بسم الله الرحمن الرحيم

Sudan University of Science and Technology

College of Graduate Studies

Serological and Molecular Detection of Hepatitis B virus in Elmanagil District-Algazeira State.

الكشف المصلي والجزيئي لفيروس التهاب الكبد الوبائي في منطقة المناقل-ولاية الجزيره

A Dissertation submitted in partial fulfillment for the requirement of M.Sc degree in Medical Laboratory Science (Microbiology)

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Dedication

To my dears:

Mother

Father

Brothers

And Sisters

Acknowledgments

First of all thanks to **ALMIGHTY ALLAH** for every things, and for giving me chance and power to start and complete this study. Thanks to my supervisor: **Prof. Yousif Fadlalla** for his instruction and suggestion that help me to complete this work with remarkable flexibility. I wish to thank **Suhair Ramadan Rehan** Sudan University of Sciences and Technology for technical help in the laboratory and **Abdalla Ahmed Abdalla** for his help in collecting samples and I wish to thank **Amel Jafar** for materials supplying. Finally I acknowledge after Allah my family, who stimulating me to continue this work and provide me with encouragement and support that make this study possible.

Abstract

The aim of the study was to determine prevalence of Hepatitis B virus among population at Sharafat village on Almanagil province and to compare between ICT and ELISA for surface antigen detection and to detect HBV DNA by PCR. Randomly collected a total of ninety two subjects (n=92) were enrolled in this study. The serum samples were examined by ICT to detect HBsAg, only 6 out of 92 (6.5%) samples were HBsAg positive. ELISA test showed that 30 out of 92 (32.6%) samples were found positive for HBsAg. These 30 samples which was HBsAg positive by ELISA tested by nested PCR and all of them were found positive. The study indicated insignificant (P>0.05) association between HBV infection and risk factors including age, gender, marital status, history of blood transfusion and history of surgical operation. The distribution of positive cases in Gender was 16 (17.4%) of cases males and 14 (15.2%) were females, in Age groups the highest result was (8.7%) in group (5-15) years and there were no infections in age group (56-65), in marital status was 18 (19.6%) married and 12 (13%) were not married, No infections noticed in those had blood transfusion history, in those whose had history of surgical operation it was 9 (9.8%) of cases had history of surgical operation and 21 (22.8%) had no.

The study conclude that prevalence of HBV infection among population at Sharafat village on Almanagil province exceeds the estimated nationwide prevalence. However, that should not ignore this high prevalence and giving special attention to prevent outbreak of hepatitis B.

ملخص الدراسه

كان الهدف من الدراسه هو تحديد نسبة انتشار فيروس التهاب الكبد البائي في مواطني قرية شرفت ريف المناقل في ولاية الجزيره ومقارنه اختبار ICT واختبار ELISA في تشخيص الاصابه بمرض فيروس التهاب الكبد البائي وتحديد الحمض النووي للفيروس عن طريق تفاعل البلمره المتعدد. كان عدد العينات المستخدم في الدراسه (92) جمعت عشوائيا تم فحص عينات المصل بواسطة الشريحه لتحديد البروتين السطحي الفيروس وجد فقط 6 (6.5%) من اصل 92 موجبه ثم اختبرت باستخدام اختبار الايزا حيث كانت النتيجه موجبه ل30 (32.6%)عينه من اصل 92 عينه تم اخدها بطريقه عشوائيه انها مصابه بالفيروس كما تم تاكيد هده النتيجه باستخدام تقنيه تفاعل البلمره المتعدد وجميعها (30) اعطت نتائج موجبه.

كانت (القيمه المعنويه اكبر من 0.05) وتوضح عدم وجود علاقه بين الاصابه والعوامل المؤثره اي ليس هناك ارتباط مع العمر والنوع والحاله الزوبجية ونقل الدم والعمليات الجراحيه, وكان توزيع الحالات الموجبه بين الذكور 16 (17.4%) والاناث 14 (15.2%) حاله وبين الاعمار كانت عالية (8.7%) في المجموعه (5-15) سنه ولم توجد حالة اصابه في المجموعه (56-65), بين المتزوجين وغير المتزوجين كانت نسبة الاصابه 18 (19.6%) و 12(13%) علي التوالي, لم توجد اصابه بين من لهم تاريخ نقل الدم , بين من سبق لهم الخضوع لعمليات جراحيه ومن لم يخضعو لعمليات جراحيه كانت نسبه الاصابه 9 (9.8%) و 21 (22.8%) علي التوالي .

ما تحصلنا عليه من هذه الدراسه ان نسبه الاصابه بفيروس التهاب الكبد الوبائي ب في قريه شرفت ريف المناقل قد زادت كثيرا عن النسب المحدده عالميا لدلك يجب النظر وعدم تجاهل نسبة الانتشارالعاليه (32.6) واعطاء المشكله اهتمام خاص لمنع وتدارك وباء انتشار فيروس الكبد البائي.

