activating signaling pathways. One of the regions which overlaps with the X gene in the genome of HBV (nucleotides 1374 to 1836), is the basal core promoter (BCP) (nucleotides 1742 to 1849) (Cho et al., 2011). The overlapping region is the binding site of cellular and viral transcription factors thus, have a key role in regulating gene expression of the virus and host. Sequence change of this site is effective in increasing viral replication and progression of liver disease (Wei et al., 1999). Precore and basal core promoter (BCP) mutant are the most common in HBV mutants. Double mutations in BCP A1762T/G1764A have been reported related to development of HCC (Lupberger and Hildt, 2007). This mutation A1762T/G1764A decrease the expression of HBeAg but increase the replication of HBV genome (Yang et al., 2008). One study showed that the incidence of HCC was higher in genotype C than genotype B, with variant precore G1896 (wild-type) higher than G1896A variant, and the BCP double mutation A1762T/G1764A is significantly higher compared with the wild-type variant of the BCP. The highest risk occurs when infected with
HBV genotype C and the wild-type variant for the precore 1896, and mutant for BCP variants A1762T/G1764A (Yang et al., 2008).

1.2.5 HBV Genotypes

1.2.5.1 History and classification of genotypes:

In 1988, Okamoto and colleagues compared full nucleotide sequences of 18 HBV strains, they found that these clustered into four groups, A to D, with more than 8% divergence between the groups. This degree of divergence has since become the definition for HBV genotype. Since the first description of four genotypes (A-D) of HBV in 1988, four more have been identified, designated E and F (Norder et al., 1994), G (Stuyver et al., 2000) and H (Arauz-Ruiz et al., 2002). Moreover, subgenotypes with distinctive sequence characteristics and a divergence in the complete genome of >4% have been found within genotypes A (Kimbi et al., 2004), B (Sugauchi et al., 2004), C (Huy et al., 2004) and F (Norder et al., 1994). Thus the classification of HBV into eight genotypes through phylogenetic analysis is defined by an inter-group divergence of >8% in the complete genome sequence and of >4% in the S gene (Norder et al., 1992). In a larger study, Norder and colleagues compared the S gene sequences from 122 strains and confirmed the existence of the two new groups, E and F (Norder et al., 2004). A highly divergent (15%) strain from Brazil has been reported by Naumann which expressed the adw4 phenotype and constitutes genotype F. It has often been used
as an out-group in phylogenetic studies of HBV, as it is the most divergent human-derived genotype reporte(Naumann et al., 1993).

1.2.5.2 Genotypes of HBV:

Nine genotypes of HBV, A – I, with a distinct geographic distribution have been recognized (Kramvis et al., 2005; Norder et al., 2004; Yuet al., 2005). A tenth genotype, J, has been proposed but was found only in one person (Tatematsu et al., 2009). Genotype A, D and E circulate in Africa (Kramvis et al., 2007). Genotype A prevails in southern, eastern and central Africa. Genotype D is the dominant genotype in northern Africa, whereas in western Africa genotype E predominates. Subgenotypes have also been identified within genotypes A and D (Kramvis et al., 2005; Norder et al., 2004).

Phylogenetic clustering within subgenotypes has been proposed to be denoted clades, representing strains with less than 4% diversity (Kramvis, 2008)(Figure5).

The nomenclature defining genotypes and sub classification has lately been debated and universal criteria pursued. A nucleotide divergence of 75% based on completed genomes has been suggested for genotypes, to better match phylogenetic clusters (Kramvis et al., 2008; Kurbanov et al., 2005; Schaefer et al., 2009).
Figure (5): HBV genotypes phylogenetic tree showing the 9 genotypes of HBV. (Rajoriya et al., 2017)

Figure (6): Geographical distribution of the HBV genotypes and sub-genotypes. Genotype I and J are not shown as they have not been ratified by the ICTV; genotype I is found in Southern China and Vietnam whilst genotype J was identified from a Japanese World War II person who lived in Borneo (S. Locarnini, M, 2013).
1.2.5.3 Genotype and Clinical outcome:

There have been substantial efforts to link genotypes to different clinical outcomes. Genotype A has been associated with a higher tendency to cause chronic infection and for the better, transition into the inactive carrier state after HBeAg seroconversion, in comparison with genotype D (Rodriguez-Frias et al., 2006; Sanchez-Tapias, et al., 2006). However, in a European study comparing genotype A and D, no difference was found in the degree of liver damage (Rodriguez-Frias et al., 2006). Subgenotype D1 have been associated with higher frequency of chronic liver disease compared to other D subgenotypes (Chandra et al., 2009). The clinical impact of genotypes with treatment efficiency has also been studied. Genotype A and B has been associated with a better response to interferon treatment compared to genotype C and D (Janssen et al., 2005; Kao et al., 2000; Wai et al., 2002). In regions where both genotype B and C prevail, several reports have shown that genotype C infections have worse clinical outcome compared with genotype B in terms of severe inflammation, cirrhosis (Chan et al., 2002; Kao et al., 2000; Kao et al., 2002; Lindh et al., 1997; Lindh et al., 1999; Nakayoshi et al., 2003) and prevalence of hepatocellular carcinoma (Chan et al., 2004, Chan et al., 2003). In accordance, genotype B has been associated with an earlier and a higher rate of HBeAg seroconversion than genotype C (Chu et al., 2002; Chu, Liaw ,2005; Furusyo et al., 2002). On the other hand, genotype B has also been
associated with a higher rate of severe icteric flares as compared with patients in Hong Kong carrying genotype C (Chan et al., 2002). In comparison with C2, subgenotype C1 has been associated with a higher frequency of developing basal core promoter mutations (Chan et al., 2005).

1.2.6 Viral life cycle

1.2.6.1 Entry and uncoating:
The initial phase of HBV infection involves the attachment of mature virions to host cell membranes, likely involving the pre-S domain of the surface protein (Figure 6). However, early events of the viral life cycle, including entry, uncoating, and delivery of the viral genome into the nucleus, are not well understood (Klingmuller et al., 1993).

1.2.6.2 Replication of HBV genome:
Recent studies reported that HBV virion interacts with the hepatocyte surface heparansulfateproteoglycans, which functions as primary attachment receptor for HBV (Schulze et al., 2007). Incoming HBV virions are bound by cell-surface receptors, the identity of which remains unknown. After membrane fusion, cores are presented to the cytosol and translocated to the nucleus (Rabe et al., 2006). There, their genomic DNA is maturated to a covalently closed circular (ccc) form, which serves as a transcriptional template for host RNA polymerase II. The cccDNA molecules play in the HBV life cycle the role analogous to that of
integrated proviral DNA in retroviral replication (Jun-Bin, 2003). The resulting RNAs are transported to the cytoplasm where they are translated to give rise to the viral envelope, core, X and polymerase proteins. Next, nucleocapsids are assembled in the cytosol, and during this process a single molecule of pregenomic RNA is incorporated together with the polymerase into the assembling viral core. Within this structure, viral DNA synthesis is sequentially initiated: after negative-stranded synthesis and concomitant degradation of the RNA template, positive-stranded DNA synthesis occurs (Seeger et al., 2007).

Figure (7): Schematic diagram of HBV life cycle (Ghany and Liang, 2007)
Some cores bearing the newly synthesized genome are recycled back to the nucleus to maintain a stable intranuclear pool of transcriptional templates. However, most cores bud into intracellular membranes (endoplasmic reticulum or proximal Golgi) to acquire their glycoprotein envelope containing the viral surface antigens. Enveloped virions are then secreted through the constitutive pathway of vesicular transport.

1.2.7 HBV infection:

HBV itself is considered as non-cytopathic, and the liver injury caused by HBV infection is mainly immunity-mediated. Enhanced viral replication can lead to a vigorous and extensive immune response which results in massive liver injury. There is a wide spectrum of liver disease caused by HBV. These include acute hepatitis, chronic hepatitis, LC, and HCC. The clinical manifestation is very diverse and characterized by different clinical, biochemical, serological, virological and histological findings. A better understanding of the natural history of HBV infection would help to develop effective management strategies for hepatitis B infection. HBV infection begins with the attachment of the virion to the surface of the hepatocyte. Subsequently, the cccDNA generated within the host cell nucleus is transcribed to pregenomic and subgenomic viral RNAs by host RNA polymerase. Translation of this pregenomic RNA leads to the production of pol and core proteins. This pregenomic RNA is encapsidated into viral nucleocapsid core
particles. The encapsidation mechanism is highly selective for genomic RNA, and depends on a region located near the 5′ end of the genome called the ε signal. (Brunetto et al., 2010) The ε region at the 5′ end of the pregenomic RNA interacts with the pol protein. (McMahon et al., 2009) This pol-ε interaction is stabilized by the host’s heat shock protein 90 (Hsp 90). The core proteins dimerize around the pregenomic RNA pol complex and form the viral nucleocapsids. Once the pregenomic RNA is packed into a nucleocapsid, it serves as a template for reverse transcription and minus-strand DNA synthesis. The pol protein is bound to the 5′-ε end and starts synthesizing the first three nucleotides of the minus-strand DNA. This nascent DNA chain is in turn translocated to the 3′ end of the pregenomic RNA, binding to a 12-nucleotide complementary region called direct repeat 1 (DR1). Subsequently, minus-strand DNA synthesis continues, and the template pregenomic RNA is degraded by the RNaseH activity of the pol protein except for the last few nucleotides. These RNA oligomers are translocated to the 3′ copies of another 12-nucleotide segment called direct repeat 2 (DR2), from which the synthesis of the plus strand DNA is initiated. The nucleocapsid containing the partially double-stranded DNA is directed to the endoplasmic reticulum and Golgi apparatus for virion assembly. The characteristic partially double-stranded genome (with a large gap in the plus strand) results from premature termination of
plus-strand synthesis by the pol protein. (Kock et al., 2004; Le et al., 2005; Newman et al., 2003).

