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Evaluation of Plasma Interleukin-10 Levels among Chronic Hepatitis B Patients Attending Total Lab Care Laboratories in Khartoum State.

تقييم مستويات البلازما انترليوكين-10 في مرضى التهاب الكبد الوبائي المزمن ب الذين حضروا إلى مختبرات توتال لاب كير بولاية الخرطوم

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الآيـة

قال تعالى:

{۞اللَّهُ نُــورُ السَّــمَاوَاتِ وَالْأَرْضِ ۚ مَثَــلُ نُــورِهِ كَمِشْــكَاةٍ فِيهَــا مِصْــبَاحٌ ۖ الْمِصْبَاحُ فِـي زُجَاجَـةٍ ۖ الزُّجَاجَـةُ كَأَنَّهَـا كَوْكَـبٌ دُرِّيٌّ يُوقَـدُ مِـن شَـجَرَةٍ مُّبَارَكَـةٍ زَيْتُونَـةٍ لَّا شَـرْقِيَّةٍ وَلَا غَرْبِيَّـةٍ يَكَـادُ زَيْتُهَـا يُضِـيءُ وَلَـوْ لَـمْ تَمْسَسْـهُ نَـارٌ ۚ نُّـورٌ عَلَــىٰ نُــورٍ ۗ يَهْـدِي اللَّهُ لِنُــورِهِ مَــن يَشَــاءُ ۚ وَيَضْــرِبُ اللَّهُ الْأَمْثَــالَ لِلنَّــاسِ ۗ وَاللَّهُ بِكُلِّ شَيْءٍ عَلِيمٌ۞}

صدق الله العظيم

DEDICATION

To my beloved parents who made me what I'm today

To my respectful brothers and sister

To my lovely friends

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Piously my gratitude and prayers to **ALMIGHTY ALLAH** for the mercy that followed me during the long way of this research. I owe so much to my supervisor **Dr. Wafa Ibrahim Elhag** for her immense effort not only to accomplish this work but also to inculcate the researcher's soul on me. Special thank to my colleges **Ustaz: Hosam El-din Elsir, Yosra Ibrahim** and **Nosiba Sultan** for their efforts and boundless support. I am thankful for **Ustaz: Salah Ismail** and **MR. Mohammad Abobakr** for their boundless support and helpful advices. Special thanks For Total Lab Care Laboratories and especially to **Mr. Hatim Elbdairy** for their greatest efforts in the accomplishment of this thesis. I am thankful for all the patients who agreed to participate in this research. Eventually I would like to thank anyone who contributed by any means to this research from commence, during the collection, processing of specimens or the final touches.

ABSTRACT

One of the most important factors playing role in chronic hepatitis B pathogenesis is cytokine release and one of the cytokines with anti-inflammatory characteristic is interleukin-10 (IL-10). The aim of the present study was to estimate IL-10 levels and to evaluate the utility of using it as biomarker for monitoring the progression and treatment of chronic hepatitis B infection.

Sixty patients with chronic hepatitis B disease confirmed by persistence expression of HBsAg for more than 6 months and 30 healthy controls were included in the study during the period from January to May 2017. Serum IL-10 level was investigated by Enzyme Linked Immunosorbent assay (ELISA) technique and HBV viral load was investigated by Real Time PCR. In the control group, thirty healthy individuals with age group (23-58 years) similar to the patient population were included. Controls and patients groups were compared and data were statistically analyzed.

Interleukin-10 levels of the patients was as the following from total of 60 patients 37 who represents (61.6%) of the total patients were in the levels of 1-10 ng/ml, 7 (11.7%) were in the levels of 11-20 ng/ml, 6 (10%) were in the levels of 21-40 ng/ml and 10 (16.7 %) were in the levels of more than 40 ng/ml.

Interleukin-10 levels of the controls was as the following from total of 30 controls 26 who represents (86.6%) of the total controls were in the levels of 1-10 ng/ml, 3 (10%) were in the levels of 11-20 ng/ml, 1 (3.4%) were in the levels of 21-40 ng/ml and zero of the controls were in the levels of more than 40 ng/ml.

According to HBV viral load the 60 patients had been divided into two groups, the first consists of 41 (68%) patients with HBV DNA level of 6–2000 IU/mL, the second consists of 19 (32%) patients with HBV DNA level of > 2000 IU/mL.

Interleukin-10 levels of the first group was compared with the control group and the levels in chronic hepatitis B group were statistically significantly higher (*P*=0.01). Interleukin-10 levels of the second group were compared

with the control group and the levels in chronic hepatitis B group were statistically significantly higher (*P*= 0.01).

When chronic hepatitis B patients were compared, IL-10 levels increased as HBV DNA levels increased and the result was statistically significant (*P*=0.02). IL-10 and HBV viral load were compared and positive correlation was detected (*P*= 0.01).

The present study suggests that decreasing IL-10 levels by using various techniques may have important contributions on chronic hepatitis B infection, disease progression and treatment of the disease. Moreover, IL-10 levels may be an important biomarker for hepatitis B infection monitoring and evaluation of the treatment response.

المستخلص

أحد أهم العوامل التي تلعب دوراً في التهاب الكبد الوبائي الفيروسي "ب" المزمن هي افراز السيتوكينات وأحد هذه السيتوكينات والذي له خاصية مضادة للالتهاب "الانترليوكين- 10" . الهدف من اجراء هذه الدراسة كان قياس مستويات الانترليوكين-10 وتقييم امكانية استخدامه كموسم لمتابعة تطور المرض ومدى فعالية العلاج.

تم تضمين 60 مريضاً يعانون من التهاب الكبد الوبائي المزمن "ب" تم تأكيد إصابتهم عن طريق الوجود الدائم للانتيجين السطحي للفيروس لمدة تزيد على 6 شهور وتم تضمين 30 شخصاً سليماً في الدراسة خلال الفترة من يناير وحتى مايو 2017 وتم قياس مستوى الانترليوكين-10 عن طريق تقنية الانزيم المناعي المرتبط "اليزا" وقياس مستويات الحمض النووي للفيروس عن طريق تقنية التفاعل الكمي المتسلسل حقيقي الوقت " ريل تايم بي سي ار" في المجموعة السليمة كان هناك 30 شخصاً مع متوسط عمر (58-23 سنة) مشابه لمجموعة المرضى وتم مقارنة كلا المجموعتين وتحليل البيانات احصائياً.

كانت مستويات الانترلوكين-10 في المرضى كما يلي من مجموع 60 مريضا 37 الذين يمثلون (61.6٪) من مجموع المرضى كانوا في مستويات 1-10 نانوجرام / مل، و 7 مرضى يمثلون (11.7٪) كانوا في مستويات 11 -20 نانوغرام / مل، و 6 مرضى يمثلون (10٪) كانوا في مستويات 21-40 نانوغرام / مل و 10 مرضى يمثلون (16.7٪) كانت في مستويات أكثر من 40 نانوغرام / مل.

وكانت مستويات الإنترليوكين-10 في المجموعة السليمة على النحو التالي من مجموع 30 عنصر كان 26 الذين يمثلون (86.6٪) من المجموعة السليمة في مستويات 1-10 نانوغرام / مل، و 3 مرضى يمثلون (10٪) كانوا في مستويات 11 -20 نانوغرام / مل، و مريض واحد يمثل (3.4٪) كان في مستوى 21-40 نانوغرام / مل ولم يكن هناك أي شخص من المجموعة السليمة في مستوى أكثر من 40 نانوجرام / مل.

بحسب مستويات الحمض النووي للفيروس تم تقسيم ال 60 مريضاً الى مجموعتين الأولى تتكون من 41 (68%) مريضاً كان مستوى الفيروس لديهم 6-2000 وحدة عالمية لكل مليليتر وفي المجموعة الثانية كان هناك 19 (38%) مريضاً كان مستوى الفيروس لديهم أكثر من 2000 وحدة عالمية لكل مليليتر.

تم مقارنة مستوى الانترليوكين-10 في (68%) 41 مريضاً الذين كانت نتيجة مستوى الفيروس لديهم بين 6-2000 وحدة عالمية لكل مليليتر مع المجموعة السليمة وكان مستوى الانترليوكين-10 في المجموعة المريضة أعلى احصائياً (القيمة ب = 0.01) . وتم أيضاً مقارنة مستوى الانترليوكين-10 في 19 مريضاً الذين كانت نتيجة مستوى الفيروس لديهم أكبر من 2000 وحدة عالمية لكل مليليتر مع

المجموعة السليمة وكان مستوى الانترليوكين-10 في المجموعة المريضة أعلى احصائياً (القيمة ب =0.01) .

عندما تم مقارنة كلا المجموعتين المريضتين كانت نتيجة الانترليوكين-10 تزداد بازدياد مستوى الفيروس وكان الفرق ذو دلالة احصائية (القيمة ب = 0.02)

وعندما تم مقارنة الانترليوكين-10 و مستوى الفيروس كان الارتباط بين الانترليوكين 10 و مستوى الفيروس موجباً و ذو دلالة احصائية (القيمة ب = 0.01).

مستوى الانترليوكين-10 يزداد بازدياد مستوى الفيروس و يعتقد أن انقاص مستوى الانترليوكين-10 باستخدام مختلف التقنيات له تأثير كبير على التهاب الكبد الوبائي المزمن "ب" ويمكن استخدام الانترليوكين-10 كموسم بايولوجي لمرض التهاب الكبد الوبائي المزمن "ب" ومتابعة مختلف مراحل المرض وعلاجه.

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List of Abbreviations:

HBV	Hepatitis B Virus
IL-10	Interleukin 10
IFN-ɣ	Interferon gamma
TH cells	T helper cells
CD 5	Cluster of differentiation 5
MHC	Major histocompatibility molecules
IV	Intravenous
IM	Intramuscular
SC	Subcutaneous
HBsAg	Hepatitis B surface antigen
HBcAg	Hepatitis B core antigen
HBeAg	Hepatitis B envelope antigen
AIDS	Acquired immune deficiency syndrome
nm	Nanometer
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
CCC	Covalently closed circular
ER	Endoplasmic reticulum
KB	Kilo base
pgRNA	pre-genomic
anti-HBs	Anti-hepatitis B surface antigen
ALT	Alanine aminotransferase
anti–HBC	Anti-hepatitis B core antigen
IgG	Immunoglobulin G
IgM	Immunoglobulin M
anti-HBeAg	Anti-hepatitis B envelope antigen
CHB	Chronic Hepatitis B
HCC	Hepatocellular carcinoma
AFP	Alfa-feto protein
PCR	Polymerase chain reaction
ELISA	Enzyme linked immune sorbent assay
FDA	Food and Drug Administration
Peg-IFN-α	Pegylated interferon alfa
TNF	Tumor necrosis factor
ALT	Alanine aminotransferase

CHAPTER ONE
INTRODUCTION

1. Introduction:

Hepatitis B virus (HBV) infection is a severe public health burden, and approximately one third of the world population has serological evidence of resolved or ongoing infection. HBV infection causes a broad spectrum of liver diseases ranging from acute to chronic hepatitis B infection with no biochemical evidence of liver injury to progressive chronic hepatitis B, which may advance to liver cirrhosis, liver failure, and hepatocellular carcinoma (Xueping *et al.,* 2016).

Worldwide, more than 350 million people are estimated to be chronically infected with this pathogen that results in more than one million deaths annually (Parkin,2006).

There are approximately 400 million HBV carriers in the world complications such as fulminant hepatic failure, cirrhosis and hepatocellular carcinoma develop annually in 250 000 of them. While the majority of adult infections remit, chronicity develops in 5−10% of cases .Adaptive immune response, which develops against HBV (a hepatotropic non-cytopathic virus) plays a key role in infection control. After the virus is taken into the liver cells infection ensues and host immune response is initiated which result in cytokines secretion and designation of immune response according to them (Ozguler *et al.,* 2014).

The cytokine interleukin-10 (IL-10) has pleiotropic effects on a number of different cell types. In general it is regarded as a suppressor of immune responses because it inhibits the secretion of pro-inflammatory and antiviral cytokines such as TNF-α and interferon-γ (IFN-γ). IL-10 inhibits the development and activation of T-helper lymphocytes with a Th1 (IFN- γ secreting) phenotype. Interleukin-10 secreting antigen specific regulatory cells may contribute to viral persistence (Yousri *et al*., 2008).

Interleukin-10, an anti-inflammatory Th2 cytokine, is one of the key coordinators of the inflammatory responses. (IL)-10 is mainly involved in regulation of inflammatory responses it's produced by macrophages, T-helper 2 cells, and B lymphocytes (CD5 subset) and can both stimulate and suppress the immune response (saxena *et al.,* 2014).

1.2 Rationale:

Hepatitis B virus is considered as one of the most common viruses which can transmit through blood, blood products transfusion and organ transplantation. The infection may lead to serious disease and complications like liver cirrhosis, hepatocellular carcinoma and high rate of mortality and morbidity (Law, 1998).

Results of several studies suggest possible role of Interleukin-10 (IL-10) in the modulation of immune response in autoimmunity, cancer and infections, but its role in hepatitis B infection and progression of the infection from acute into long term persistent infection have not been well established, therefore the aim of this study was to estimate the viral load and plasma interleukin-10 levels among hepatitis B chronically infected patients and to evaluate the utility of using Interleukin-10 as biomarker for monitoring the progression and treatment of chronic hepatitis B infection.

1.3 Objectives:

1.3.1 General Objective:

To evaluate the viral load and plasma interleukin-10 levels among chronic hepatitis B patients in Khartoum State using molecular and serological techniques.

1.3.2 Specific objectives:

1. To estimate Hepatitis B virus DNA levels "viral load" in plasma of chronic hepatitis B patients using real time PCR.
2. To estimate Interleukin-10 levels in plasma of chronic hepatitis B patients and healthy controls using ELISA.
3. To correlate between plasma Interleukin-10 levels and HBV viral load among chronically infected hepatitis B patients.
4. To evaluate the utility of using Interleukin-10 as biomarker for monitoring progression and treatment of chronic hepatitis B infection.

CHAPTER TWO

LITERATURE REVIEW

2. Literature review:

2.1 Background:

Hepatitis is a general term meaning inflammation of the liver and viral hepatitis is caused by infection with five distinctly different human hepatitis viruses A, B, C, D and E, which cannot be distinguished from one another without serologic testing (Heymann, 2008).

These five very different viruses make up the classical etiological agents responsible for acute or chronic viral hepatitis in humans and they share only a common tropism for the liver, with the hepatocyte representing the dominant site of viral replication and either acute or chronic forms of hepatitis representing the major clinical manifestations associated with infection (Hollinger and liang, 2001).

HBV infection is mainly occurred during early childhood or at birth in highly endemic area and the development of chronic disease occurs in approximately in 90%, 30% and 6% of persons infected prenatally, in early childhood and after 5 years of age, respectively (Saldnha, 2001).

Approximately 360 million people are chronic carriers of viral hepatitis B and more than one million die every year as a result of acute fulminant hepatic failure (Carlson and Perl, 2014).

The virus may be transmitted from individuals having acute or chronic infection, hepatitis B virus is found mainly in the blood, vaginal secretions, semen and serous fluids of an infected individual (CDC, 2012).

Transmission occurs by intravenous (IV), intramuscular (IM), subcutaneous (SC) or intradermal and premucosal exposure to infective body fluids (Heymann, 2008).

Infection with HBV can be prevented by vaccination with HBV surface antigen (HBsAg), which induces HBs-specific antibodies and T cells. A complete 3 dose course of the vaccine induces anti-HBs antibodies in more than 95% of healthy infants and in more than 90% of healthy adults, which are considered protective upon HBV exposure (Venters *et al.*, 2004).

2.2 Hepatitis B Virus biology:

Primary HBV infection of susceptible adults results from sexual contact with an infected host or from prenatal exposure to virus-containing blood or blood products, primary infection may be asymptomatic or may result in varying degrees of acute liver injury (acute hepatitis), although such hepatitis can be severe, in most adults, the primary infection resolves (Hirsch, 1991). Host immune responses to viral antigens result in the clearance of infected cells from the liver and the removal of virions from the bloodstream, lasting immunity to clinically evident reinfection typically results (Heise, 1999).

However, in a small proportion of infected adults generally less than 5%, the primary infection does not resolve, these individuals go on to a persistent infection characterized by active viral replication in hepatocytes and variable but usually substantial levels of viremia. As in primary infection, the clinical manifestations of the persistent infection vary greatly, many patients are relatively symptom free, whereas others have varying grades of chronic liver injury and inflammation, most of the morbidity and mortality of hepatitis B virus infection results from the persistent infection, some subsets of symptomatic patients (e.g., those with severe chronic active hepatitis B) have five year survival rates of less than 50%, with most deaths resulting from liver failure and its complications (Law, 1998).

Chronic hepatitis B patients who survive 25 to 30 years of viral persistence also have a markedly increased risk for developing hepatocellular carcinoma that is usually fatal, the asymptomatic carriers are the major epidemiologic reservoir of infection, it is principally from them that spread of HBV to susceptible hosts occurs (Law, 1998).

The reasons that some individuals resolve HBV infection whereas others do not remain poorly understood. Much correlative clinical evidence suggests that variations in host immune responses are a critical variable. For example, individuals with overt deficits in cell-mediated immunity (e.g., transplant recipients, patients with acquired immunodeficiency syndrome [AIDS]) are more likely to become chronic carriers than are fully immunocompetent hosts. The most biologically important example of this phenomenon is seen in the vertical transmission of HBV from pregnant mother to newborn baby.

HBV transmission to babies usually takes place at the time of delivery, when the newborn is exposed to large quantities of viremic maternal blood during passage through the birth canal. The cellular immune system of the neonate is known to be incompletely developed at birth; in this context, 80% to 90% of HBV exposures result in persistent infections (Vierling, 2007).

2.3 Virological properties:

HBV is a member of the Hepadnaviridae family, it is a 42-nm enveloped virion (known as Dane particle), with an icosahedral nucleocapsid core containing a partially double stranded circular DNA genome, Its genome is the smallest of all known animal DNA viruses. The envelope contains a protein called the surface antigen (HBsAg), which is important for laboratory diagnosis and immunization, within the core is a DNA-dependent DNA polymerase. The genome contains four genes (four open reading frames) that encode, surface envelope protein, core (nucleocapsid) protein, DNA polymerase, and X protein, an activator of viral RNA transcription. DNA polymerase has both RNA-dependent (reverse transcriptase) and DNA-dependent activity (Stefan and Francis, 2005).

Electron microscopy of a patient's serum reveals three different types of particles, a few 42-nm virions and many 22-nm spheres and long filaments 22 nm wide, which are composed of surface antigen, HBV is the only human virus that produces these spheres and filaments in such large numbers in the patient's blood in addition to HBsAg, there are two other important antigens, the core antigen (HBcAg) and the envelope antigen (HBeAg), both of which are located in the core (nucleocapsid) proteins but are antigenically different (Stefan and Francis, 2005).

2.4 Viral replication:

The HBV posses unique replication strategy which differ from the other DNA viruses. In the beginning the virion binds to a specific receptors at the surface of hepatocyte number of candidate receptors has been identified, including the transferrine receptor ,the glycoprotien receptor molecule , and human liver endonexin, the mechanism of HBsAg binding to a specific

receptor to enter cells has not been established yet (Ganem and Schneider, 2001).
Viral nucleocapsids enter the cell and reach the nucleus ,where the viral genome is delivered, in the nucleus, second strand DNA synthesis is completed and the gaps in both strands are repaired to yield a covalently closed circular (ccc) super coiled DNA molecule serve as a template for transcription of viral RNAs.These transcripts are polyadenylated and transported to the cytoplasm, where they are translated into the viral nucleocapsid and pre-core antigen (C, pre-C), polymerase (P), envelope L (large), M (medium), S (small) and transcriptional transactivating proteins (X) .The envelope proteins insert themselves as integral membrane proteins into the lipid membrane of the endoplasmic reticulum (ER), the 3.5 kb species ,spanning the entire genome and termed pre-genomic RNA (pgRNA), is packaged together with HBV polymerase and a protein kinase into core particles where it serves as a template of reverse transcription of negative strand DNA (Ganem and Schneider, 2001).

The new, mature viral nucleocapsids can then follow two different intracellular pathways, one of which leads to the formation and secretion of new virions, whereas the other leads to amplification of the viral genome inside the cell nucleus.In the virion assembly pathway, the nucleocapsids reach the ER, where they associate with envelope proteins and bud into the lumen of the ER, from which they are secreted via the Golgi apparatus out of the cell.
In the genome amplification pathway, the nucleocapsids deliver their genome to amplify the intranuclear pool of covalently closed circular DNA (cccDNA).The X protein contributes the efficiency of HBV replication by interacting with different transcription factors, and is capable of stimulating both cell proliferation and cell death (Ganem and Schneider, 2001).
The HBV polymerase is a multifunctional enzyme, the product of the P gene are involved in multiple functions of the viral life cycle, including a priming activity to initiate minus strand DNA synthesis, a polymerase activity, which synthesis DNA by using either RNA or DNA templates, a nuclease activity which degrades the RNA strand of RNA-DNA hybrids and the packaging of

the RNA pre-genome into nuleocapsids, nuclear localization signals on the polymerase mediate the transport of covalently linked viral genome through the nuclear pore (Ganem and Schneider, 2001).

2.5 Antigenic structure:

All three coat proteins of HBV contain HBsAg, which is highly immunogenic and induces anti-HBs (humoral immunity). Structural viral proteins induce specific T-lymphocytes capable of eliminating HBV infected cells.HBsAg is heterogeneous antigenically, with a common antigen designated a, and two pairs of mutually exclusive antigens, d, y, w (including several sub determinants) and r, resulting in 4 major subtypes, adw, ayw, adr, ayr (Hollinger and liang, 2001).

The distribution of subtypes varies geographically, because of the common determinants, protection against one subtypes appears to confer protection to other subtype, and no difference in clinical features have been related to subtypes. The c antigen (HBcAg) is present on the surface of core particles, HBcAg and core particles are not present in the blood in a free form , but are found only as internal components of virus particles. The core antigen shares it sequences with the e antigen (HBeAg), identified as soluble antigen but no cross reactivity between the two proteins are observed (Robinson, 1999).

2.6 Viral pathogenesis and immunity:

The course of hepatitis B may be extremely variable; hepatitis B infection has different clinical manifestations depending on the patient's age at infection and immune status, and the stage at which the disease is recognized. As the Virus considered non cytopathic virus the damage that occur appear to be mediated by the immune system attack of infected hepatocyte in order to clear the infection, during the incubation phase of the disease 6 to 24 weeks, patients may feel unwell with possible nausea, vomiting, diarrhea, anorexia and headache. Patients may then become jaundiced although low grade fever and loss of appetite may improve. Sometimes HBV infection produces neither jaundice nor obvious symptoms (Hollinger and liang, 2001).

The asymptomatic cases can be identified by detecting biochemical or virus specific serologic alterations in their blood. They may become silent carries of the virus and constitute a reservoir for further transmission to others. Most adult patients recover completely from their HBV infection, but about 5 to 10% will not clear the virus and will progress to become asymptomatic carriers or develop chronic hepatitis possibly resulting in cirrhosis and/or liver cancer and rarely, others may develop fulminant hepatitis and die (Robinson, 1999).

People who develop chronic hepatitis may develop significant and potentially fatal disease. In general, the frequency of clinical disease increases with age, whereas the percentage of carriers decreases, persistent or chronic HBV infection is among the most common persistent viral infections in humans. More than 350 million people in the world today are estimated to be persistently infected with HBV, a large fraction of these are in eastern Asia and Sub-Saharan Africa, where the associated complications of chronic liver disease and liver cancer are the most important health problems. A small number of long established chronic carriers apparently terminate their active infection and become HBsAg negative, Survivors of fulminant hepatitis rarely become infected persistently, and HBsAg carriers frequently have no history of recognized acute hepatitis (Robinson, 1999).

2.7 Hepatitis B mediated liver diseases:

The infecting dose of virus and the age of the person infected are important factor that correlate with the severity of acute or chronic hepatitis B (Mahoney and Kane, 1999).

Primary HBV infection may be associated with little or no liver disease or with acute hepatitis of severity ranging from mild to fulminant, HBV infection is transient in about 90% of adult and 10% of newborn, and persistent in the remainder. Most cases of acute hepatitis are subclinical, and less than 1% of symptomatic cases are fulminant (Robinson, 1999).

2.7.1 Acute hepatitis B infection:

The acute form of the disease often resolves spontaneously after a 4-8 week illness. Most patients recover without significant consequences and without recurrence. However, a favorable prognosis is not certain, especially in the elderly who can develop fulminating, fatal cases of acute hepatic necrosis. Young children rarely develop acute clinical disease, but many of those infected before the age of seven will become chronic carriers, the incubation period varies usually between 45 and 120 days, with an average of 60 to 90 days. The variation is related to the amount of virus in the inoculums, the mode of transmission and host factors (Chisari and Ferari, 1997).

The hallmark of acute viral hepatitis is the striking elevation in serum transaminase (aminotransferase) activity. The increase in aminotransferase especially ALT, during acute hepatitis B varies from mild to moderate increase of 3 to 10 fold to striking increase of more than 100 fold.

In patients with clinical illness, the onset is usually insidious with vague abdominal discomfort, nausea and vomiting, sometimes arthralgias and rash, often progressing to jaundice, fever may be absent or mild (Chisari and Ferari, 1997).