1.2.7.1 Acute HBV infection:

Primary infection in susceptible hosts can either be asymptomatic or symptomatic, and in adults, this is usually self-limited with viral clearance and the development of immunity (Wright and Lau, 1993). If symptomatic disease occurs, clinical symptoms usually range from non-specific symptoms such as fatigue and nausea, to more specific symptoms of liver enlargement and right upper quadrant tenderness. The clinical incubation period of acute hepatitis B averages 2-3 months, followed by a prodromal period of symptoms such as fatigue, anorexia and nausea, there is also a rapid rise in serum ALT levels and HBsAg and HBV DNA levels are present at very high numbers (Liang, 2009). This phase is followed by a decrease in viral levels, together with a clearance of HBsAg and undetectable levels of HBV DNA. In approximately 1% of patients acute liver failure occurs (Berk and Popper, 1978). The presence of fulminant hepatitis is marked by a sudden appearance of fever, abdominal pain, vomiting, jaundice, confusion and coma (Liang, 2009).
The first serologic marker of HBV infection to appear is HBsAg. The HBsAg usually persists in serum throughout the period of clinical illness, and is commonly used to diagnose infection with HBV. HBsAg and HBV DNA are usually cleared in 3 to 6 months. The disappearance of HBsAg and the appearance of anti-HBsAg (anti-HBs) mark the resolution of acute infection. The anti-HBc generally appears at approximately the same time as HBsAg. HBc IgM develops initially and is eventually replaced by HBc IgG (Figure 8). Anti-HBs is undetectable in 10-15% of recovered patients, who may have detectable antibody to HBcAg (anti-HBc) alone, suggesting past infection. Occasionally, approximately 0.1-0.5% of patients with acute hepatitis B may develop acute liver failure (ALF) (Fattovich, 2003), which is typically marked by jaundice, followed by evidence of coagulation abnormalities and mental alternations. In this case, HBsAg and HBV DNA levels generally drop drastically, and some patients may be HBsAg negative. It has been reported that 45% adult patients with ALF in USA recovered spontaneously, 25% required liver
transplantation, while 30% died without liver transplantation (Nguyen and Vierling, 2011).

**1.2.7.2 Chronic Hepatitis B:**

Following acute HBV infection, 5% of adults, 30% of children and almost 90% of newborn to chronically infected mothers, become chronic carriers of the virus, probably because of failure of induction or activity of cytotoxic T lymphocytes (CTL). In these patients despite the high level of viremia there is no inflammatory necrosis of infected hepatocytes (immune tolerant phase). (Chu et al., 1985). During the following years of infection, the immune tolerant phase is replaced by increased hepatitis activity presumably reflecting immune activation (immune clearance phase) (Tsai et al., 1992; Hyams, 1995). Either because of CTL lysis of infected cells or of cytokine production by CD4-positive lymphocytes, hepatocytes infected with HBV are cleared during the seroconversion or immune clearance phase. About 5% of HBeAg positive chronic infections may seroconvert to anti-HBe per year (figure 9). In the majority of these HBeAg(-)/anti-HBe(+) patients, HBs antigenemia and small amounts of HBV DNA are detected in the serum by polymerase chain reaction (PCR), despite normal liver function tests, indicating that viral replication is still occurring at a low level (Carman et al., 1989). In some patients, particularly those infected at birth or in the early years
of life from infected family members, there is emergence of an HBeAg-negative variant. This virus, along with the HBeAg-positive strain, can be detected in virtually all patients after HBeAg/anti-HBe seroconversion (Carman et al., 1989). The A1896 variant is present in very small amounts during the latter period of the HBeAg positive phase of the disease and is selected at, or after, seroconversion to anti-HBe, whilst the HBeAg-producing strain is gradually being cleared (Okamoto et al., 1990). This process can take years, during which time a mixture of both strains is usually seen (Loriot et al., 1995). Some patients after seroconversion have high viraemia and develop further inflammatory liver disease (Hadziyannis and Vassilopoulos, 2001).

Figure (9): Characteristics of progression to chronic hepatitis B infection (Mahoney, 1999).
1.2.7.2. Chronic hepatitis B phases

1.2.7.2.1 Immune tolerance:

It is characterised by the presence of HBeAg, high serum levels of HBV DNA, normal or minimally elevated serum alanine aminotransferase (ALT) and minimal histological activity with scant fibrosis (Fattovich et al., 2008; Michielson, 2005). HBeAg induces immunological tolerance with consistent high levels of viral replication (usually ≥10^8 copies /ml) and is therefore associated with chronic infection (Francois et al., 2001; Heathcote, 2007). It is primarily observed as a perinatally acquired infection and can last for decades (Valsakamis, 2007). It usually lasts for 2-4 weeks in healthy adults but often several decades in those infected neonatally or in early childhood (Wright, 2006; Pungpapong, 2007).

1.2.7.2.2 Immune clearance:

In this phase the HBV DNA levels fluctuate but progressively decrease with elevated ALT and hepatic necroinflammation. Hepatic inflammation is believed to be associated with immune system and high levels of liver enzymes indicate the severity of liver damage (Hoofman and Thio, 2007). In this phase patients which were asymptomatic may start to show signs and symptoms which are suggestive of hepatitis flares (Linand Kao, 2008). An important outcome is the seroconversion
of HBeAg to anti-HBe but some patients may develop mutations in the precore/core region inhibiting the expression of HBeAg (Hoofman, Thio, 2007).

1.2.7.2.3 Low replicating phase:
The third phase is characterised by HBeAg negativity and anti-HBe positivity, low or undetectable levels of HBV DNA, normal ALT levels and inactive liver histology with minimal fibrosis (Fattovich et al., 2008). It may lead to a decline in HBV replication where HBsAg becomes undetectable (Michielson et al., 2005). HBV carriers in this phase usually confer a favourable prognosis, but in Asian countries the carriers may develop complications even after HBsAg clearance (Lin and Kao, 2008). The immune system minimizes active viral replication and thus lowering HBV DNA levels. It has been shown that 50% of patients at this stage clear HBeAg within 5 years of diagnosis and 70% within 10 years (Wright, 2006).

1.2.7.2.4 Reactivation stage:
The patients who present in the HBeAg negative stage can be further divided into the CHB inactive HBsAg carriers and the CHB patients with biochemical and intermittent virological activity. Patients who present in the latter group account for one third of CHB patients. These patients present with periodic stages of fluctuating ALT and HBV DNA levels and active hepatitis and fibrosis (Shi, 2009). This transition to virological activity may be due to the occurrence of nucleotide
substitutions in the BCP/precore region that decrease HBeAg expression. These patients are associated with active liver diseases and an increased risk of cirrhosis and HCC (Carman et al., 1989).

1.2.7.2.5 Fulminant hepatitis B:

Fulminant hepatitis B occurs in about 0.1% to 0.5% of patients acutely infected with HBV and there is lysis of infected hepatocytes by the immune system (Wright, 2008). It is marked by sudden appearance of fever, abdominal pain, vomiting and jaundice accompanied by disorientation, confusion and coma (Liang, 2009). It usually develops after immunesuppression (i.e. chemotherapy), and initially it was believed to be caused by heightened immune response such in the case of HBV Infection with HDV or HCV co-infection (Loriot et al., 1995).