The icteric phase of acute viral hepatitis begins usually within 10 days of the initial symptoms with the appearance of the dark urine followed by a pale stools and yellowish discoloration of the mucus membrane, conjunctiva, sclera, and skin. Jaundice becomes apparent clinically when the total bilirubin level exceeds 2 to 4 mg/dl. It is combined by hepatomegaly and splenomegaly. About 4-12 weeks thereafter, the jaundice disappears and the illness resolves with the development of natural, protective antibodies (anti-HBs), in about 95% of adults (Hollinger and liang, 2001).

Acute hepatitis B is characterized by presence of anti–HBC IgM serum antibodies converting to IgG with convalescence and recovery, and the transient presence of HBsAg, HBeAg, and viral DNA, with clearance of these markers followed by seroconversion to anti-HBsAg and anti-HBeAg. More than 90% of adult onset infection cases fall in to this category. The

remaining 5 to 10 % of adult onset infection and over 90% of cases of neonatal infection become chronic and may continue for the life span of the patient (Mahony and Kane, 1999).

2.7.2 Chronic hepatitis B infection:

Chronic HBV infection is defined as persistence of hepatitis B surface antigen (HBsAg) for more than six months, high levels of HBV DNA, and presence of hepatitis B e Antigen (HBeAg) in the serum. Chronic HBV infection occurs in approximately five to ten percent of individuals with acute HBV infection. Long-term effects of chronic HBV infection include cirrhosis, liver failure, and hepatocellular carcinoma. HBV infections acquired by infants or children are significantly more likely to progress to chronic HBV infections as compared to adults (Keeffe *et al*, 2008).

Chronic hepatitis can cause serious destruction to the liver and it contributes greatly to the world wide burden of the disease states. Surprisingly, some of the patients infected persistently may have no clinical or biochemical evidence of liver disease, while other may show signs of easy fatigability, anxiety, anorexia, and malaise (Mahony and Kane, 1999).

Three phases of viral replication occur during the course of HBV infection, especially with chronic hepatitis B. High replicative phase, in this phase HBsAg, HBeAg, and HBV DNA are present and detectable in the sera, aminotransferase levels may increase, and moderate inflammatory activity is histologically apparent and the risk of evolving to cirrhosis is high. Low replicative phase, this phase is associated with the loss of HBeAg, or loss of HBV DNA concentrations, and with the appearance of anti-HBe. Histologically, a decrease in inflammatory activity is evident (Gitlin, 1997).

Non replicative phase, markers of viral replication are either absent or below detection level, and the inflammation is diminished. However, if cirrhosis has already developed, it persists indefinitely. In cirrhosis, liver cells die and are progressively replaced with fibrotic tissue leading to nodule formation. The internal structure of the liver is deranged leading to obstruction of blood flow and decrease in liver function. This damage is caused by recurrent immune responses stimulated by the presence of the virus. Because liver inflammation

can be totally symptom less, progression of inflammation to cirrhosis can occur without the knowledge of the patient (Gitlin, 1997).

Phases of chronic hepatitis B infection (Gitlin, 1997).

	ALT	HBV DNA	HBeAg	Liver Histology
Immune-tolerant phase	Normal	>1million IU/ml	Positive	Minimal inflammation and fibrosis
HBeAg-Positive immune active phase	elevated	≥2000 IU/ml	Positive	Moderate-to-severe inflammation or fibrosis
Inactive CHB phase	Normal	< 2000 IU/ml	Negative	Minimal necroinflammation but variable fibrosis
HBeAg-Negative Reactivation phase	elevated	≥2000 IU/ml	Negative	Moderate-to-severe inflammation or fibrosis

2.8 Laboratory diagnosis of Hepatitis Virus:

The current diagnostic repertoire of HBV serum markers includes viral antigen and antibodies detected or measured by immunoassay and viral DNA measured by Polymerase chain reaction (PCR) based amplification assays. In addition, we might detect both viral antigens (HBsAg, HBcAg) and viral nucleic acid in liver tissue by immunohistochemistry and PCR. When selecting the most appropriate assay it is important to note that their relevance is related to the biological properties of the particular marker they detect (Ferruccio *et al.,* 2010).

HBsAg forms part of the envelope of the virion that contains the viral nucleocapsid and nucleic acid, but also exist in large quantities within the serum, in the form defective particles (nucleocapsid and nucleic acid free) that circulate in the blood, individuals with HBsAg in their serum have overt

hepatitis infection, but do not necessarily have active liver disease and great majority of inactive HBV carriers have normal livers. In general, the disappearance of HBsAg and antibody to HBsAg (anti-HBS) seroconversion follow recovery and represent the closet to cure in both acute and chronic HBV infection (Ferruccio *et al.,* 2010).

Occult HBV infection is defined as the persistence of HBV DNA in the liver with detectable (200 IU/ml) or undetectable HBV DNA in serum of individuals who are HBsAg-negative, which defined by the indefinite persistence of antibodies to HBV core antigen (anti-HBc) ; thus anti-HBc is considerd as overall marker of exposure to HBV infection. In absence of HBsAg serum Anti-HBs is indicate protective immunity against HBV acquired by vaccination (anti-HBc-negative) or natural infection (anti-HBc-positive) (Ferruccio *et al.,*2010).

Detection of HBeAg in the serum is the hallmark of first phase of infection with HBV virus and indicates active virus replication but it does not provide any diagnostic indications about the liver disease. HBV DNA represents the direct product and hallmark of viral replication; however, levels of HBV DNA in patients with CHB do not correlate directly with the degree of HBV induced liver disease. Indeed, the highest HBV DNA levels are usually found in HBeAg-positive individual with normal liver characterizes the immune tolerant phase of CHB infection. By contrast. The lowest HBV DNA levels are persistently found in inactive HBeAg-negative, anti-HBe positive, HBsAg carriers where viral replication is kept under effective immune control. Detection of intermediate levels of HBV DNA levels in both HBeAg positive and HBeAg negative is strongly indicative of chronic hepatitis B infection (Ferruccio *et al.,* 2010).

2.9 Interleukin-10 (IL10):

IL-10 is an inhibitor of activated macrophages and dendritic cells and is thus involved in the control of innate immune reactions and cell-mediated immunity. IL-10 is an inhibitor of host immune responses, particularly responses involving macrophages. IL-10 has a four-a-helical globular domain structure and binds to a type II cytokine receptor. IL-10 is produced mainly

by activated macrophages, and because it inhibits macrophage functions, it is an excellent example of a negative feedback regulator. It is not clear whether different stimuli may act on macrophages to induce the production of a regulatory cytokine like IL-10 and effector cytokines like TNF and IL-12, or whether the same stimuli elicit production of all these cytokines but with different kinetics. T lymphocytes also secrete IL-10, and it is produced by some non-lymphoid cell types as well (Moore *et al*., 2001).

The biologic effects of IL-10 result from its ability to inhibit many of the functions of activated macrophages. Macrophages respond to microbes by secreting cytokines and by expressing co-stimulators that enhance T cell activation and cell mediated immunity. IL-10 acts on the activated macrophages to terminate these responses and return the system to its resting state as the microbial infection is eradicated. IL-10 inhibits the production of IL-12 by activated macrophages and dendritic cells. Because IL-12 is a critical stimulus for IFN-ɣ secretion and is an inducer of innate and cell mediated immune reactions against intracellular microbes, IL-10 functions to downregulate all such reactions. In fact, IL-10 was discovered as an inhibitor of IFN-ɣ production. IL10 inhibits the expression of co-stimulators and class II MHC molecules on macrophages and dendritic cells. Because of these actions, IL-10 serves to inhibit T cell activation and terminate cell-mediated immune reactions. Knockout mice lacking IL-10 develop inflammatory bowel disease, probably as a result of uncontrolled activation of macrophages reacting to enteric microbes. These mice also show excessive inflammation and tissue injury in response to chemical irritants. The Epstein-Barr virus contains a gene homologous to human IL-10, and viral IL-10 has the same activities as the natural cytokine. This raises the intriguing possibility that acquisition of the IL-10 gene during the evolution of the virus has given the virus the ability to inhibit host immunity and thus a survival advantage in the infected host (Moore *et al*., 2001).

2.10 IL-10 and Hepatitis B Virus:

Hepatitis B virus (HBV) is the major cause of chronic liver disease throughout the world, with 55-80% of patients developing chronic hepatitis

after infection with the virus. The outcomes of chronic HBV infections are extremely variable. The majority of cases are associated with insidious and progressive liver disease that may eventually lead to cirrhosis and hepatocellular carcinoma. However, the pathogenesis of liver damage during chronic HBV infections is poorly understood. There is suggestive evidence that T-cell immunoregulatory cytokines may play a key role in influencing the persistence of HBV infection and the extent of liver damage (Jiang *et al*., 2002).

In the course of HBV-related hepatitis, some Th1 phenotype cytokines are positively correlated with hepatic inflammatory activity. Activated CD4+ T cells can be divided into 2 subsets based on their cytokine secretion profiles. The T helper type 1 (Th1) subset produces interferon-gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), and (IL)-2, and participates in cell-mediated immune responses. The T helper type 2 (Th2) subset produces IL-4 and IL-10, and mediates humoral immune responses as well as anti-parasitic and allergic responses (Frydas *et al.,* 2004).

The Th1/Th2 cytokine balance is likely important in determining the rate of HBV infection chronicity and HBV induced liver injury. In fact, some studies have suggested that a preferential shift towards either Th1 or Th2 response may influence the clinical outcome and disease progression. IL-6 and IL-18 are defined as pro-inflammatory cytokines, particularly as IL-6 plays a role in immune responses that may lead to viral clearance and as IL-18 levels are correlated with metabolic and viral hepatic diseases. The viral infections and some chronic injuries are known to stimulate the production of IL-10 and subsequently suppress the immune system. While the pathogenesis of chronic HBV infection has not been clearly defined yet, many studies suggest that cytokines play important roles in both immunoregulation and immune impairment (Jiang *et al*., 2002).

2.11 Previous Studies:

In the past few years so many researches were conducted to estimate the association of IL-10 and hepatitis B virus and to correlate between IL-10 and chronicity development. IL-10 was found to inhibit T cell activity, which is developed against T cell response and viral infections. It shows its effects by

decreasing molecular expression of antigen presenting cells, and so by decreasing cytokine production, it may prevent T cell activation by interrupting T cell maturation. Despite negative regulatory functions, it has been shown in several studies that IL-10 can stimulate proliferation of NK cells, CD8 T cells, B cells and antibody production (Foulds *et al.,* 2006; Kang and Allen, 2005).

In addition to HBV and HCV infections, IL-10 is a cytokine with a key role in regulation of cellular immune response against Epstein-Barr virus, herpes simplex virus and HIV. Inappropriate release of cytokines such as IL-10 in chronic HCV infected patients was correlated with HCV clearance, fibrogenesis and treatment resistance to interferon (Ozguler *et al*., 2015).

Besides, IL-10, which is generally an immunosuppressive, can have effects on pathogen specific immune response through various mechanisms. In persistent viral infections, it has been determined that many cell types produce IL-10 (Belkaid and Tarbell, 2009; Rouse and Masopust, 2006).

Additionally, suppression states during persistent infections occur because IL- 10 limits various immune parameters, so the infection cannot be cleared. Modulation of cells expressing IL-10, which inhibits antiviral activity, is biologically and therapeutically important (Slobedman *et al*., 2009).

In many studies, it was shown that IL-10 was important in sensitivity to inflammatory diseases, in response to HBs antigen vaccination, in HBV carriers, in HBeAg seroconversion, and in hepatocellular carcinoma related to HBV. Its production can be regulated at transcriptional, post-transcriptional and translational levels (Vicari and Trinchieri, 2004).

In many studies, it was determined that control of cytokine polymorphism has an important role in HBV infection outcomes. The correlation between IL-10, which is produced by HBV antigen stimulated (HBcAg, HBsAg, pre-S1Ag) peripheral blood monocytic cells and chronic hepatitis B disease activity has not been understood yet. However, it was observed in previous studies that HBV antigens could stimulate IL-10 production. It was shown that IL- 10 secretion from monocytes was increased by pro-inflammatory cytokine IFN-γ; increased secretion of IL-10 might suppress immune

response in HBV infection, and it prevented serious liver damage (Wirth *et al.,* 2000).

2.12 Prevention and Control:

Vaccine for prevention of hepatitis B virus infection has been routinely recommended for infant since 1991 in the United State. It can also given to those who are at high risk like health care workers. Most vaccines are given in three doses over a course of months. A protective response to vaccine is defined as an anti-HBs concentration of more than 10 IU/ml in the recipient's serum. Hepatitis B immunoglobulin may be used to protect persons who are exposed to hepatitis B. It is particular efficacious within 48 hours of the incident. It may also be given to neonates who are at increased risk of contracting hepatitis B i.e. whose mothers are HBsAg and HBeAg positive (Hollinger and liang, 2001).

2.13 Treatment:

The U.S. Food and Drug Administration (FDA) have approved seven agents for the treatment of CHB. The first licensed agent for the treatment of chronic HBV infection was the conventional form of interferon alfa which have antiviral, anti-proliferative, and immunomodulatory effects. Pegylated interferon alfa (PEG-IFN-α), an agent that is almost identical to that of standard IFN-α, was licensed in 2005. Other agents that are currently in use are nucleoside and nucleotide analogues which are pure anti-virals that act via suppression of HBV replication through inhibition of the reverse transcriptase and DNA polymerase activities. Lamivudine, a nucleoside analogue, was the first among them to be licensed in 1998. During the past decade, two other nucleoside analogues; Entecavir (in 2005) & Telbivudine (in 2006), and two nucleotide analogues; Adefovir (in 2002) and Tenofovir Disoproxil fumarate (in 2008) were licensed (Norah *et al.,* 2015).

The ultimate goal of CHB treatment is to prevent or decrease the development of cirrhosis, hepatic failure and HCC. These endpoints are reached by the suppression of viral replication, which are monitored through parameters such as reduction in HBV DNA to undetectable levels; reduction of serum ALT to normal levels; loss of HBeAg with or without detection of

anti-HBe; and improvement in the histological findings. But, viral eradication is nearly unachievable because of the tendency HBV to integrate into the host genome or remain latent as cccDNA Considering the extensive cost, the risk of adverse events and the drug resistance with long-term treatment, the most important question that arises is, which CHB patients need to be treated now and which patients can be monitored and have treatment deferred. And, as the efficacy and the optimal timing to initiate antiviral strategies are greatly influenced by the dynamic course of the disease and the above-mentioned host, viral, and environmental factors associated with progression of CHB; we have tried to focus on the current therapeutic strategies on two separate grounds based on the HBeAg status (Norah *et al.,* 2015).

CHAPTER THREE

MATERIALS AND METHODS

3. Materials and Methods:

3.1 Study design:

This was descriptive comparative cross sectional study.

3.2 Study area:

Total Lab Care laboratories in Khartoum State.

3.3 Study duration:

This study was conducted during the period from January to May 2017.

3.4 Study population:

Study was conducted in chronically infected hepatitis B patients and healthy individuals as control.

3.5 Sample size:

Total of Ninety (n=90) blood samples were collected in EDTA containers, 60 chronic hepatitis B patients and 30 healthy controls.

3.6 Sampling technique:

Chronically infected hepatitis patients were randomly selected**.**

3.7 Inclusion criteria:

Chronically infected hepatitis B patients with persistent positive ELISA test for HBsAg for more than 6 months were included.

3.8 Exclusion criteria:

Non Chronically infected hepatitis B patients with positive ELISA test for HBsAg for less than 6 months were excluded.

3.9 Data collection:

Data was collected using self-administrated questionnaire.

3.10 Ethical consideration:

Approval had been taken from Sudan University of Science and Technology, College of graduate studies and Medical Microbiology Department., approval had been taken from Total lab Care laboratories and verbal consent had been taken from patients.

3.11 Laboratory work:

3.11.1 Specimens collection and preservation:

Four milliliters (ml) of blood was collected from hepatitis B patients with positive ELISA testing for HBsAg for more than 6 months into EDTA containers for molecular and serological procedures and was centrifuged at 3000 rpm for 5 minutes to obtain plasma which transferred into Plain containers and stored at -20 °C until processing.

3.11.2 Real time PCR for Hepatitis B virus:

Hepatitis B virus DNA levels was studied with Roche Cobas ®Taqman® 48 analyzer and High Pure system viral nucleic acid kit PCR Template Preparation Kit was used for hepatitis B virus DNA extraction (Roche, Germany).

Specimen and Control Preparation and DNA extraction:

First 625 µL of Lysis/Binding Working Solution was pipetted into each well of the Lysis Rack.

By Opening one well at a time, 500 µL of specimen and control was pipetted into the appropriate well.

By vortexing the filled Lysis Rack was Mixed for approximately 10 seconds.

The Lysis Racks was placed into a preheated 50°C (± 2°C) water bath and incubated for 10 minutes.

The Lysis Rack was centrifuged for 10-20 seconds at a setting of 4600 xg in the micro-titer plate centrifuge.

Then 250 µL of isopropanol was pipetted into each well.

Specimens was mixed by inverting the rack three times, then vortexing the rack for approximately 10 seconds and Centrifuge the Lysis Rack for 10-20 seconds at a setting of 4600 x g in the micro-titer plate centrifuge.

Then 750 µL of specimen or control mixture was transferred to the corresponding wells of the Filter Tube Rack with affixed Waste Rack.

The Filter Tube Rack assembly was centrifuged for 2 minutes at 4600 xg in the micro-titer plate centrifuge.

The remaining specimen or control mixture was transferred to the corresponding wells of the Filter Tube Rack and the Lysis Rack was discarded appropriately.

The Filter Tube Rack assembly was centrifuged for 2 minutes at 4600 x g in the micro-titer plate centrifuge.

The Filter Tube Rack was removed from the Waste Rack and Waste Rack was discarded and replaced with a new Waste Rack.

Then 400 µL of Inhibitor Removal Buffer (IRB) was pipetted down the side of each well.

The Filter Tube Rack assembly was centrifuged for 2 minutes at 4600 xg in the micro-titer plate centrifuge.

Then 700 µL of Wash Buffer (WASH) was pipette down the side of each well.

The Filter Tube Rack assembly was centrifuged for 2 minutes at 4600 x g in the micro-titer plate centrifuge.

The Filter Tube Rack was removed from the Waste and the Waste Rack was replaced with a new Waste Rack.

Then 700 µL of Wash Buffer was pipetted down the side of each well.

The Filter Tube Rack assembly was centrifuged for 3 minutes at 4600 xg in the micro-titer plate centrifuge.

The Filter Tube Rack was removed from the Waste Rack and placed onto the Elution Rack.

Then 75 µL of the pre-warmed Elution Buffer (ELB) was pipetted onto the center of each filter without touching the filter.

The Elution Rack was incubated at room temperature for a minimum of 3 minutes after Elution Buffer was added to the last well.

The Filter Tube Rack assembly was centrifuged for 3 minutes at 4600 x g in the micro-titer plate centrifuge.

The Filter Tube Rack was removed from the Elution Rack.

The Cover Rack was placed onto the Elution Rack.

Then 50 µL of Working MMX was added into each K-tray well.

Then 50 µL of each processed specimen and control was added to the appropriate K-tray wells containing Working MMX using a micropipettor with an aerosol barrier or positive displacement tip and gently each specimen or control was mixed up and down three times with the micro pipettor without generating bubbles.

Working MMX Preparation:

For 24 tests, 191 µL of CTM Mn2+ was added to one vial of HBV MMX. The bottle was capped and mixed well by inverting 10 times and used within 2 hours.

For 12 tests, 660 µL of HBV MMX was removed and placed in a 2 mL tube. 90 µL of CTM Mn2+ was added to the 2 mL tube containing HBV MMX. The bottle was capped and mixed by inverting 10 times and used within 2 hours.

Amplification and Detection

The Systems Icon in the System Tab was selected; Open button was clicked to open the Thermal Cycler. When the Thermal Cycler Cover was completely opened and "Ready to Load" is seen in the Systems window. Using the K-tray carrier Transporter, the loaded K-tray carrier containing the capped K-tray with Working Master Mix and specimens and controls were transferred into the Thermal Cycler. The Thermal Cycler lid was closed.

Start button on the Systems window below the TC icon was clicked to close the Thermal Cycler Cover and start the run.

Amplification and detection were automatically performed by the COBAS® TaqMan® 48 Analyzer.

3.11.3 Enzyme Linked Immune Sorbent Assay (ELISA) for Interleukin-10:

Commercial **BioLegend** Human IL-10 ELISA MAX^{TM} Deluxe Set was used to detect IL-10 according to manufacturer instructions.

ELISA Procedure summary:

Day (1):

Diluted Capture antibody solution was added to each well; the plate was sealed and incubated overnight between 2◦C and 8◦C.

Day (2):

The Plate was washed 4 times, and blocked by adding 200 μL1X assay diluents A to each well, and then the plate was sealed and incubated at room temperature for 1 hour with shaking at 500 rpm.

The Plate was washed 4 times, 100 μL diluted standard and samples was added to the appropriate wells.

The plate was sealed and incubated at room temperature for 2 hours with shaking.

The Plate was washed 4 timesand 100 μL diluted detection antibody solution was added to each well then he plate was sealed and incubated at room temperature for 1 hour.

The Plate was washed 4 times, and 100 μL diluted Avidin-HRP solution was added to each well, the plate was sealed and incubated at room temperature for 30 minutes.

The Plate was washed5 times, with soaking for 30 seconds to 1 min per wash, then 100 μLof freshly mixed TMB substrate solution was added to each well and incubated in the dark 30 minutes.

100 μL stop solution was addedto each well and absorbance was readed at 450 nm.

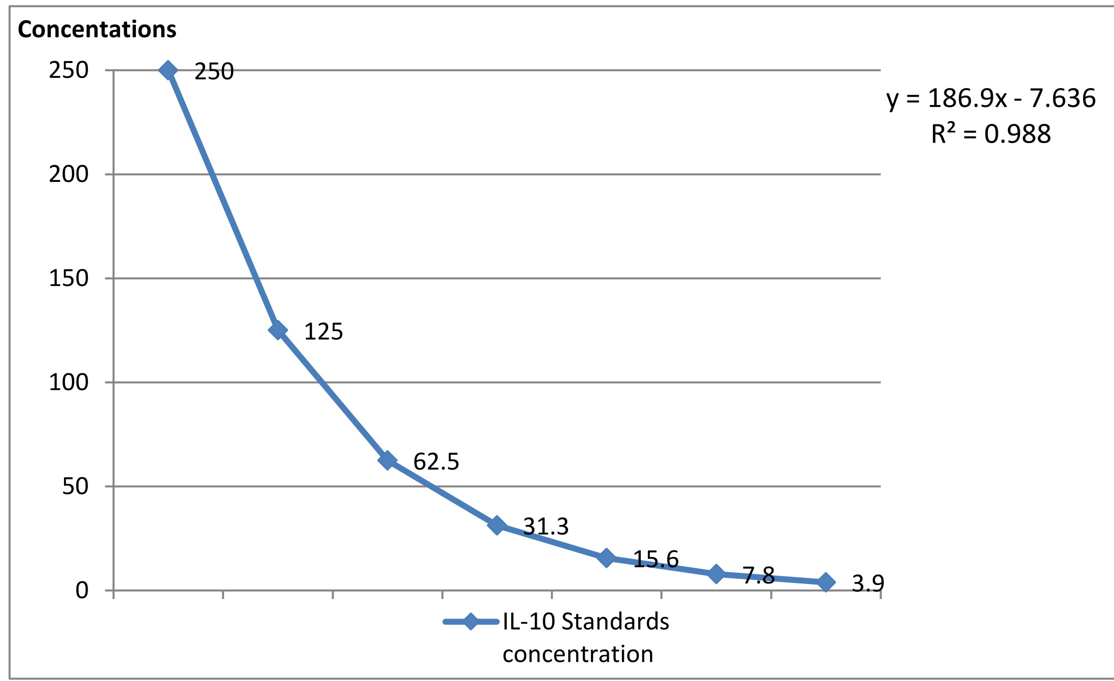
Note: the plate washing was performed with 300 μL of wash buffer per well.

Interpretation of Results:

Sirio S™ Reading device were used to estimate IL-10 absorbance in each well then, the absorbance was converted into concentrations by drawing an absorbance/concentrations curve from the given 6 standard. All the calculations were carried out using Excel program depending on the following Equation according to manufacturer's instructions.

Y= 186.9X-7.636

Y= Concentration.
X= Absorbance.
R^2= Linearity of the result "indicate pipetting accuracy"
*The accepted R^2 value range is (0.8-1.0)



The Concentration of the six IL-10 standards.

- **3.11 Data analysis:**

  Collected data was analyzed by using SPSS program version 19.

CHAPTER FOUR
RESULTS

4. RESULTS:

A total of 60 patients and 30 healthy controls who had matched in age group (23-58 years) had recruited to participate in this study during the period from January 2017 to May 2017. Among patients group there were 40 male and 20 female in ratio 2:1 and among controls group there were 20 male and 10 female in ratio 2:1 (figure 1).

Patients group had been divided into two groups according to HBV DNA levels the first group consist of 41 (68%) patients with HBV DNA level of 6–2000 IU/mL, the second group consist of 19 (32%) patients with HBV DNA level of > 2000 IU/mL. Interleukin-10 levels of 30 healthy participants in the controls group were compared with those in the patients groups.

When the controls group and the first group were evaluated, it was determined that IL-10 levels were higher in the first group and the result was statistically significant (P= 0.01).