1.2.8 Disease progression:

Liver diseases caused by HBV usually progress through a multi-step and long-term process from CHB to LC and/or HCC. Progressive fibrosis may ultimately develop into LC and/or HCC (Hyams, 1995). For CHB patients, 15-40% of them are estimated at high risk to develop to advanced liver diseases, including LC, liver failure or HCC (Fattovich et al., 2008). Several longitudinal studies have reported that untreated CHB patients have a high 5-year cumulative rate of developing LC, ranging from 8% to 20%. Moreover, for untreated patients with compensated LC, the 5-year cumulative rate of hepatic decompensation is about 20%. For those with
decompensated LC, the 5-year survival rate ranges from 14% to 25% (Fattovich, 2003; Hadziyannis and Papatheodoridis, 2006; McMahon, 2009). HBV-related HCC develops at an annual rate of 2% to 5% in LC patients (Fattovich et al., 2004). Over 0.5-1 million and 5-10% cases of liver transplantation patients annual death resulted from HBV-related LC decompensation and HCC. In addition, the risk of developing HCC cannot be ruled out in any phases of the natural course of CHB infection (Ganem and Prince, 2004; Hoofnagle et al., 2007; Liaw, 2005). The risk factors associated with disease progression appear to be: host-related (age, gender, immune status and genetic background) or HBV-related (HBV DNA replication that reflected by viral load, HBeAg status, HBV genotype and HBV mutations) or others (co-infection with HCV or HIV). Predicting disease progression remains a key challenge in management of CHB infection, and more work are encouraged to better understand the molecular, immunological, cellular as well as genetic basis of HBV-related disease. Liver cancer, which has a high mortality, is the fifth most common cancer in male and the seventh most common in female worldwide. HCC, the most common form of liver cancer, is one of the most severe sequelae of chronic HBV infection. Every year, HCC is diagnosed in more than 500,000 people worldwide. Most cases of HCC (approximately 80%) occur in Eastern Asia and sub-Saharan Africa, where HBV infection is endemic, with a high incidence rate (more than 20 per 100,000 individuals) (El-Serag, 2011).
HBV infection as the major etiology of HCC in humans. It is recently estimated that over 50% of HCC worldwide were attributed to HBV infection (El-Serag, 2012). Compared to populations without HBV infection, chronic HBV carriers have a 10- to 25-fold higher lifetime risk of developing HCC (Sherman, 2009). It is estimated that HCC incidence rate in patients with chronic HBV infection in East Asia was 0.002 person-years in inactive carriers, 0.6 person-years in those patients with chronic HBV without LC, and 3.7 person-years in those with compensated LC (Fattovich et al., 2008). The risk of developing HCC remains high even in patients with occult HBV infection (Pollicino et al., 2004) and after HBsAg seroclearance. Antiviral therapy that controls HBV replication can reduce but not eliminate the risk of development of HCC in CHB patients, and its effect may only be obvious after several decades(Ahn et al., 2005; Yuen et al., 2008).

1.2.9 Genetic variability of HBV:

The viral polymerase enzyme of HBV lacks the proofreading mechanism and uses an RNA intermediate during replication that lead to genetic variability due to random errors as a result of mutations occurring within the entire genome (Datta, 2008). The mutations in the preS/S, preC/C, P and X genes arise during acute or fulminant and chronic HBV infections (Francois, 2001; Lin et al., 2005).

1.2.9.1 Precore mutations:
It has been well known that some chronic HBV carriers with active liver disease are HBeAg negative and that HBeAg is not necessary for viral replication. In 1989 it was shown that a TAG stop codon (G1896A) in the precore region could explain the absence of HBeAg in patients with high viremia levels. Later it was found that this mutation was frequent also in HBeAg negative patients with low HBV DNA levels, and that other mutations in the precore region, e.g. in the start codon or codon 2, also may abolish the production of HBeAg (Lindh et al., 1996). Further, the configuration of the nucleotides 1858 of HBV precore region was found to determine the development of the stop codon mutation at nt 1896 due to the requirements for stability of the stem-loop structure of the encapsidation signal (Li et al., 1993; Lindh et al., 1995; Lok et al., 1994). Thus, the precore stop mutation (G1896A) develops only in strains having T at position 1858, explaining that the G1896A mutation was rare in genotype A, which always carries C-1858. This also explained the lack of stop codon mutations in part of the genotype C strains from East Asia, which were found to carry T-1858, either in combination C-1856 or T-1856. The latter variability has putative clinical importance because having TCC instead of CCC at position 1856 to 1858 has been associated with higher ALT levels and higher rate of liver cirrhosis (Chan et al., 2006). Because precore mutations may abolish the production of HBeAg they are often considered as the cause of loss of HBeAg during the course of infection. In a longitudinal study,
Moriyama *et al.* (1994) however observed that loss of detectable HBeAg preceded the appearance of precore mutations. From this one may conclude that loss of HBeAg primarily is due to immune mediated reduction of HBV DNA levels, probably in combination with anti-HBe production, and that the emergence of precore mutants is a linked but parallel phenomenon resulting from immune selection. Still, a HBeAg negative status is explained by the precore sequence for the proportion of patients who retain high HBV DNA levels (>1 million copies/mL) in the HBeAg negative phase. (*Moriyama et al.* 1994).

**1.2.9.2 Surface mutations:**

The genomic surface region is divided into the S, Pre S1 and PreS2 regions with S gene coding for HBsAg made up of 226 amino acids. The S domain or HBsAg is the most important protein in virions together with spherical and filamentous particles (*Francois et al.*, 2001; *Yokosuka, Arai*, 2006). The disulphides bonds are essential in stabilising its three dimensional structure. HBsAg is important and contains major neutralising epitopes used in commercial hepatitis B vaccines. Forming HBsAg is the major hydrophilic region (MHR) with the “a” determinant important for antigenicity. The MHR is divided into five sections, HBs1, HBs2, HBs4 and HBs5 (*Francois et al.*, 2001). The middle part of HBsAg has 55 amino acids and depending on the genotype has additional 119 or 108 amino acids (*Yokosuka, Arai*, 2006; *Bruss.*, 2007). Mutations in the S gene are important.
since they affect the immunogenecity of HBsAg especially the ‘a’ determinant which might lead to failure of antibodies to neutralize HBV. HBsAg mutations include G145R, D144A, P142S, Q129H, I/T126N/A, M133L associated with immune escape and viral persistence (Huang et al., 2006). The G145R found in the HBs4 region is a stable mutation has been reported in several individuals who were infected while having been vaccinated or received anti-HBs immunoglobulin (Karthigesu et al: 1994).

1.2.9.3 X gene mutants:

HBV X is the smallest of four kinds of HBV functional genes, it expresses a 154-amino-acid multifunctional protein (HBx). It is called X protein due to its uncertainty of its function during infection. (Block et al., 2007). The primary structure contains more than 60% of hydrophobic residues and its functional domain has been divided into two domains, namely the N and C terminals (Feitelson et al. 2005; Murakami., 1999). The N terminal domain is responsible for transforming activity, anchorage-independent proliferation activity and can overcome oncogene induced senescence (OIS). It has been shown that the N terminal domain and its variability are involved in contributing to differential pathogenic potentials of HBV genotypes/subtypes. The C terminal domain exercises a proapoptotic activity to counter balance the proliferative and transforming activities of N terminal domain (Datta, 2008). The gene also seems to
be associated with modulation of a wide range of cellular functions, leading to HCC (Karthigesu *et al.*, 1994). Moreover, of the four open reading frames, the X gene remains enigmatic. There are conflicting suggestions about the functional activity of HBx. HBx induced HCC in certain transgenic mice in vivo (Kim *et al.*, 1991; Yu *et al.*, 1999; Gottlob., *et al.*, 1998; Schaefer *et al.*, 1998). Nevertheless, there are other transgenic lineages in which HBx does not lead to HCC development (Billet *et al.*, 1995; Perfumo *et al.*, 1992).

1.2.10 HBV Diagnosis and staging:

The diagnosis of HBV infection is based on a collection of clinical, biochemical, histological, and serologic findings.

1.2.10.1 HBV serological markers:

Previous HBV infection is characterized by the presence of antibodies (anti-HBs and anti-HBc). Immunity to HBV infection after vaccination is characterized by the presence of only anti-HBs. CHB is defined as the persistence of HBsAg for more than 6 months. Recently, quantitative HBsAg level determination has been proposed to differentiate inactive HBsAg carriers from persons with active disease (Brunetto *et al.*, 2010).

1.2.10.2 HBeAg and anti-HBe Ab:

It also needs to be established whether the person is in the HBeAg-positive or HBeAg-negative phase of infection, though both require lifelong monitoring, as
the condition may change over time. In persons with CHB, a positive HBeAg result usually indicates the presence of active HBV replication and high infectivity. Spontaneous improvement may occur following HBeAg-positive seroconversion (anti-HBe), with a decline in HBV replication, and normalization of ALT levels. This confers a good prognosis and does not require treatment. HBeAg can also be used to monitor treatment response, as HBeAg (anti-HBe) seroconversion in HBeAg-positive persons with a sustained undetectable HBV DNA viral load may be considered a potential stopping point of treatment. However, this is infrequent even with potent NA therapy. Some HBeAg-negative persons have active HBV replication but are positive for anti-HBe and do not produce HBeAg due to the presence of HBV variants or pre-core mutants (Valsakamis, 2007; Hoofman, Thio, 2007).

1.2.10.3 Virological evaluation of HBV infection:
Serum HBV DNA concentrations quantified by real-time polymerase chain reaction (PCR) correlate with disease progression (McMahon, 2009; Lok, 2009; Chen, 2007) and are used to differentiate active HBeAg-negative disease from inactive chronic infection, and for decisions to treat and subsequent monitoring. Serial measures over a few months or longer are preferable, but there remains a lack of consensus regarding the level below which HBV DNA concentrations are indicative of “inactive” disease, or the threshold above which treatment should be
HBV DNA concentrations are also used for optimal monitoring of response to antiviral therapy, and a rise may indicate the emergence of resistant variants. WHO standards are now available for expression of HBV DNA concentrations. Serum HBV DNA levels should be expressed in IU/mL to ensure comparability; values given as copies/mL can be converted to IU/mL by dividing by a factor of 5 to approximate the conversion used in the most commonly used assays (i.e. 10 000 copies/mL = 2000 IU/mL; 100 000 copies/mL = 20 000 IU/mL; 1 million copies/mL = 200 000 IU/mL). The same assay should be used in the same patient to evaluate the efficacy of antiviral therapy. Access to HBV DNA testing remains very poor in resource-limited settings (Saldanha et al., 2001; Shyamala et al., 2004).