When the controls group and the second group were evaluated IL-10 levels was higher in the second group and the result was statistically significant (P= 0.01).

When the two groups with high HBV DNA levels were compared, IL-10 level was higher in the second group, and the difference was statistically significant (P= 0.02) Table (1).

The lowest IL-10 levels (1-10 ng/ml) were detected in (61.6%) of the patients population and (86.6%) of the Controls and the highest IL-10 levels (More than 40 ng/ml) were detected in (16.7%) of Patients and 0 % of Controls, the majority of controls IL-10 levels were less than (5 ng/ml) and the majority of patients IL-10 levels were more than (5 ng/ml) Table (2, 3) and Figure (3, 4).

When IL-10 levels and Hepatitis B viral load levels were evaluated for correlation, positive correlation was detected and the result was statistically significant (P= 0.01) Figure (2).

Table (1): *P*-value when IL-10 levels were compared within the different groups.

Comparisons of groups	*P*-value
Comparison of control and first groups	0.01
Comparison of control and second groups	0.01
Comparison of first and second groups	0.02

**P*-value is significant at $P < 0.05$.

* First group means patients with HBV DNA level 6–2000 IU/m.

* Second group means patients with HBV DNA level more than 2000 IU/m.

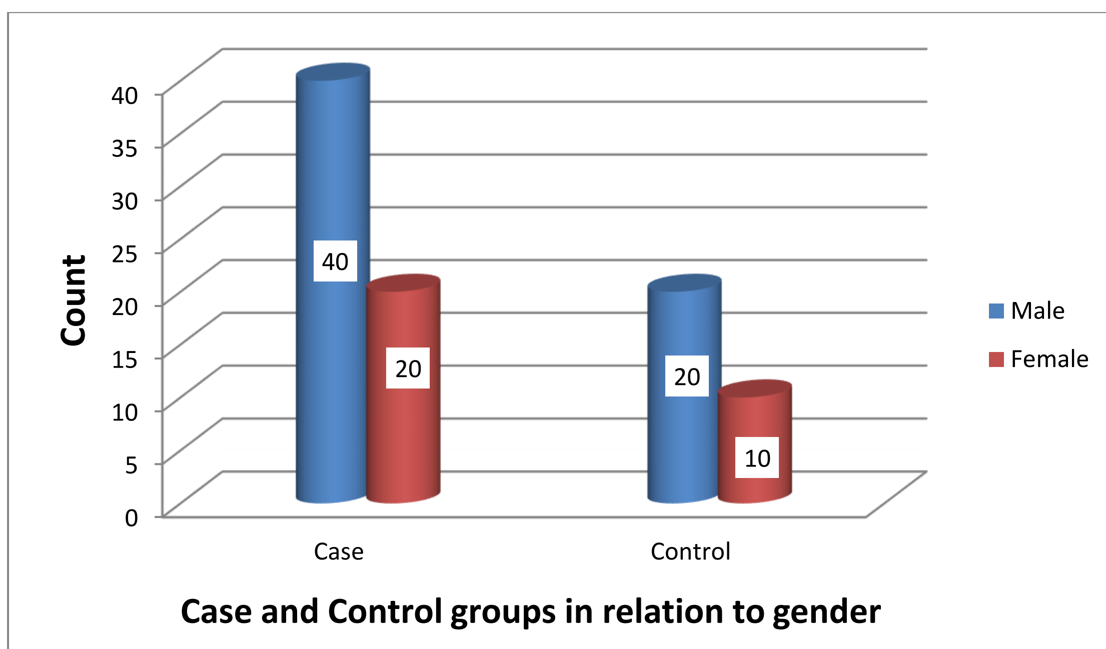


Figure (1): Distribution of study participants among case and control groups according to gender.

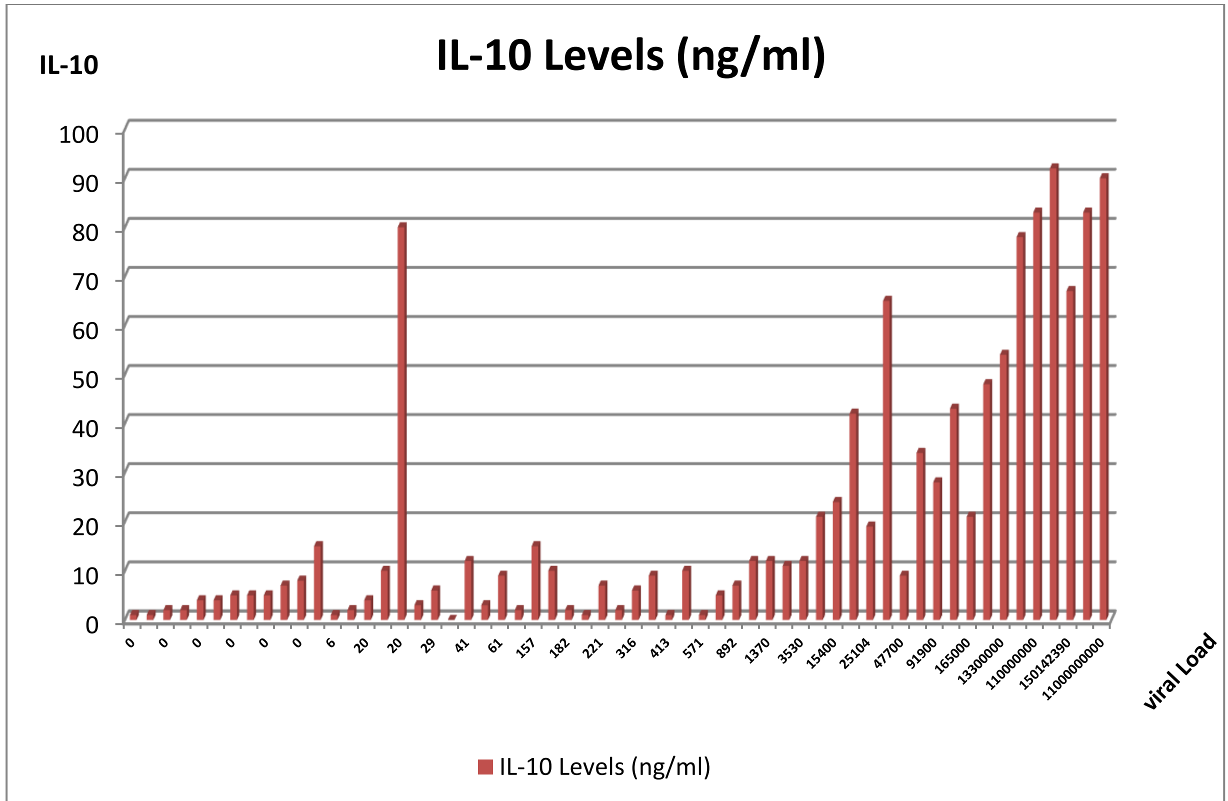


Figure (2): IL-10 levels expressed in (ng/ml) in correlation with viral load levels.

Table (2): Distribution of IL-10 levels (ng/ml) among patients and controls.

IL-10 Levels (ng/ml)	Patients	Controls
1-10	37 (61.6%)	26 (86.6%)
11-20	7 (11.7%)	3 (10%)
21-40	6 (10%)	1 (3.4%)
More than 40	10 (16.7%)	0
Total	60	30

Table (3): Distribution of Age groups and IL-10 levels among patients population.

IL-10 levels groups(ng/ml)	Age groups			Total
	23-33	34-44	45-58	
1-10	9	12	16	37
11-20	3	2	2	7
21-40	2	3	1	6
41-100	3	4	3	10
Total	17	21	22	60

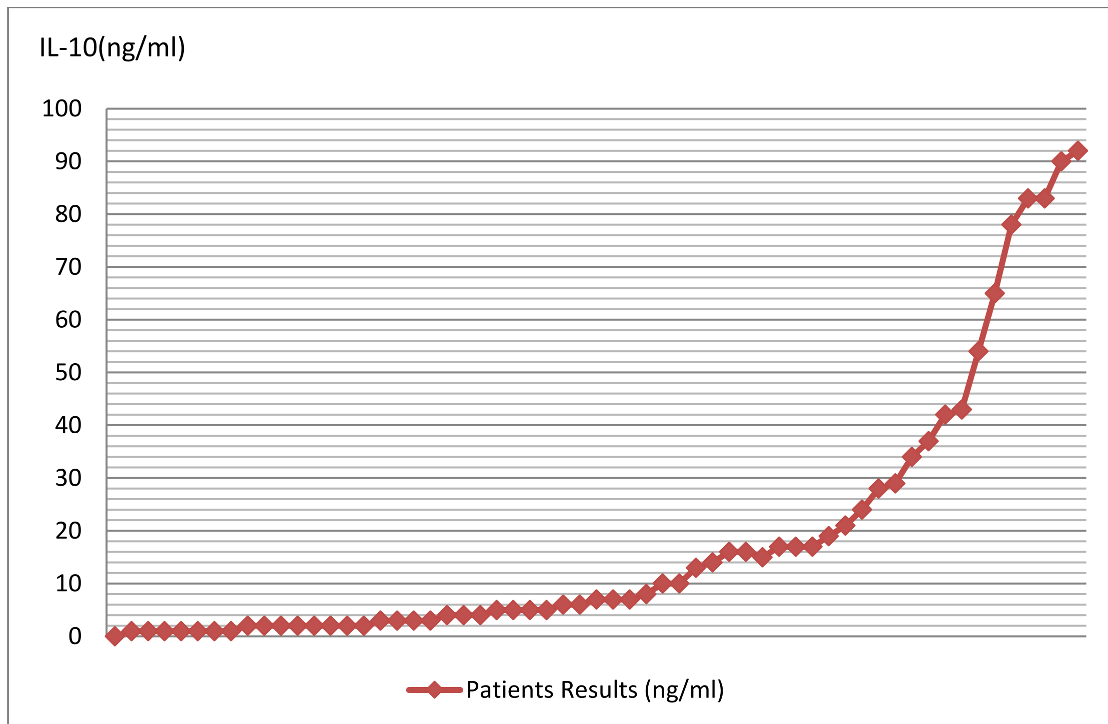


Figure (3): IL-10 levels among chronic hepatitis B patients.

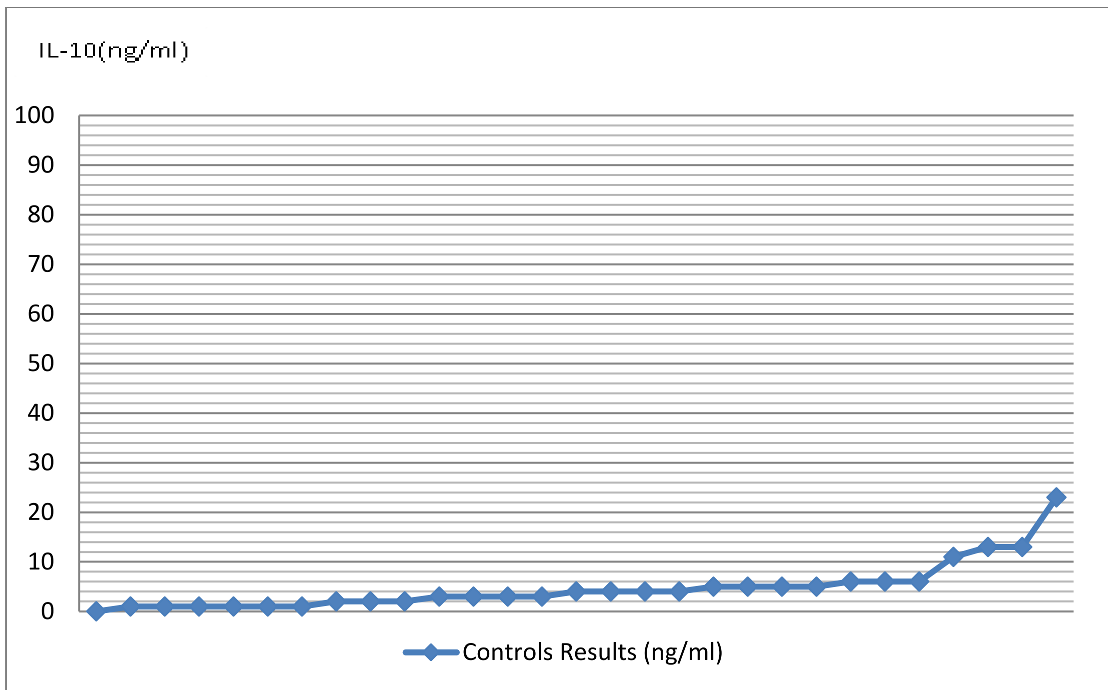


Figure (4) IL-10 Levels among healthy control group.

CHAPTER FIVE

DISCUSSION

5.1 Discussion:

Interleukin-10 was known as an inhibitor of cytokine synthesis and it can cause T cell dysfunction by various mechanisms, it was first defined as a Th2 cytokine produced by CD4 cells. Currently, it is known that many cells including liver cell types such as hepatocytes, sinusoidal endothelial cells, Kuppfer cells, hepatic satellite cells and lymphocytes associated with the liver can produce it. Additionally, suppression states during persistent infections occur because IL-10 limits various immune parameters, so the infection cannot be cleared (Pestka *et al.,* 2004).

The present study estimated the presence of correlation between HBV viral load and IL-10 and evaluated the utility of using IL-10 as biomarker for monitoring of hepatitis B infection and progression of the chronic infection in correlation with hepatitis B viral load.

In the present study, IL-10 was detected in plasma of all study participants in varying levels and its levels were significantly higher in chronic hepatitis B patients than in healthy individuals this finding was similar to Ozgular *et al*., (2015) study in which significant variation was also detected. In Arababadi *et al.,* (2010) study, IL-10 levels of 352 patients with occult HBV infection were estimated, and it was found that the levels were significantly higher when compared to controls, this support the present study finding.

The present study showed that IL-10 levels increased in close temporal correlation with viral load increase, this finding was similar to those of Das *et al*., (2012) and Li *et al.,* (2011) which also linked the IL-10 levels increase to the peak of liver inflammation. Li *et al.,* study also detected positive correlation between IL-10 levels and ALT enzyme.

Another study conducted by Kaymakoğlu *et al.,* (1999) reported that IL-10 levels in both asymptomatic carriers and chronic hepatitis B patients were significantly higher than those in the control group a finding similar to the present study result. However, no significant difference was determined when asymptomatic carriers and chronic hepatitis B patients were compared while in Yıldız *et al.,* (2007) study, IL-10 levels were determined significantly higher in chronic hepatitis B patients when compared with inactive carriers.

The present study findings was different from the study of Xueping *et al.,* (2016) which concluded that IL-10 levels were not related to the severity of liver injury, progression, and prognosis of HBV related chronic liver diseases.

Das *et al.,* (2012) study suggests that IL-10 may serve a physiological role in attenuating inflammation, but may inadvertently dampen adaptive immune responses and cause viral persistence.

Park *et al.,* (2011) reported that IL-10 levels were significantly higher in chronic hepatitis B patients resistant to lamivudine than the responsive ones, and suggested that it might be an indicator in monitorization of treatment response in chronic hepatitis B patients.

Also Ozgular *et al*., (2015) reported significant correlation between the fibrosis index and IL-10 levels and IL-10 was found to be increased with increased liver fibrosis, this finding support the present study finding. In the similar study of Li *et al.,* (2011) it was reported that IL-10 levels were significantly higher in patients with liver cirrhosis than patients diagnosed with chronic hepatitis B. This support that IL-10 could be an important marker for progression of chronic hepatitis B into liver cirrhosis.

5.2 Conclusion:

In conclusion, IL-10 levels may be an important biomarker for hepatitis B infection monitoring and evaluation of the treatment response. Also the results suggest that high IL-10 levels could be the reason of inability to clear the virus and therefore, decreasing IL-10 levels by using various techniques may have important contributions on chronic hepatitis B infection.

5.3 Recommendations:

Further studies may be conducted considering:

1. The increase of sample size and patients population (chronic carriers, patients on antiviral therapy, patients has not starting antiviral treatment yet, patient with liver cirrhosis and hepatocellular carcinoma) is likely to enhance our understanding of IL-10 roles and biological activities.
2. The use of other markers like HBeAg and Anti-HBe in correlation with IL-10 will give us important information about the status of IL-10 during HBeAg seroconversion and viral replication status.
3. Estimating the histological activity indices and fibrosis scores in liver biopsies, and ultrasound findings will yield great information about liver status and the effect of IL-10 during the different disease phases.
4. Finally, taking this work into the next levels the genomic levels and estimating the effects of different mutation and gene polymorphisms using a number of molecular and bioinformatics tools will expanse our knowledge about the role of IL-10 in the pathogenesis of hepatitis B virus infection and so many other diseases.

6. Referances:

Arababadi M, Pourfathollah A, Jafarzadeh A and Hassanshahi G. (2010). Serum levels of IL-10 and IL-17A in occult HBV-infected south-east Iranian patients. *Hepat Mon*; **10:** 31−5.

Belkaid Y and Tarbell K. (2009). Regulatory T cells in the control of host-microorganism interactions. *Annu Rev Immunol*; **27**: 551−89.

Carlson A and Perl T. (2014). Healthcare Workers as Source of hepatitis B and C viruses transmission. *Clin Liver Dis*: **34**(12) 153-168.

CDC. (2012). Chapter 9 Hepatitis b Epidemiology and Prevention of Vaccine preventable disease. The pink Book: updated 12thEdition: 15-138.

Chisari G and Ferari H. (1997). Hepatitis B virus infection: epidemiology and vaccination. *Epidemiologic Reviews*. **28**(1): 112-125.

Das A, Ellis G, Pallant C, Lopes AR, Khana P and Peppa D. (2012). IL-10- producing regulatory B cells in the pathogenesis of chronic hepatitis B virus infection. *J Immunol*. **189**: 3925−35.

Ferruccio B, Teerha P, Maurizia RB, Yun FL. (2010). Diagnostic markers of chronic hepatitis B infection and disease. *International medical press.***15** (3):35-44.

Foulds KE, Rotte MJ and Seder RA. (2006). IL-10 is required for optimal CD8 T cell memory following Listeria monocytogenes infection. *J Immunol*. **177**: 2565−74.

Frydas S, Karagouni E, Hatzistilianou M, Kempuraj D, Comani S, Petrarca C, Iezzi T, Verna N, Conti P and Castellani ML. (2004).Cytokines and allergic disorders: revisited study. Int J *Immunopathol Pharmacol*.**17**: 233-235.

Ganem D and Schneider RJ. (2001). Hepadnaviridea the viruses and their Replication in: knipe DM*et al*, eds. Fields Virology, 4thed .Philadelphia, Lippincott Williams and Wilkins. 2923-2969.

Gitlin N. (1997). Hepatitis B diagnosis, prevention and treatment. *Clin chem*. **43**(8):1500-1506.

Heise K. (1999). Identification of Hepatitis B virus putative inters genotype recombinants by using fragment typing. *J Gen Virol* .**87**: 2203-2215.

Heymann DL. (2008). Viral Hepatitis B. In: Control of Communicable Disease Manual 19th ed. Elsevier, Philadelphia.284-293.

Hirsch T. (1991). Hepatitis B biology clinical review. *J Med Virol.* **81**: 435-440.

Hollinger FB and liang TJ. (2001). Hepatitis B virus. In: knipe DM *et al.*, eds. Feilds Virology, 4th ed. Philadelphia, Lipponcott Williams and wilkins. 2971-3036.

Jiang R, Feng X, Guo Y, Lu Q, Hou J, Luo K, and Fu N. (2002). T helper cells in patients with chronic hepatitis B virus infection. *Chin Med J* .**115**:422-424.

Kang S and Allen PM. (2005). Priming in the presence of IL-10 results in direct enhancement of CD8+ T cell primary responses and inhibition of secondary responses. *J Immunol*. **174**: 5382−9.

Kaymakoğlu S, Gürel N, Demir K, Çakaloğlu Y, Badur S, Çevikbaş U., *et al*. (1999). Relationship between serum levels of interleukin-10, interleukin-2 and soluble intercellular adhesion molecule-I and liver injury in chronic hepatitis B virus infection. *Turk J Gastroenterol*; **10:** 243−8.

Keeffe EB, Dieterich DT and Han SH. (2008). A treatment algorithm for the management of chronic hepatitis B virus infection in the United States. *Clin Gastroenterol Hepatol*. **6**(12):1315-1341.

Law D. (1998). The different epidemic and evolution of HBV genotypes. *Yi Chuanviro* .**34**: 666-672.

Li C, Xing SJ, Duan XZ, Wan MB and Wang HF. (2011). The study on frequency distribution of regulatory T cells and its functional markers in peripheral blood of chronic hepatitis B. Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi. **25:** 33−5.

Mahoney D and Kane R. (1999). Liver biopsy assessment in chronic viral hepatitis. *J Viral Hepat*. **14**; 16-21.

Moore W, De Waal R, Coffman L and O'Garra A. (2001) .Interleukin-10 and Il-10 receptors. *Annu Rev Immunol.***19**:683-765.

Norah AT, Natalie HB, Kyong MC, Jessica P, Maureen MJ, and Hassan M. (2015). AASLD Guidelines for Treatment of Chronic Hepatitis B. *J of Hepatology*. **9**:1-23.

Ozguler M, Akbulut H, and Akbulut A.(2015).Evaluation of Interleukin-10 Levels in Patients Diagnosed with Chronic Hepatitis. *West Indian Med J*. **64** (2): 71-75.

Parkin M. (2006). The global health burden of infection associated cancers in the year 2002. *Int J Cancer*. **118** (2): 30–44.

Park Y, ParkY, Han KH, and Kim HS. (2011). Serum cytokine levels in patients with chronic hepatitis B according to lamivudine therapy. *J Clin Lab Anal*. **25:** 414−21.

Pestka S, Krause CD, and Walter MR. (2004). Interferons, interferon-like cytokines, and their rcptors. *Immunol Rev*. **202**: 8−32.

Robinson. (1999). Hepatitis B antigenic structure and different subtype distribution. *World J Hepatol*.**35** (2):74-81.

Rouse BT and Masopust D. (2006). Waking up T cells to counteract chronic infections. *Trends Immunol*. **27**: 205−217.

Saldnha V. (2001). The global risk of hepatitis B infection. *Int J Cancer*. **118**(2):30-44.

Saxena R , Yogesh Kumar Ch, Indu V, and Jyotdeep K. (2014).Association of interleukin-10 with hepatitis B virus mediated disease progression in Indian population. *Indian J Med Res.***139** (5)*:*737-745.

Slobedman B, Barry PA, Spencer JV, Avdic S and Abendroth A. (2009). Virus encoded homologs of cellular interleukin-10 and their control of host immune function. *J Virol*. **83**: 9618−29.

Stefan H and Francis M. (2005). Diagnostic markers for chronic hepatitis B infection. *J Med Viro*. **64**: 271–277.

Venters C, Grahm W, and Cassidy W. (2004). Recombinant HBV vaccine: prescriptive past, Present and Future. *Expert Rev Vaccines*. **3**:119 -129.

Vicari AP and Trinchieri G. (2004). Interleukin-10 in viral diseases and cancer. *Immunol Rev* .**202**: 223−36.

Vierling JM. (2007). The immunology of hepatitis B virus. *Clin Liver Dis*. **11**:727–59.

Wirth S, van den Broek M, Frossard CP, Hügin AW, Leblond I, Pircher H and Hauser C. (2000). CD8 (+) T cells secreting type 2 lymphokines are defective in protection against viral infection. *Cell Immunol*. **202**: 13–22.

Yu X, Zheng Y, Deng Y, Li J, Guo R, Su M, Ming D, Lin Z, Zhang J and Su Z. (2016). Serum Interleukin (IL)-9 and IL-10, but not T-Helper 9 (Th9) Cells, are Associated With Survival of Patients With Acute-on-Chronic Hepatitis B Liver Failure. *Medicine j*. **95**(16): 1-8.

Yıldız F, Irmak H, Tuncer G, Yetkin MA, Onde U and Bulut C *et al*. (2007). Interleukin-6 and interleukin-10 levels in patients with chronic hepatitis B virus infection. *Flora J Infect Clin Microbiol*.**12:**46−51.

Yousri M, Mohamed E, Amal F, Mohamed H, Mohamed A and Haidy E. (2008). Role of Serum Interleukin–10 as a Marker of T-helper 2 Activity in Egyptian Chronic Hepatitis C Patients. *Bull Egypt. Soc. Physiol. Sci.* **28** (1): 141-152.

- **Appendices:**

1. **COBAS® TaqMan® HBV Test For Use With The High Pure System .**

INTENDED USE

The COBAS® TaqMan® HBV Test For Use With The High Pure System (HPS) is an *in vitro* nucleic acid amplification test for the quantitation of Hepatitis B Virus (HBV) DNA in human serum or plasma, using the High Pure System Viral Nucleic Acid Kit for manual specimen preparation and the COBAS® TaqMan® 48 Analyzer for automated amplification and detection.

The COBAS® TaqMan® HBV Test is not intended for use as a screening test for blood or blood products for the presence of HBV or as a diagnostic test to confirm the presence of HBV infection. .

PRINCIPLES OF THE PROCEDURE

The COBAS® TaqMan® HBV Test is based on two major processes: (1) manual specimen preparation to obtain HBV DNA; (2) automated PCR amplification of target DNA using HBV specific complementary primers, and detection of cleaved dual fluorescent dye-labeled oligonucleotide detection probes that permit quantitation of HBV target amplified product (amplicon) and HBV Quantitation Standard DNA, which is processed, amplified, and detected simultaneously with the specimen.