1.2.10.4 Other lab tests:

There are other supplementary tests that can be performed, and these include biochemical testing of the functioning of the liver and histological evidence of liver damage. Liver function tests include bilirubin, AST, ALT, alkaline phosphatase, gamma-glutamyl transpeptidase, albumin and globulin. However, ALT and AST are the two most important indicators of hepatocellular damage in viral hepatitis, and may fluctuate or may remain consistently elevated in chronic active HBV. Other liver function tests may include bilirubin, alkaline phosphatase
and gammaglutamyl transpeptidase albumin. Globulin plays an important role in assessing the synthetic functioning of the liver (Hollinger, 1996).

1.2.11 HBV therapy:

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

1- Status of HBV markers (HBsAg, HBeAg, anti-HBc, anti-HBc-IgM).

2- HBV viral load.

3- Liver function enzymes (ALT; Alanine Transaminase, AST; Aspartate transaminase)

4- Immune status of the infected patient.

5- Presence of liver cirrhosis.

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

1.2.11.1 Treatment with alpha interferon (IFN-α):

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

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

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

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

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

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

1.2.11.3 Treatment with nucleotide analogues:

Adefovir, which also emerged from the HIV research, was an alternative to lamivudine and was recommended for lamivudine-resistant HBV variants. Adefovir became an approved treatment for HBV in the United States in September 2002 and in the European Union in March 2003. Adefovir is an acyclic phosphonate, which acts as a nucleotide analogue of adenosine (De Clercq et al., 2005). However, it turned out to be a sub-optimal treatment option, as it does not evolve the desired effect in all patients, beside the fact that it was shown to be nephrotoxic, even in small amounts. Tenofovir disoproxil (Viread®) is the last approved drug (2008) against HBV. It provides a slightly modified form of adefovir with an additional methyl group, making it less nephrotoxic than adefovir (Reynaud et al., 2009).

1.2.12 Previous Studies:

HBV-X protein is associated with the pathogenesis of HBV related diseases, specially in hepatocellular carcinomas. Genetic variability of the X gene includes genotypic specific variations and mutations emerging during chronic infection. Its coding sequence overlaps important regions for virus replication, including the
basal core promoter. Differences in the X gene may have implications in biological functions of the protein and thus, affect the evolution of the disease. A study done in Buenos Aires, Argentina by Barbini et al., (2012) aimed to describe the diversity of HBV-X gene in chronic hepatitis patients infected with different genotypes, according to liver disease. They found that mutations at any of 127, 130 and/or 131 amino acid positions and HBeAg(−) status were associated with mild liver disease. Another study was done in Seoul, Korea by Jong-Hang Lee et al., (2011) about the impact of hepatitis B virus (HBV) X gene mutations on hepatocellular carcinoma development in chronic HBV infection. Their studied population were chronic hepatitis B (CHB) patients, liver cirrhosis (LC) patients, and HCC patients. The results of their study showed that HBx131, HBx130, HBx5, HBx94, and HBx38 amino acid mutations were common in HCC patients and various mutations, e.g. HBx130HBx131 (double) mutations and HBx5HBx130HBx131 (triple) mutations were significantly high in HCC patients. Also, double and triple mutations increased the risk for HCC by 3.75-fold (95% confidence interval) and 5.34-fold (95% CI), respectively, when HCC patients were compared to CHB patients.

In addition, a study done by Fatimawali et al., (2014) in Indonesia for the identification X gene mutations, genotype and subgenotype of hepatitis B virus. Concluded that the genotype C was the most prevalent genotype followed by genotype B and found the mutation in x protein is related significantly to the
clinical severity of the liver and hepatocellular carcinoma (HCC), ie V5L in subgenotype C2, and I127T and H94Y in subgenotype C5.

The association between hepatitis B virus X gene mutations and clinical status in patients with chronic hepatitis B infection was studied by Cho et al., in Iksan, Korea. They found that each of the mutations G1386M, C1485T, C1653T, T1753V, A1762T, and G1764A was significantly associated with the patient’s clinical status. The T1753V and A1762T/G1764A mutations were found to be significantly more frequent in Hepatitis B e antigen (HBeAg)-negative than in HBeAg-positive patients \((P<0.001)\). Specific X gene mutations (G1386M, C1653T, and A1762T/ G1764A) were significantly more prevalent in patients with liver cirrhosis and HCC than in chronic hepatitis patients and C1485T mutations were significantly more prevalent in HCC patients than in chronic hepatitis patients \((p<0.001)\). Only the prevalence of the T1753V mutation increased as the HBV infection progressed from liver cirrhosis to HCC \((P=0.023)\) (Cho et al., 2011).

In Iran another study was done to investigate the frequency of mutations at nucleotides 1762, 1764 and 1766 of HBV X gene in patients with chronic hepatitis B and hepatitis B-related cirrhosis. It concluded that A1762T / G1764A mutations in the overlapping region of the X gene C-terminal with BCP, leads to disease progression from the chronic stage to cirrhosis, by changing the amino acid sequence of the X protein (Salarneia et al., 2016). In south Korea Hong Kim et al.,
(2016) studied X region mutations of hepatitis B virus related to clinical severity. They found that the A1762T/G1764A BCP mutation is one of the most frequently encountered HBx mutations and plays a significant role in liver disease progression in chronic patients with HBV infections. They found these mutations to contribute to disease progression by inducing mutations of other HBx mutations related to clinical severity, such as G1386A/C (V5M/L), C1653T (H94Y), T1753V (I127V) and HBx C terminal deletion or insertion. Moreover, T1753V (I127L,T,N,S) and C1653T (H94Y) mutations in the EnhII/BCP region and A1383C, G1386A/C (V5M/L) and C1485T (P38S) in the negative regulation domain were significantly related to severe types of liver diseases, including HCC. Furthermore, deletions or insertions affecting the C-terminal region of HBx also contribute to the clinical outcome and severity in chronic patients (Hong Kim et al., 2016). Al-Qahtani et al., in (2017) in their study in Saudi Arabian among inactive HBV carriers and patients with active carriers, cirrhosis and HCC groups, found that male sex, age > 50 years, and high serum alanine aminotransferase level were associated with risk of progressive liver disease and I127T, V131I, and F132Y/I/R mutations showed a significant increasing trend associated with the disease progression to HCC. H94Y and K130M mutations were also significantly associated with severe liver disease. One double mutation (K130M+V131I) and two triple mutations (I127T+K130M+V131L and K130M+V131I+F132Y) were observed, with
significant rising prevalence through progressive clinical phases of liver disease to HCC.

In south Africa, Lesibana aimed to characterise HBV X gene and the overlapping basal core promoter region at a molecular level in order to determine their genetic variability at nucleotide and amino acid level. The study included 20 HBV positive samples with different serological profiles of HBV markers (HBsAg, anti-HBs, anti-HBc and HBeAg) to establish HBV infection. The study found that HBx substitutions which circulate in different genotypes may affect the different pathogenic potential due to complex interactions of the virus with the host. Also genotype D isolates displayed most mutations/substitutions in the C terminal of HBx which is responsible for transactivation and patients with this genotype may be at high risk of HCC.Furthermore triple mutations of T1753M/A1762T/G1764A within basal core promoter region were detected mostly in HBeAg negative samples. However further analysis of HBV X gene variability is needed(Lesibana,2010).

Mukhlidet al.,(2013) in his study which was done among Sudanese patients with different liver diseases found that the prevalent genotypes wereD(59%), E(30%), putative D/E recombinants(2.5%) and A(8.5%). They concluded that patients infected with genotype E had higher frequency of hepatitis B e antigen-positivity and higher viral loads compared to patients infected with genotype D. Basic core
promoter/precore region mutations, including the G1896A in 37% of HBeAg-negative individuals, could account for hepatitis B e antigen-negativity. Pre-S deletion mutants were found in genotypes D and E.

1.3 Rationale:

Hepatitis B virus (HBV) is a serious problem worldwide causing various liver diseases such as acute, chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) which can develop as a complication of advanced cirrhosis. The way in which cancer develops is not fully understood. It is thought, however, the HBV DNA somehow becomes incorporated into liver cells DNA of the patients. The mutations on the HBV genes exactly X gene may induce amino acids changes in this gene and also can affect other genes and modify HBV expression (Buckwold et al., 1996; Baumert et al., 1998; Scaglioni et al., 1997). In addition, differences in the X gene may have implications in the biological functions of the protein and thus, affect the evolution of the disease (Kreutz et al., 2002; Datta et al., 2008; Kidd-Ljunggren et al., 1997; Tanaka, Mizokami, 2007). The genetic variability of HBx also can leads to genotypic-specific variants and mutants that emerge during chronic infection (Lin et al., 2005). Studies focusing only on X gene and its variants are scarce in Sudan. Consequently, X gene variability may reveal interesting and novel mutations, sequence motifs or residues that are important for chronic liver diseases or HCC carcinogenesis. Also the differences between HBV genotypes are
the mainstay to severity, complications and treatment of HB. Knowledge of the
genotypes prevailing in HB patients, may be is important in treatment
management, as well as disease prognosis because genotypes may play a role in
both of these aspects (Khaled et al., 2010; Dokanehiifard and Bidmeshkipour,
2009).

1.4 Objectives of the study

1.4.1 General Objective:

To detect and characterize HBV-X gene in Sudanese patients with chronic hepatitis B.

1.4.2 Specific objectives:

a) To detect HBV X gene in HBV infected Sudanese patients.

b) To sequence HBV X gene PCR positive products.

c) To analyze and compare generated sequence data set with GenBank HBV X
gene sequences.

d) To determine the common gene mutations in relation with the outcomes of liver
diseases.

e) To determine the prevalent genotypes of HBV strains among patients with
chronic liver diseases

f) To detect the prevalence of HBeAg among the studied populations.
Chapter (2)
Materials & Methods
Materials and Methods

2.1 Study design:

This is a cross-sectional, hospital-based study.

2.2 Study area:

The study was conducted in gastroenterology unit at Ibn Sina hospital, Khartoum Isotope center and the Military Hospital at Khartoum state, Sudan.

2.3 Study Duration:

The study was done during the period from February 2013 to October 2017.

2.4 Study population:

The Study included HBsAg positive patients who attended gastroenterology unit at Ibn Sina hospital, Khartoum Isotope center and the Military Hospital between February 2013 to October 2017. The studied populations were classified as asymptomatic carriers (ASCs) if they had normal ALT or chronic hepatitis (CH) patients if they had abnormal ALT or if they use anti HBV regimen therapy, liver Cirrhosis (LC) and hepatocellular carcinoma (HCC) patients. Clinical and ultrasonographic evidence were used to diagnose cirrhotic (CR). HCC patients were diagnosed using histopathological techniques and alpha fetoprotein level.
Alanine amino transferase (ALT) levels were determined using spectrophotometer (Biosystem S.A., Barcelona.Spain).

2.4. 1Inclusion Criteria:

HBsAg positive patients including asymptomatic carriers, chronic hepatitis, liver cirrhosis and hepatocellular carcinoma, who attended the study areas during the study period. The participants were classified according to their clinical recorded data on patient’s files.

2.4. 2 Exclusion Criteria:

Patients suffering from HIV or co-infected with the other hepatitis viruses’ infections were excluded from this study.

2.4.3 Sample size:

A total of 185 patients were included in this study. The sample size was calculated using the following formula:

\[ n = \frac{z^2 pq}{d^2} \]

Where:

n is the number of sample size.

z is the value of the standard normal variable (z= 1.96).
P is the incidence of HBV Infection (p = 0.14) and (q = 0.86).

q= 1-p

d is a marginal error (d =0.05)

2.4.4 Data collection:

The participants collected data included age, gender, place of residence, date of diagnosis time of disease discovery and treatments.

2.4.5 Specimens collection:

Under aseptic condition (wearing gloves, tourniquet applying and 70% alcohol for cleaning venipuncture site) 5 ml of blood samples were collected from participants and drawn into plain containers, allowed to clot, then centrifuged and the serum samples were separated into cryogenic tubes and freezed at -20°C until analysed.

2.5 Laboratory methods:

2.5.1 Serological assays for HBV markers:

Immunochromatography test was used to detect HBsAg where Enzyme Linked Immunosorbent Assay kits was used to detect HBeAg and anti HBc Ab in serum specimens.
2.5.1.1 HBsAg:

The HBsAg was tested in serum specimens using Immunochromatography test (InTec. co.China) with advanced quality (sensitivity 99.4%; specificity 99.8%). The principle of the test is that unique combination of monoclonal and polyclonal antibodies are used to sandwich HBsAg from the specimen. Monoclonal antibody is conjugated with colloidal gold and is impregnated at the sample pad. The polyclonal antibodies are selectively immobilized at the test band area. On dipping Dipstick in the specimen, the test sample flows through the sample pad by capillary action. If the serum contains HBsAg it will form a complex with Anti-HBsAg colloidal gold conjugate and allows this to be trapped by the test line. The unbound colloidal gold particles continue to move along the strip by capillary action until they come in contact with the control line and are trapped, giving the red line demonstrating the validity of the test.

2.5.1.2 Anti-HBcAb:

ELISA test (DIALAB, Austria) was used for the detection of HBcAb in serum. The test is based on solid phase, one step incubation competitive principle ELISA. The test was conducted following manufacturer’s instructions and the microplates read at a wavelength of 450 nm, using the ELISA reader. The presence or absence of HBcAb was determined by relating the absorbance of the specimens to the cutoff
value. The cutoff value is the mean of the absorbance value (OD) of the three negative controls multiplied the factor 0.5. Specimens with OD values greater than or equal to the cutoff value established with the negative control were considered positive while those with ODs lower than the cutoff value were recorded as negative.

2.5.1.3 HBeAg:

ELISA kit (AccuDiaTM, CA, USA) was used for the detection of HBeAg in participants’ sera. The test is based on the double antibody sandwich method of detection HBeAg in serum. The test was conducted following manufacturer’s instructions and the microplates read at a wavelength of 450 nm, using the ELISA reader. The presence or absence of HBeAg was determined by relating the absorbance of the sample to the cutoff value. The cutoff value is the mean of the absorbance value (OD) of the negative control multiplied the factor 2.1. Specimens with OD values greater than or equal to the cutoff value were considered positive while those with ODs lower than the cutoff value were recorded as negative.
2.5.2 HBV PCR DNA assay

2.5.2.1 HBV DNA extraction:

Chelex extraction method was used to extract DNA from serum samples. Sterile technique was done to prevent the contamination of DNA extracts. 10% chelex was prepared and preserved in refrigerator at 4 - 8 °C. After removal of chelex from refrigerator, 500µl of every serum sample was added to the microcentrifuge tubes. Then centrifuged for 10 minute at 10.000 rpm. After that the supernatant was removed. Then 50 - 70µl of 10% chelex added and incubated at 95°C for 20 minutes on a heat block to allow viral lysis to occur. During the incubation, the mixture was vortexed every 5 min to ensure all viruses were lysed. After incubation the mixture was centrifuged at 10.000 rpm and supernatant which contain virus DNA was separated and stored until used for PCR reaction.

2.5.2.2 Amplification of HBV DNA

2.5.2.2.1 PCR Optimization:

The study followed the protocol of nested PCR by Han Lee et al., (2011) that used two sets of forward and reverse primers. In each run, positive and negative controls were included for quality control. Primers used for the first round were (5-CATGCGTGGAACCTTTGTG-3; positions 1233 to 1251) and (5-CTTGCCCTKAGTGCTGTATGG-3; positions 2072 to 2053).
(5-T CCTCTGC CGATCC A CTG-3; positions 1254 to 1263) and
(5-C AGAAG CTCC AAATTCTTTA TA-3; positions 1937 to 1916) were used for
the second PCR amplifications. First round PCR was performed with 1 - 3 µL of
extracted DNA which was amplified in a 25µl master mix containing the
following:10X reaction buffer, 25mM MgCl2, 25mM dNTPs and taq polymerase
(iNtRON biotechnology, Gyeonggi, Korea) 0.5µl of 10µM of sense and anti-sense
primers (Macrogen, Geumcheon, Seoul, Korea). PCR was performed as follows:
initial denaturation at 94° C for 5 min. Denaturation of 94° C for 1 min,
annealing temperature of 55° C 1 min, extension at 72° C 2 min for 35 cycles;
and final elongation at 72° C for 10 min. For the second round, 1-2 µL of the
first round PCR product was re-amplified using the same reaction mixture
composition, except that internal primers were used. Second round PCR was
performed as follows: initial denaturation of 94° C 5 min; denaturation of 94° C
30 sec, annealing temperature of 51° C 1 min, extension at 72° C 2 min for 30
cycles; and final elongation at 72° C for 10 min. PCR products were run for 30
min on agarose gelstained with ethidium bromideand evaluated under UV light.
Table(1): sequences of HB X gene primers that include outer and inner primers.