The Master Mix reagent contains primer pairs and probes specific for both HBV DNA and HBV Quantitation Standard DNA. The Master Mix has been developed to ensure equivalent quantitation of genotypes A through G of HBV. The detection of amplified DNA is performed using target-specific and Quantitation Standard-specific dual labeled oligonucleotide probes that permit independent identification of HBV amplicon and HBV Quantitation Standard amplicon. The quantitation of HBV viral DNA is performed using the HBV Quantitation Standard. The HBV Quantitation Standard is a non-infectious linearized plasmid that contains the identical primer binding sites as the HBV DNA target and a unique probe binding region that allows HBV Quantitation Standard amplicon to be distinguished from HBV target amplicon. The HBV Quantitation Standard is incorporated into each individual specimen and control at a known copy number and is carried

through the specimen preparation, PCR amplification and detection steps along with the HBV target. The COBAS® TaqMan® 48 Analyzer calculates the HBV DNA titer in the test specimen by comparing the HBV signal to the HBV Quantitation Standard signal for each specimen and control. The HBV Quantitation Standard compensates for effects of inhibition and controls for the preparation and amplification processes to allow the accurate quantitation of HBV DNA in each specimen.

Specimen Preparation

The COBAS® TaqMan® HBV Test processes plasma and serum specimens and isolates HBV DNA through a generic manual specimen preparation based on nucleic acid binding to glass fibers. The HBV virus particles are lysed by incubation at an elevated temperature with a protease and chaotropic lysis/binding buffer that releases nucleic acids and protects the released HBV DNA from DNases in plasma and serum. A known number of HBV Quantitation Standard DNA molecules are introduced into each specimen along with the lysis reagent. Subsequently, isopropanol is added to the lysis mixture that is then centrifuged through a column with a glass fiber filter insert. During centrifugation, the HBV DNA and HBV Quantitation Standard DNA are bound to the surface of the glass fiber filter. Unbound substances, such as salts, proteins and other cellular impurities, are removed by centrifugation. The adsorbed nucleic acids are washed and eluted with an aqueous solution. The disposables allow for a parallel processing of 12 specimens or multiples thereof. The processed specimen, containing HBV DNA and HBV Quantitation Standard DNA, is added to the amplification/detection mixture. The HBV target DNA and the HBV Quantitation Standard DNA are then amplified and detected on the COBAS® TaqMan® 48 Analyzer using the amplification and detection reagents provided in the Test kit.

PCR Amplification

Target Selection

Selection of the target DNA sequence for HBV depends on identification of regions within the HBV genome that show maximum sequence conservation among all genotypes. Accordingly, the appropriate selection of the primers and probe is critical to the ability of the test to detect all clinically relevant

genotypes of HBV. A region of the partly single-stranded circular DNA genome of HBV has been shown to have maximum conservation of DNA sequences among genotypes. The COBAS® TaqMan® HBV Test uses PCR amplification primers that define a sequence within the highly conserved pre-Core/Core region of the HBV genome.

Target Amplification

Processed specimens are added to the amplification mixture in amplification tubes (K-tubes) in which PCR amplification occurs. The Thermal Cycler in the COBAS® TaqMan® 48 Analyzer heats the reaction mixture to denature the double stranded DNA and expose the specific primer target sequences on the HBV circular DNA genome and the HBV Quantitation Standard DNA. As the mixture cools, the primers anneal to the target DNA. The thermostable *Thermus specie* Z05 DNA polymerase (Z05) in the presence of Mn2+ and excess deoxynucleotide triphosphates (dNTPs), including deoxyadenosine, deoxyguanosine, deoxycytidine, and deoxyuridine (in place of thymidine), extends the annealed primers along the target template to produce a double-stranded DNA molecule termed an amplicon. The COBAS® TaqMan® 48 Analyzer automatically repeats this process for a designated number of cycles, with each cycle intended to double the amount of amplicon DNA. The required number of cycles is preprogrammed into the COBAS® TaqMan® 48 Analyzer. Amplification occurs only in the region of the HBV genome between the primers; the entire HBV genome is not amplified.

Selective Amplification

Selective amplification of target nucleic acid from the specimen is achieved in the COBAS® TaqMan® HBV Test by the use of AmpErase (uracil-N-glycosylase) enzyme and deoxyuridine triphosphate (dUTP). The AmpErase enzyme recognizes and catalyzes the destruction of DNA strands containing deoxyuridine26, but not DNA containing deoxythymidine. Deoxyuridine is not present in naturally occurring DNA, but is always present in amplicon due to the use of deoxyuridine triphosphate as one of the dNTPs in the Master Mix reagent; therefore, only amplicon contains deoxyuridine. Deoxyuridine renders contaminating amplicon susceptible to destruction by the AmpErase enzyme prior to amplification of the target DNA. Also any nonspecific product formed after initial activation of the Master Mix by

manganese is destroyed by the AmpErase enzyme, thus improving sensitivity and specificity. The AmpErase enzyme, which is included in the Master Mix reagent, catalyzes the cleavage of deoxyuridine-containing DNA at the deoxyuridine residues by opening the deoxyribose chain at the C1-position. When heated in the first thermal cycling step the amplicon DNA chain breaks at the position of the deoxyuridine, thereby rendering the DNA non-amplifiable. The AmpErase enzyme is inactive at temperatures above 55°C, i.e. throughout the thermal cycling steps, and therefore does not destroy target amplicon formed during amplification.

Detection of PCR Products in a COBAS® TaqMan® Test

The COBAS® TaqMan® HBV Test utilizes real-time27,28 PCR technology. The use of dual-labeled fluorescent probes provides for real-time detection of PCR product accumulation by monitoring of the emission intensity of fluorescent reporter dyes released during the amplification process. The probes consist of HBV and HBV Quantitation Standard-specific oligonucleotides labeled with a reporter dye and a quencher dye. In the COBAS® TaqMan® HBV Test, the HBV and HBV Quantitation Standard probes are labeled with different fluorescent reporter dyes. When the dual fluorescent dye-labeled probes are intact, the reporter fluorescence is suppressed by the proximity of the quencher dye due to Förster-type energy transfer effects. During PCR, the probe hybridizes to a target sequence and is cleaved by the 5' → 3' nuclease activity of the thermostable Z05 DNA polymerase. Once the reporter and quencher dyes are released and separated, quenching no longer occurs, and the fluorescent activity of the reporter dye is increased. The amplification of HBV DNA and HBV Quantitation Standard DNA are measured independently at different wavelengths. This process is repeated for a designated number of cycles, each cycle effectively increasing the emission intensity of the individual reporter dyes, permitting independent identification of HBV DNA and HBV Quantitation Standard DNA. The intensity of the signals is related to the amount of starting material at the beginning of the PCR.

Fundamentals of COBAS® TaqMan® HBV Test Quantitation

The COBAS® TaqMan® HBV Test accurately provides quantitative results over a very wide dynamic range since the monitoring of amplicon is

performed during the exponential phase of amplification. The higher the HBV titer of a specimen, the earlier the fluorescence of the reporter dye of the HBV probe rises above the baseline fluorescence level (see Figure 1). Since the amount of HBV Quantitation Standard (QS) DNA is constant between all specimens, the fluorescence of the reporter dye of the HBV QS probe should appear at the same cycle for all specimens. In cases where the QS amplification and detection is affected by inhibition or poor specimen recovery, the appearance of fluorescence will be delayed, thereby enabling the calculated titer of HBV target DNA to be adjusted accordingly. The appearance of the specific fluorescent signal is reported as a critical threshold value (Ct). The Ct is defined as the fractional cycle number where reporter dye fluorescence exceeds a predetermined threshold (the Assigned Fluorescence Level), and starts the beginning of an exponential growth phase of this signal. A higher Ct value indicates a lower titer of initial HBV target DNA. A 2-fold increase in titer correlates with a decrease of 1 Ct for target HBV DNA, while a 10-fold increase in titer correlates with a decrease of 3.3 Ct.

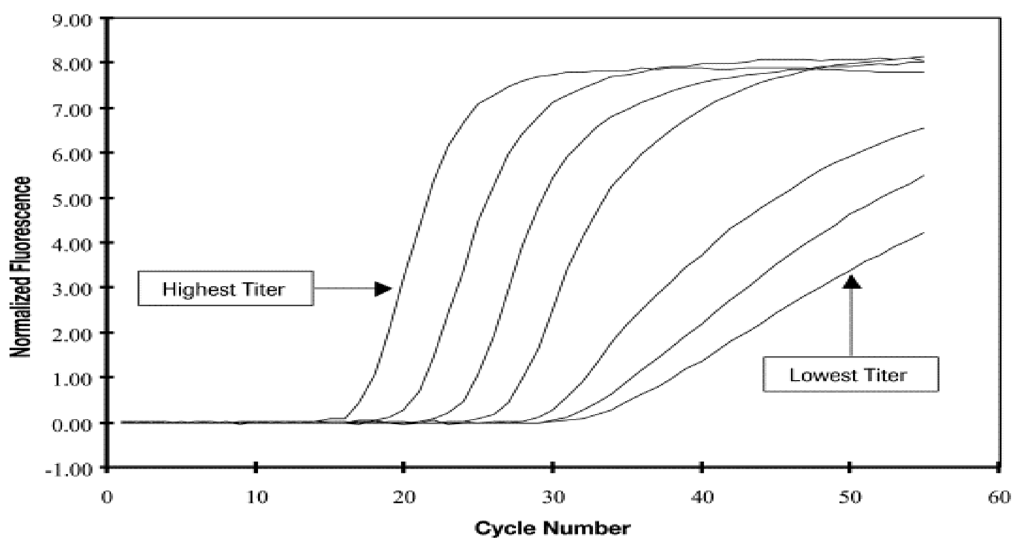


Figure 1 depicts the target growth curves for a dilution series of virus spanning over a 5-log10 range. As the concentration of the virus increases, the growth curves shift to earlier cycles. Therefore the leftmost growth curve corresponds to the highest viral titer level whereas the rightmost growth curve corresponds to the lowest viral titer level.

HBV DNA Quantitation:

The COBAS® TaqMan® HBV Test quantitates HBV viral DNA by utilizing a second target sequence (HBV Quantitation Standard) that is added to each test specimen at a known concentration. The HBV Quantitation Standard is a non-infectious linearized plasmid DNA construct, containing fragments of HBV sequences with primer binding regions identical to those of the HBV target sequence. The HBV Quantitation Standard also generates an amplification product of the same length and base composition as the HBV target DNA. The detection probe binding region of the HBV Quantitation Standard has been modified to differentiate HBV Quantitation Standard amplicon from HBV target amplicon. During the annealing phase of the PCR on the COBAS® TaqMan® 48 Analyzer, the specimens are illuminated and excited by filtered light and filtered emission fluorescence data are collected for each specimen. The readings from each specimen are then corrected for instrumental fluctuations. These fluorescence readings are sent by the instrument to the AMPLILINK software and stored in a database. Pre-Checks are used to determine if the HBV DNA and HBV Quantitation Standard DNA data represent sets that are valid, and flags are generated when the data lie outside the preset limits. After all Pre-Checks are completed and passed, the fluorescence readings are processed to generate Ct values for the HBV DNA and the HBV Quantitation Standard DNA. The lot-specific calibration constants provided with the COBAS® TaqMan® HBV Test are used to calculate the titer value for the specimens and controls based upon the HBV DNA and HBV Quantitation Standard DNA Ct values. The COBAS® TaqMan® HBV Test is standardized against the WHO HBV International Standard for NAT Testing 97/74629 and titer results are reported in International Units (IU/mL).