<table>
<thead>
<tr>
<th>Primer Sequence</th>
<th>Position</th>
<th>Outer/Inner</th>
<th>F/R</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-CATGCCTGGAACCTTTGTG-3;</td>
<td>1233 to 1251</td>
<td>Outer</td>
<td>sense</td>
<td>Han Lee et al., 2011</td>
</tr>
<tr>
<td>5-CTTGCTKAGTGCTGTATGG-3</td>
<td>2072 to 2053</td>
<td>Outer</td>
<td>Anti sense</td>
<td>Han Lee et al., 2011</td>
</tr>
<tr>
<td>5-TCCTCTGCCGATCCATACTG-3</td>
<td>1254 to 1263</td>
<td>Inner</td>
<td>Sense</td>
<td>Han Lee et al., 2011</td>
</tr>
<tr>
<td>5-CAGAAGCTCCAATTCTTTA TA-3</td>
<td>1937 to 1916</td>
<td>Inner</td>
<td>Anti sense</td>
<td>Han Lee et al., 2011</td>
</tr>
</tbody>
</table>

2.5.2.3 Detection of the amplified HBV X gene PCR products:

PCR products were run on agarose gel for 30 min, stained with ethidium bromide, and evaluated under UV light. Detection of the amplified PCR products was performed using two microliters (µl) of the second round PCR product on 1.5% agarose gel electrophoresis with ethidium bromide. The gel was run for 30 minutes in the (TE) buffer at 95 Voltage with positive and negative controls. The expected bands (683bp) were detected against 1000bp DNA ladder using ultraviolet (UV) BDA system (Biometra Inc)(figure10).
Figure(10): Agel electrophoresis diagram showing X gene products with 1000bp DNA ladder. The PCR amplification generated a DNA fragment of expected size (683 bp).

2.5.2.4 HB X gene sequencing and sequence analysis:

HBV X gene positive products were sequenced in Canada by Macrogen, Inc. (Canada), and (Korea) using sanger DNA sequencing (Macrogen, Geumcheon, Seoul, Korea). The nucleotide sequences from position 1374 to 1838, located in the region of the X gene, were examined, and the sequences were verified in both directions (Figure11). Nucleotides sequences comparisons and alignments were done and translated into proteins using software BioEdit (figure12). Mutations were identified by comparing X gene sequences with corresponding sequences of HBV genotypes isolates.
2.6 Statistical analysis methods:
Data were expressed as means. All statistical analyses and tests were performed with the SPSS statistical package (SPSS 20.0, Chicago, IL, USA) and p<0.05 was regarded as statistically significant. Significant differences were determined using t-tests.

2.7 Ethical consideration
Ethical approval was obtained from Research Committee, Sudan University of Science and Technology and the National Ethics Committee at Ministry of Health, Khartoum State. Information about the Study was delivered to patient and consent obtained then verbally interview was conducted, however, in case of patients under 18 Y.Old parental consent was obtained. All Participants were given the rights to withdraw at any time.
Figure(11): Chromatogram of the X gene
Chapter (3)

Results
Results

During the period from February 2013 to October 2017, a total of 185 patients were recruited to participate in this study. The participants mean age was 43.5 ±16.02 (range from 17 to 80 years). The majority of the patients (75.7%) were males, (Table.2). Almost one half of the studied population (47.5%) were ASC, followed by CH(21.0%), LC(20.5%) and HCC(10.8%) (Table3). The mean age of the patients with HCC was 53.76±16.91 which is significantly higher than in the other clinical groups (P.value:0.01). All the participants were positive for HBcAb. HBeAg was positive in 34(18.3%) of the participants (Table.4).

Out of the 185 hepatitis B patients, HBV DNA of 19(10.3%) patients was successfully amplified using nested PCR. The low percentage of successful amplifications of gene may be due to the fact that most of the studied population were on treatment that expected to affect the viral load. Of the 19 patients, 16 (84.2%) were males. Out of the 19 positive PCR samples, HBeAg was positive in 4(21%) patients and 15(79%) were HBeAg-negative.

HBx30, HBx88, HBx127, HBx130 and HBx131 were the most frequently detected mutations while other types of mutations were not common. The double mutations K130M and V131I were detected in 9 (47.3%) patients out of 19 patients, eight of them with hepatocellular carcinoma (3) and liver cirrhosis(5) and one patient with chronic hepatitis. Mutation at position aa127(I127T/ N/P/G) was the most frequent
among patients with HCC (66.7%) followed by those with liver cirrhosis (57.1%). Mutation F30L/V more frequent in HCC and LC (100% and 85.7%) respectively. Mutation at position 88 (I88F/S/C/V) was most frequent among asymptomatic carriers patients (83.3%) followed by those with chronic hepatitis (66.7%) (Table.5).
**Table (2):** Distribution of participants according to the gender.

<table>
<thead>
<tr>
<th>Gender</th>
<th>Number</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>140</td>
<td>75.7</td>
</tr>
<tr>
<td>Females</td>
<td>45</td>
<td>24.3</td>
</tr>
<tr>
<td>Total</td>
<td>185</td>
<td>100</td>
</tr>
</tbody>
</table>

**Table (3):** Distribution of the participants according to the liver disease.

<table>
<thead>
<tr>
<th>Liver disease</th>
<th>Number</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatocellular carcinoma</td>
<td>20</td>
<td>10.8</td>
</tr>
<tr>
<td>Liver cirrhosis</td>
<td>38</td>
<td>20.5</td>
</tr>
<tr>
<td>Chronic hepatitis</td>
<td>39</td>
<td>21.0</td>
</tr>
<tr>
<td>Asymptomatic carriers</td>
<td>88</td>
<td>47.5</td>
</tr>
<tr>
<td>Total</td>
<td>185</td>
<td>100</td>
</tr>
</tbody>
</table>
**Table (4):** Laboratory findings of the participants.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HCC(20)</th>
<th>LC(38)</th>
<th>CH(39)</th>
<th>ASC(88)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ALT e±SD</td>
<td>46.25± 27.10</td>
<td>52.9 ± 38.73</td>
<td>68.18 ± 56.90</td>
<td>22.10 ± 7.92</td>
</tr>
<tr>
<td>HBeAg+</td>
<td>2(10%)</td>
<td>7(18.4%)</td>
<td>11(28.2%)</td>
<td>14(15.9%)</td>
</tr>
<tr>
<td>HBcAb</td>
<td>20(100%)</td>
<td>38(100%)</td>
<td>39(100%)</td>
<td>88(100%)</td>
</tr>
</tbody>
</table>

**Table (5):** Prevalence of different types of mutations in relation to the hepatitis B clinical status

<table>
<thead>
<tr>
<th>Clinical Status</th>
<th>NO of total patients</th>
<th>No of Patients with+ve products</th>
<th>Types of Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>HBx30 Frequency(%)</td>
</tr>
<tr>
<td>HCC</td>
<td>20</td>
<td>3</td>
<td>3(100%)</td>
</tr>
<tr>
<td>LC</td>
<td>38</td>
<td>7</td>
<td>6(85.7%)</td>
</tr>
<tr>
<td>CHB</td>
<td>39</td>
<td>3</td>
<td>1(33.3%)</td>
</tr>
<tr>
<td>ASC</td>
<td>88</td>
<td>6</td>
<td>2(33.3%)</td>
</tr>
<tr>
<td>Total</td>
<td>185</td>
<td>19(10.3%)</td>
<td>12(63.2%)</td>
</tr>
</tbody>
</table>
For the purpose of mutation analysis, patients were classified into two groups, Hepatocellular carcinoma plus Liver cirrhosis as group one, and Asymptomatic carriers plus Chronic hepatitis classified as group two. Direct sequencing of the 19 samples revealed different mutations in the full length of HBx gene. The variability of the amino acid level was determined and analyzed. HBx30, HBx130 and HBx131 mutations were common among group one (HCC+LC) than group 2 (CH+ASC).

HBx 30 mutation was detected in 9 (90%) patients of group one which is significantly higher than group 2 (P.value:0.04). Double mutations HBx130/131 were significantly higher in group one (P.value:0.01) in which it was detected in 8 (80%) patients. HBx127 was found in 6 (60%) patients of group one and 3 (33.3%) patients of group 2. HBx 88 mutation was detected in 5 (50%) patients of group one. (Table 6). Several other mutations were detected in this study which include HBx 31, 94, 101, 69 and 73, but in low frequencies.
Table 6 The frequency and percentage of common mutations among HBV positive patients according to the clinical group.

<table>
<thead>
<tr>
<th>Clinical group</th>
<th>Patients No</th>
<th>Types of Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HBx30 Frequency(%)</td>
</tr>
<tr>
<td>HCC&amp; LC</td>
<td>10</td>
<td>9(90%)</td>
</tr>
<tr>
<td>ASC&amp; CH</td>
<td>9</td>
<td>3(33.3%)</td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
<td>12(63.1%)</td>
</tr>
</tbody>
</table>

Key: HCC; hepatocellular carcinoma, LC: liver cirrhosis, ASC; a symptomatic carriers, CH: chronic hepatitis.
Figure (12): Alignment of HB X gene amino acid compared with corresponding reference sequence.
3.4 Distribution of mutations in the HBV X gene in relation to HBeAg detection:

The following table (Table 7) shows the most prevalent mutations in relation to HBeAg serostatus.

**Table 7: The frequencies of various mutations according to HBeAg serostatus.**

<table>
<thead>
<tr>
<th>HBeAg status</th>
<th>Total Number</th>
<th>HBx127</th>
<th>HBx130</th>
<th>HBx130</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>frequ</td>
<td>%</td>
<td>frequ</td>
</tr>
<tr>
<td>HBeAg+</td>
<td>4</td>
<td>1</td>
<td>25%</td>
<td>2</td>
</tr>
<tr>
<td>HBeAg-</td>
<td>15</td>
<td>8</td>
<td>53.3%</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
<td>9</td>
<td>47.4%</td>
<td>10</td>
</tr>
</tbody>
</table>

3.5 Genotypes and phylogenetic analysis:

HBV genotyping was done by using the NCBI genotyping BLAST tool. The HB x gene sequences was compared with reference sequence. The most frequent genotypes were genotype D 9(47.4%), Genotype E 8(42.1%) and genotype A 2(10.5%) (Table 8). Some mutations were genotype specific, these were substitutions at position aa46 and 102 which were present only in genotype D. Mutation at position aa30 was found in all patients with genotype E and the 2 (100%) patients with genotype A, while in genotype D it was found in just 2(22.2%) patients out of 9. Double mutations at position aa130/131 were found in 3(33.3%)
out of the 9 patients with genotype D, 4(50%) patients out of 8 in genotype E and in the 2(100%) patients with genotype A. Mutation at position aa127 was found in 5(55.6%) patients out of the 9 with genotype D, 3(37.5%) patients out of 8 with genotype E and one(50%) patients out of 2 with genotype A. Also mutation at position aa 88 was found in all patients with genotype D, 2 (25%) patients out of 8 with genotype E and one(50%) patients out of 2 with genotype A which mean that it was more prevalent in genotype D compared to genotype E and A(Table9).

Out of the the 3 patients with HCC, genotype A was the commonest 2 (66.7%), followed by E 1(33.3%). Out of the 7 patients with LC, genotype E was detected in 4(57%) patients, while genotype D was detected in 3 (43%) patients. In the group of patients with CHB, genotype D was found in 6(67%) patients and genotype E in 3(33%) patients(Table10).

Patients with genotype E had ALT elevation exceeding 1.5 times than those with genotype D. Of the 8 patients with genotype E, 4 (50%) had cirrhosis, 1 (12.5%) had HCC and 3(37.5%) CHB. Of the 9 patients with genotype D, 3 (33.3%) had cirrhosis and 6 (66.6%) had CHB.
Table 8: The frequency of HBV genotypes among patients with positive HBV DNA

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Frequency</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2</td>
<td>10.5</td>
</tr>
<tr>
<td>D</td>
<td>9</td>
<td>47.4</td>
</tr>
<tr>
<td>E</td>
<td>8</td>
<td>42.1</td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Table 9: Prevalence of mutations in relation to the different HBV genotypes:

<table>
<thead>
<tr>
<th>HBV genotype</th>
<th>Total</th>
<th>HBx30</th>
<th>HBx130/131</th>
<th>HBx127</th>
<th>HBx88</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Freq</td>
<td>%</td>
<td>Freq</td>
<td>%</td>
<td>Freq</td>
</tr>
<tr>
<td>D</td>
<td>9</td>
<td>2</td>
<td>3</td>
<td>33.3%</td>
<td>5</td>
</tr>
<tr>
<td>E</td>
<td>8</td>
<td>8</td>
<td>4</td>
<td>50%</td>
<td>3</td>
</tr>
<tr>
<td>A</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>100%</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
<td>12</td>
<td>9</td>
<td>(63.2%)</td>
<td>9</td>
</tr>
</tbody>
</table>

Table 10: Distribution of HBV genotypes in relation with Liver diseases:

<table>
<thead>
<tr>
<th>HBV genotype</th>
<th>Total</th>
<th>HCC</th>
<th>LC</th>
<th>CH</th>
<th>ASC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Freq</td>
<td>%</td>
<td>Freq</td>
<td>%</td>
<td>Freq</td>
</tr>
<tr>
<td>D</td>
<td>9</td>
<td>0</td>
<td>3</td>
<td>42.9%</td>
<td>2</td>
</tr>
<tr>
<td>E</td>
<td>8</td>
<td>1</td>
<td>4</td>
<td>57.1%</td>
<td>1</td>
</tr>
<tr>
<td>A</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
<td>3</td>
<td>7</td>
<td>36.8%</td>
<td>3</td>
</tr>
</tbody>
</table>
Figure (13): Prevalence of genotypes among LC group
Figure (14): Prevalence of genotypes among HCC group
Figure (15): Prevalence of genotypes among CHB group
The phylogenetic trees were constructed with sequences of all HBV samples in the MEGA 7 software. Phylogenetic analysis was performed using Maximum Likelihood and Neighbour Joining methods. Robustness of the phylogenetic analysis was evaluated by a 1000 bootstrap resampling. Genetic distances were calculated using the Tamura Nei model of nucleotide substitution.

**Figure (16)** A rooted phylogenetic tree of 19 complete X sequences of HBV obtained from Sudanese liver disease patients using neighbour-joining. Bootstrap statistical analysis was performed using 1000 datasets.
Figure(17): The sequences are labeled by their accession numbers and country.
Table 11: Different mutations with and without biological functions in relation to genotype, disease and HBeAg serostatus.

<table>
<thead>
<tr>
<th>patient</th>
<th>Age</th>
<th>Liver Dis</th>
<th>ALT</th>
<th>HBeAg</th>
<th>Genotype</th>
<th>Common Mutations</th>
<th>Other additional mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>49</td>
<td>Cirrhosis</td>
<td>137</td>
<td>-</td>
<td>E</td>
<td>F30V, I127T, K130M, V131I</td>
<td>G22S, T36D, T47A,</td>
</tr>
<tr>
<td>02</td>
<td>31</td>
<td>Chronic</td>
<td>144</td>
<td>-</td>
<td>E</td>
<td>F30V</td>
<td>G22S, T36D, L123S, T47A</td>
</tr>
<tr>
<td>03</td>
<td>67</td>
<td>Chronic</td>
<td>49</td>
<td>-</td>
<td>D</td>
<td>I127N, K130M, V131I, I88F</td>
<td>P46S</td>
</tr>
<tr>
<td>04</td>
<td>63</td>
<td>Cirrhosis</td>
<td>44</td>
<td>-</td>
<td>E</td>
<td>F30L, K130M, V131I, I88V</td>
<td>G22S, T36D, L123S, T47A</td>
</tr>
<tr>
<td>05</td>
<td>25</td>
<td>HCC</td>
<td>24</td>
<td>-</td>
<td>A</td>
<td>F30V, I127T, K130M, V131I</td>
<td>S31A, T36A, T47S</td>
</tr>
<tr>
<td>06</td>
<td>48</td>
<td>Cirrhosis</td>
<td>39</td>
<td>-</td>
<td>D</td>
<td>I88FL, 127T, K130M, V131I</td>
<td>P46S</td>
</tr>
<tr>
<td>07</td>
<td>43</td>
<td>Cirrhosis</td>
<td>28</td>
<td>+</td>
<td>D</td>
<td>F30V, I127T, K130M, V131I, I88F</td>
<td>P46S</td>
</tr>
<tr>
<td>08</td>
<td>41</td>
<td>HCC</td>
<td>74</td>
<td>-</td>
<td>E</td>
<td>F30V, I127T, K130M, V131I</td>
<td>G22S, T36D, L123S, T47A</td>
</tr>
<tr>
<td>09</td>
<td>48</td>
<td>HCC</td>
<td>36</td>
<td>-</td>
<td>A</td>
<td>F30L, K130M, V131I, I88S</td>
<td>S31A, T47S</td>
</tr>
<tr>
<td>10</td>
<td>63</td>
<td>ASC</td>
<td>38</td>
<td>-</td>
<td>D</td>
<td>I88F, V131L</td>
<td>S31L, T36A, T47P, P46S</td>
</tr>
<tr>
<td>17</td>
<td>21</td>
<td>ASC</td>
<td>33</td>
<td>-</td>
<td>D</td>
<td>I88F</td>
<td>P46S</td>
</tr>
<tr>
<td>18</td>
<td>43</td>
<td>ASC</td>
<td>29</td>
<td>-</td>
<td>D</td>
<td>K130M, V131X, I88C</td>
<td>S31L, P46S</td>
</tr>
<tr>
<td>19</td>
<td>56</td>
<td>Cirrhosis</td>
<td>35</td>
<td>+</td>
<td>E</td>
<td>F30V</td>
<td>-</td>
</tr>
</tbody>
</table>
3.6 HBeAg seroprevalence:

The prevalence of HBeAg was determined for the 127 HBsAg+ chronic patients using a double antibody sandwich ELISA method. 25 individuals of them were found to be HBeAg-positive giving an overall prevalence of 19.7%. Of those 25 individuals, 8 (23.5%) were females, and 17 (18.2%) were males (Table 12).