- **REAGENTS :**

High Pure System Viral Nucleic Acid Kit 48 Tests:

LYS 2 x 25 mL

(Lysis/Binding Buffer)

Tris

50% (w/w) Guanidine-HCl

< 1% Urea

19% (w/w) Triton X-100

CAR 2 x 2 mg

(RNA, lyophilized)

PK 2 x 100 mg

(Proteinase K, lyophilized)

≥ 64% (w/w) Proteinase K, lyophilized

IRB 1 x 33 mL

(Inhibitor Removal Buffer)

Tris

65% Guanidine-HCl

(add 20 mL Ethanol)

WASH 1 x 20 mL

(Wash Buffer)

Tris

NaCl

(add 80 mL Ethanol)

ELB 1 x 30 mL

(Elution Buffer)

RS 4 x each

(High Pure System Viral Nucleic Acid Rack Set)

Lysis Rack

Filter Tube Rack with affixed Waste Rack

Elution Rack

Cover Rack

WR 8 x each

(High Pure System Viral Nucleic Acid Waste Rack)

- **Specimen Preparation and Control Reagents:**

HBV QS 2 x 1.0 mL
(COBAS® TaqMan® HBV Quantitation Standard)
Tris-HCl buffer
EDTA
< 0.001% linearized, double stranded plasmid DNA containing an insert. The DNA insert contains HBV primer binding sequences and a unique probe binding region.
Amaranth dye
< 0.005% Poly rA RNA (synthetic)
0.05% Sodium azide

HBV H(+)C 2 x 1.0 mL
[HBV High (+) Control]
< 0.001% linearized, double stranded plasmid DNA containing HBV sequences.
Negative Human Plasma, non-reactive by US FDA licensed tests for antibody to HCV, antibody to HIV-1/2, HIV p24 antigen and HBsAg; HIV-1 RNA, HCV RNA and HBV DNA not detectable by PCR methods
0.1% ProClin® 300 preservative

HBV L(+)C 2 x 1.0 mL
[HBV Low (+) Control]
< 0.001% linearized, double stranded plasmid DNA containing HBV sequences.
Negative Human Plasma, non-reactive by US FDA licensed tests for antibody to HCV, antibody to HIV-1/2, HIV p24 antigen and HBsAg; HIV-1 RNA, HCV RNA and HBV DNA not detectable by PCR methods
0.1% ProClin® 300 preservative

CTM (–) C 4 x 1.0 mL
[COBAS® TaqMan® Negative Control (Human Plasma)]
Negative Human Plasma, non-reactive by US FDA licensed tests for antibody to HCV, antibody to HIV-1/2, HIV p24 antigen and HBsAg; HIV-1 RNA, HCV RNA and HBV DNA not detectable by PCR methods

0.1% ProClin® 300 preservative

- **Amplification and Detection Reagents:**

HBV MMX 2 x 24 Tests

(COBAS® TaqMan® HBV Master Mix) 2 x 1.4 mL

Tricine buffer

Potassium hydroxide

Potassium acetate

Glycerol

< 0.001% dATP, dCTP, dGTP, dUTP

< 0.001% Upstream and downstream primers to the Pre-Core/Core region of HBV

< 0.001% Fluorescent-labeled oligonucleotide probes specific for HBV and the HBV Quantitation Standard

< 0.001% Oligonucleotide aptamer

< 0.05% Z05 DNA Polymerase (microbial)

< 0.1% AmpErase (uracil-N-glycosylase) enzyme (microbial)

0.09% Sodium azide

CTM Mn2+ 2 x 24 Tests

(COBAS® TaqMan® Manganese Solution) 2 x 1.0 mL

< 1.2% Manganese acetate

Glacial acetic acid

0.09% Sodium azide

2- Humen IL-10 ELISA Max™ Deluxe Set:

- **ELISA reagents and procedure provided Manufacturers' sheath:**

Human IL-10 ELISA MAX™ Deluxe Set

-10 ELISA MAX™ Deluxe Set

04 (5 plates) / 430605 (10 plates) / 430606 (20 plates)

B214546

31-JAN-2018

ts Description	Quantity (5 plates)	Volume (per bottle)	Part No.	Lot No.
uman IL-10 ELISA MAX™ Capture Antibody (200X)	1 vial	300 µL	79029	B210594
Human IL-10 ELISA MAX™ Detection Antibody (200X)	1 vial	300 µL	79030	B210595
Human IL-10 Standard	2 vials	32 ng	79031	B214545
Avidin-HRP (1,000X)	1 vial	60 µL	79004	B210596
Substrate Solution A	1 bottle	30 mL	78570	B210213
Substrate Solution B	1 bottle	30 mL	78571	B210214
Coating Buffer A (5X)	1 bottle	30 mL	79008	B210941
Assay Diluent A (5X)	1 bottle	60 mL	78888	B213262
Nunc™ MaxiSorp™ ELISA Plates, Uncoated	5 plates	-	423501	-

Storage Conditions

1. Unopened set: Store set components between 2°C and 8°C. Do not use this set beyond its expiration date.
2. Opened or reconstituted components:
   - 2.1. Reconstituted standard stock solution can be aliquoted into polypropylene vials and stored at -70°C for up to one month. Avoid repeated freeze/thaw cycles.
   - 2.2 Other components: Store opened reagents between 2°C and 8°C and use within one month.

Note: Precipitation of Assay Diluent A (5X) may be observed when stored long term between 2°C and 8°C. The precipitation does not alter the performance of the assay. If heavy precipitation is observed, it can be filtered to clarify the solution.

Lot #: B214546

Absorbance (450 nm)

10.00

1.00

0.10

0.01

1

10

100

1,000

Human IL-10 (pg/mL)

This standard curve is for demonstrative purposes only.
A standard curve must be run with each assay.

his is to certify that the product was manufactured under stringent process ontrols to ensure lot to lot consistency and complete lot traceability. The roduct has been tested and meets quality control specifications.

ignature: ______ (Quality Control) Date: 2/25/16

ioLegend is ISO 9001:2008 and ISO 13485:2003 Certified

DR RESEARCH USE ONLY

oLegend | 9727 Pacific Heights Blvd | San Diego, CA 92121 U.S.A.

8-5800 | Fax: (877)-455-9587 | biolegend.com

ELISA MAX™ Deluxe Set Protocol

Materials to be Provided by the End-User

1. Phosphate-Buffered Saline (PBS): 8.0 g NaCl, 1.16 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.2 g KCl, add deionized water to 1.0 L, pH to 7.4, 0.2 µm filtered.
2. Wash Buffer: BioLegend Cat. No. 421601 is recommended, or PBS + 0.05% Tween-20.
3. Stop Solution: BioLegend Cat. No. 423001 is recommended, or acid solution, e.g. 2N H_2SO_4.
4. Plate Sealers: BioLegend Cat. No. 423601 is recommended.

Reagent Preparation

Reagents Description	Dilute with	Dilution for 1 plate
Coating Buffer A (5X)	Deionized Water	2.4 mL in 9.6 mL DI H_2O
Capture Antibody (200X)	1X Coating Buffer A	60 µL in 12 mL Buffer
Assay Diluent A (5X)	PBS	12 mL in 48 mL PBS
Detection Antibody (200X)	1X Assay Diluent A	60 µL in 12 mL Buffer
Avidin-HRP (1,000X)	1X Assay Diluent A	12 µL in 12 mL Buffer

Standard reconstitution: Reconstitute the lyophilized Human IL-10 Standard by adding 0.2 mL of 1X Assay Diluent A to make the 160 ng/mL standard stock solution. Allow the reconstituted standard to sit at room temperature for 15-20 minutes, then briefly vortex to mix completely.

To prepare 250 pg/mL top standard, perform an initial 1:10 dilution by adding 10 µL standard stock solution to 90 µL of 1X Assay Diluent A. Then add 15.6 µL to 984.4 µL of 1X Assay Diluent A. Perform six two-fold serial dilutions of the 250 pg/mL top standard with 1X Assay Diluent A in separate tubes. 1X Assay Diluent A serves as the zero standard (0 pg/mL).

Samples: For cell culture supernatant samples, the end user may need to determine the dilution factors in a preliminary experiment. Serum or plasma samples should be tested initially without any dilution. If dilution is required, samples should be diluted in 1X Assay Diluent A before adding to the wells.

TMB Substrate Solution is a mixture of equal volumes of Substrate Solution A and Substrate Solution B. Mix the two components immediately prior to use. For one plate, mix 5.5 mL Substrate Solution A with 5.5 mL of Substrate Solution B in a clean container (solution should be clear and colorless).

ELISA Procedure Summary

Day 1

Add 100 µL diluted Capture Antibody solution to each well, seal the plate and incubate overnight between 2°C and 8°C.

Day 2

1. Wash plate 4 times*, block the plate by adding 200 µL 1X Assay Diluent A to each well, seal the plate and incubate at room temperature for 1 hour with shaking at approximately 500 rpm (with a 0.3 cm circular orbit). All subsequent incubation with shaking should be performed similarly.
2. Wash plate 4 times*, add 100 µL diluted standards and samples to the appropriate wells.
3. Seal the plate and incubate at room temperature for 2 hours with shaking.
4. Wash plate 4 times*, add 100 µL diluted Detection Antibody solution to each well, seal the plate and incubate at room temperature for 1 hour with shaking.
5. Wash plate 4 times*, add 100 µL diluted Avidin-HRP solution to each well, seal the plate and incubate at room temperature for 30 minutes with shaking.
6. Wash plate 5 times*, soaking for 30 seconds to 1 minute per wash. Add 100 µL of freshly mixed TMB Substrate Solution to each well and incubate in the dark for 30 minutes.
7. Add 100 µL Stop Solution to each well. Read absorbance at 450 nm and 570 nm within 15 minutes. The absorbance at 570 nm can be subtracted from the absorbance at 450 nm.

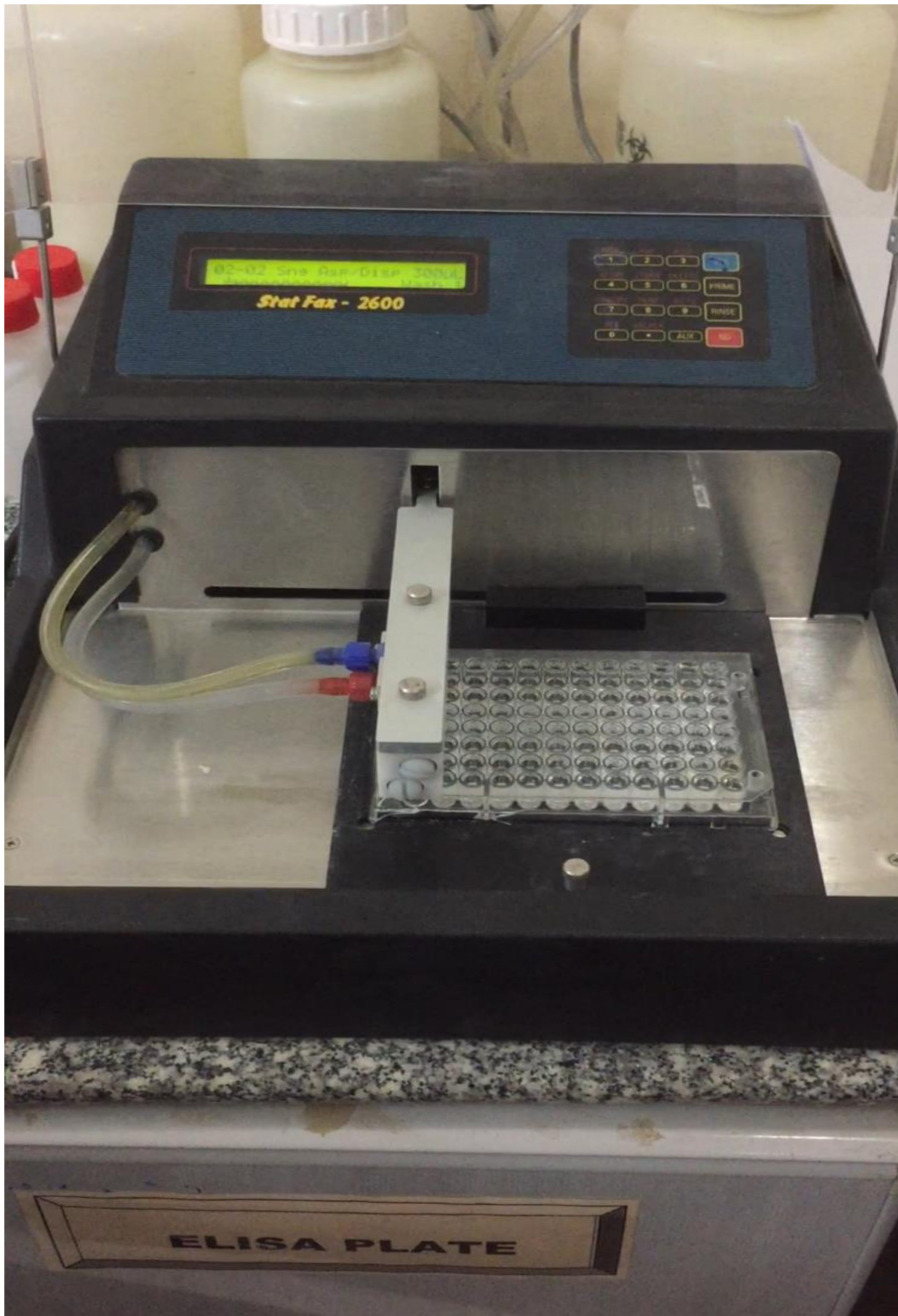
***Plate Washing**: Wash step is crucial to assay precision. Wash the plate with at least 300 µL of Wash Buffer per well and blot any residual buffer by firmly tapping the plate upside down on clean absorbent paper.

For more detailed set information, please refer to the online manual at:
www.biolegend.com/media_assets/pro_detail/datasheets/430604.pdf

BioLegend®

Part No. 78517_V02

- **ELISA washer and micro titer plate during washing steps:**



- **ELISA micro titer plate show the absorbance results of IL-10:**