**Table (12):** Incidence of HBeAg and chronic hepatitis patients according to the gender

<table>
<thead>
<tr>
<th>HB Marker</th>
<th>No</th>
<th>Male(%)</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBsAg</td>
<td>127</td>
<td>93(73.2%)</td>
<td>34(26.8)</td>
</tr>
<tr>
<td>HBeAg</td>
<td>25(19.7%)</td>
<td>17(18.2%)</td>
<td>8(23.5%)</td>
</tr>
</tbody>
</table>
Chapter (4) Discussion, Conclusion and Recommendations
Discussion

Many studies have shown that genetic variations in HBV genome influence clinical manifestations and progression of the HBV infection (Lazarevic et al., 2014; Zhang et al., 2016). HBx is known to be a multifunctional protein that not only activates transcriptional transactivation but also mediates cell growth via proliferation and apoptosis (Bouchardand Schneider, 2004). Moreover, the HBx protein is potentially oncogenic via multistep carcinogenesis, it modifies apoptosis, inhibits nucleotide excision, repairs damaged cellular DNA, and modulates transcriptional activation of cellular growth regulating genes (Birrer et al., 2004). The present study identified specific HBx gene mutations and investigated the clinical correlation of these mutations with the progression of HBV infection.

Five positional mutations within the HBx protein including HBx30, HBx88, HBx127, HBx130 and HBx131 were identified in this study, three of them (HBx30, HBx130 and HBx131) had a significant correlation with advanced liver diseases including HCC and LC. In this study, the double mutations of HBx130+HBx131 were significantly more frequent in cirrhotic and HCC patients (80%) compared to patients with chronic hepatitis (10%) (P.value=0.01). Based on that, it can be concluded that the occurrence of this mutation has an important role in liver disease progression to more severe stages. Many studies agree with the results
of our study. Chen et al. in China found that the frequency of these mutations in patients with chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) was 33%, 56% and 85%, respectively (Chen et al., 2005). In addition, a recent study done in Saudi Arabia in 2017 showed significant association of these mutations with severity of liver disease (Ahmed Qahtani et al., 2017). Our results agree with the results of study done in India which revealed an increased prevalence of these mutations in a progressive forms of HBV infection (Malik et al., 2012). The prevalence of K130M+V131I double mutation in studies done among Taiwanese, Chinese, and Indian hepatitis B patients were 85%, 64%, and 45%, respectively (Chen et al., 2005, Wang et al., 2007, Malik et al., 2012). Our result show that the prevalence of these mutations in Sudanese patients with HCC is similar to that of hepatitis B Taiwanese patients.

In our study, HBx127 mutation was detected in 60% of patients with HCC and LC which agrees with the result obtained by Ahmed Ahmed Al-Qahtani et al., (2017) who found this mutation in 50% of Saudi Arabian patients with HCC. This is maybe explained by the population movement between two countries and transfer of the HBV genotypes. HBx127 mutation has been shown to promote transactivation of cellular growth regulating genes (Lin et al., 2005). Shinkai et al., (2007) suggested this mutation is important for HBV for evading immune system leading to persistent viral replication which is important in hepatocarcinogenesis. On the other hand,
many studies were focused on the importance of combinational mutations in the HBx protein with the progression of HBV infection. Our study showed that the prevalence of I127T+K130M+V131I triple mutation in HCC patients (66.7%) and LC patients (42.8%). Ahmed Al-Qahtaniet al., found that the frequency of I127T+K130M+V131I triple mutation in HCC patients was 46.4%(Ahmed Al-Qahtaniet al., 2017). In another study done by Datta et al., found that frequency of this triple mutation in HCC patients with genotype D was 67%(Datta et al., 2014). However in our HCC patients, the type of mutation was commonly associated with genotype E and A while in Datta et al., study the mutation was associated with genotype D. Previous data have established the specific presence of mutation at position 127 along with the double mutation at positions 130 and 131 and strong association between K130M+V131I mutation and the presence of a polar mutation at position 127 (I to T) (Iavarone et al., 2003).

In our study, mutation at position aaF30L/V was detected in 90% the patients with HCC and LC patients. This mutation may be the cause of disease progression.QiWangaet al.,(2011) found this mutation in the tumor tissues from patients with HCC from China. They proved that this mutation had a significant role in the development of HCC. The finding of this mutation in serum samples of Sudanese with advanced liver diseases is the first one.
In the present study, mutation at position 88 of HBx protein was found in 77.8% of patients with chronic HB and 50% of patients with LC and HCC, with no association between liver diseases progression and this mutation. Our result is disagree with the result obtained by Ghosh et al., who found that this mutation is associated with liver cirrhosis in Indian population (Ghosh et al., 2012). Also in a study done in China by Qi Wang et al., it was found that the mutation at amino acid 88 was more frequent in HCC samples (Qi Wang et al., 2011). As suggested by Malik et al., the difference in association of mutations with severe symptoms of HBV infection varies across different ethnic populations (Malik et al., 2012).

In the present study, H94Y substitution was detected in two patients, one with chronic hepatitis and the other with LC, clustered with genotype E. However Malinga., (2010) in his study which was done in South Africa found that this substitution clustered with genotype D, which means that it’s not genotype specific. In a study done in Japan it was found that H94Y mutation was associated with HCC in patients with HBV genotype C2 (Shinkai et al., 2007).

In our study, two patients out of three with HCC had S101P substitution. One patient with LC showed S101L and two patients with Chronic HB showed S101C/F. Our Study found that this substitution occurred in genotypes A, D and E. Kwun and Jang (2004) reported that HBx variants with S101P stabilized p53 more efficiently,
probably by protecting it from the MDM2-mediated degradation. This showed different effects on the cell cycle progression, and eventually on the cell growth rate, implicating its biological significance.

In our study, P46S mutation was detected in 9 patients all of them with genotype D which means its genotype specific.

Knowledge of the genotypes prevailing in our HB patients, with and without liver disease, is important in treatment management, as well as disease prognosis because genotypes play a role in both of these aspects (Kramvis et al., 2005, Kao et al., 2002). In our study, the most prevalent HBV genotypes were, genotype D (47.4%), Genotype E (42.1%) and genotype A (10.5%). This agrees with the result of the last study done in Sudan by Muklid et al., (2013), who found the most prevalent HBV genotypes to be genotype D (48.5%), genotype E (24.2%), genotypes A (7.1%) and (2%) with a putative D/E recombinant. Our results disagree with the result of the study done by Mahgoub et al., 2011 who found genotype E (57.5%) to be the most frequent, followed by genotype D (40.5%) and genotype A (2%) (Mahgoub et al., 2011). This may due the different in studied population who were HCC, LC and Ch in Muklid et al., study and in study done by Mahgoub et al., were blood donors.

The presence of HBeAg in the serum of patients with hepatitis B virus is a reflection of active viral replication in hepatocytes and is considered a surrogate marker for the
presence of the DNA of hepatitis B virus (Yang et al., 2002). Testing for the HBeAg can also identify individuals with a high risk of developing liver cancer (You et al., 2004, Yang et al., 2002). We studied the prevalence of hepatitis B virus e antigen (HBeAg) among individuals diagnosed to be chronic hepatitis B and analyzed according to the gender. Of 127 patients with CHB, the HBeAg was detected in 25 (19.7%) patients. Our results is in agreement with the results obtained by Joseph et al., (2012). They investigate 572 Nigerian participants for HBeAg status, 110 were found to be positive (19.2%), and 462 were negative (80.8%). this may reflect the highly incidence of HBeAg positivity in African countries and may be taken as an indication of active HBV in the Sudanese population.
Conclusion

The mutation in HBx protein is related significantly to the clinical severity of the liver and hepatocellular carcinoma (HCC). The prevalence of aa30, 130 and 131 mutations in the X gene are associated with the clinical status as chronic HBV infection progresses from chronic hepatitis to cirrhosis or HCC and from cirrhosis to HCC. These finding suggests that these mutations may be useful markers for predicting the clinical course of patients with chronic hepatitis B. Genotypes of HBV isolated from blood samples of 19 HBV infecting hepatitis B patients was detected and showed that 9 patients were infected with D genotype HBV, 8 patients with E genotype. The rest of 2 patients were infected with A genotype. Although genotype D was the most prevalent one but genotype E may have the sever clinical outcome through increasing ALT level which is a marker of liver damage.
**Recommendations:**

Further studies on HBx gene with large sample size and advanced extraction methods are required to detect the mutations and their importance as risk factors for HBV liver advanced diseases.

Specialized studies on the biological functions of different mutations to provide comprehensive understanding of liver diseases.
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Appendixes
Appendix-I: The consent Form

Sudan University of Science and Technology

Faculty of Medical Laboratory Science

Characterization of HBV X gene in Sudanese patients.

Consent form for participation in the study

I am a Researcher from the faculty of Medical Laboratory Science, Sudan University of Science and Technology. We are conducting a study on the characterization of hepatitis B virus X gene (HBV X gene). We will collect a blood sample to assess genetic variability of HBV X gene. All data will remain confidential and will not be accessible. You will not get any payment to participate in this study and your participation is completely voluntarily and you could drop out of the study at any time. If you have any questions please contact Abosufyan Salama 0912470317.

I……………………………… read and understood the content of this form
Appendix-II: Questionnaire

Sudan University of Science and Technology

Faculty of Medical Laboratory Science

Study on Characterization of HBV X gene in Sudanese patients.

Questionnaire

Patients data:

Name ……………….  age……  Gender :…………Country :……………….

Place of residence :……………….

Date of diagnosis ………………….

Clinical Diagnosis…………………………………………………………………….