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Abbreviations

HBV Hepatitis B virus

ALT Alanine aminotransverase

AST Aspartate aminotransverase

cccDNA Covalently Closed Circular DNA

ICT Immunochromatography test

ELISA Enzyme linked Immune Sorbent Assay

HCC Hepato Cellular Carcinoma

OHB Occult hepatitis B

HRP Horse Radish peroxidase

MHC Major Histocompatibility Complex

SVR Sustain virological response

SPSS Statistical package for Social Science

TMB TetramethyleBenzidine

PCR Polymerase chain reaction

WFI water for injection

DNA Deoxy ribonucleic acid

CHAPTER ONE

INTRODUCTION

CHAPTER ONE

INTRODUCTION

1.1 Background

Hepatitis B, one of the major and common infectious diseases of the liver. Hepatitis B virus (HBV) chronically infects approximately 350 million people worldwide. Without intervention, approximately 15% to 40% of chronically infected individuals will eventually develop cirrhosis, end-stage liver disease or hepatocellular carcinoma, or require liver transplantation. The availability and extensive use of the HBV vaccine has dramatically reduced the number of incident infections in worldwide. Effective therapeutic agents have been and continue to be developed to treat chronic infection. The present review provides a comprehensive overview of diagnostic tests for HBV infection and immunity, and elaborates on HBV risk factors, vaccine prevention and therapeutic monitoring (Krajden *et al.*,2005). Hepatitis means inflammation of the liver. It is most common caused by one of the several viruses, such as hepatitis A virus, hepatitis B virus, hepatitis C virus and other viruses, Toxins, bacterial infections, certain drugs, and heavy alcohol use can also cause hepatitis (Jawetz *et al.*,2007). HBV is transmitted through percutaneous or parenteral contact with infected blood body fluids, and by sexual intercourse (Ganem and Schneider., 2001).

Transmission rates significantly increase if acute infection occurs at or near the time of delivery, with rates reported as high as 60 percent (Sookoian., 2006) . Mother-to-infant transmission represents a basic factor in maintaining chronic HBV infection and usually depends on the degree of maternal infectivity, especially in the prenatal period (Wang *et al.*, 2003). Exposure to the virus varied from 47%–78%, with a hepatitis B surface antigen prevalence ranging from 6.8%

in central Sudan to 26% in Southern Sudan. Studies pointed to infection in early childhood in southern Sudan while there was a trend of increasing infection rate with increasing age in northern Sudan. Hepatitis B virus was the commonest cause of chronic liver disease and hepatocellular carcinoma and was the second commonest cause of acute liver failure in the Sudan (Hatim., 2008).

1.2. Rationale

Hepatitis B virus (HBVs) infection has been recognized as a worldwide health problem. It was estimated that more than two billion people alive today have been infected and 350 million of these suffer from chronic hepatitis B (Krajden *et al.*,2005).

There are numerous of extra hepatic manifestations have been reported in patients with both acute and chronic hepatitis B (arthralgias or arthritis, skin rashes, glomerulonephritis and neuritis), all of which are present in polyarteritis nodosa (PAN) which is the most unique and spectacular extrahepatic manifestation. In the 1970s, the frequency of PAN due to the hepatitis B (HBV) reached 30%. Clinical manifestations reflect this most classic form of PAN, Hepatic manifestations including, ALT (alanine amino trasferase) AST (Aspartate amino tranferase) elevations are mild and usually overlooked.

We conducted this study among population at Sharafat village at Almanagil province on Algazeira State because there were repeated cases from this area. Therefore, this study was aimed to study the prevalence of hepatitis B among population at that area and to identify possible risk factors associated with hepatitis B infection in them.

We chosen ICT in the study because it become used for screening of HBV in most Sudan rural hospital for blood transfusion.

1.3. Objective:

1.3.1. General objective:

To determine the prevalence of hepatitis B virus among population at Sharafat village, province of Almanagil at Algazeira state

1.3.2. Specific objective:

1. To screen for HBV by ICT.
2. To determine the frequency of hepatitis B surface antigen (HBsAg) By sandwich ELISA among population at Sharafat village.
3. To compare between ICT results and ELISA results.
4. To Confirm the ELISA results by PCR.
5. The possible effect of risk factors (age, gender, married, history of surgical operation and blood transfusion).

CHAPTER TWO
LITERATURE REVIEW

CHAPTER TWO

2. Literature Review

2.1 Discovery of the Hepatitis B Virus

The history of modern research on viral hepatitis began in the year 1963, when Nobel Prize winner Baruch S. Blumberg (1925–2011) reported for the first time publicly on the discovery of a new antigen named Australia antigen (AuAg) (Blumberg.,2002). In the years following, AuAg would become the first specific marker of viral hepatitis. Thereafter, viral hepatitis type B became a driving force for the development of modern virus diagnostics and vaccines. This article will recapitulate the major advances in the field of hepatitis B research throughout the last 50 years and point out some perspectives for future research.

2.2. Classification

The hepatitis B virus is classified as the type species of the Orthohepadnavirus, which contains three other species: the Ground squirrel hepatitis virus, Woodchuck hepatitis virus, and the Woolly monkey hepatitis B virus. The genus is classified as part of the Hepadnaviridae family, which contains two other genera, the Avihepadnavirus and a second which has yet to be assigned. This family of viruses have not been assigned to a viral order (Mason*et al.*,2008).

2.3Morphology and genome structure

Elecctron microscopy of HBsAg-positive serum revealed three morphologic forms The most numerous are spherical particles measuring 22 nm in diameter, These small particles are made up exclusively of HBsAg-as are tubular or filamentous forms, which have the same diameter but may be over 200 nm long-and result from overproduction HBsAg. larger,42–nm spherical virions (originally referred to as Dane particle) are less frequently observed. The outer surface, or envelope, contains HBsAg and surrounds a 27-nm inner nucleocapsid core that contains

HBcAg.The variable lengthof a single-stranded region of the circular DNA genome results in genetically heterogeneous particles with a wide range of buoyant densities **(**Jawetz*et al* .,2010).

2.3.1. Structure

The HBV core contains besides the HBV genome the HBV polymerase with the primase (pr) and the reverse transcriptase (RT) domainand the cellular protein kinase C alpha (PKC). Note there are two start codons in the PreC/core ORF and the three start codons in the HBs ORF. **(**National ReferenceCenter for Hepatitis B and D,2013).

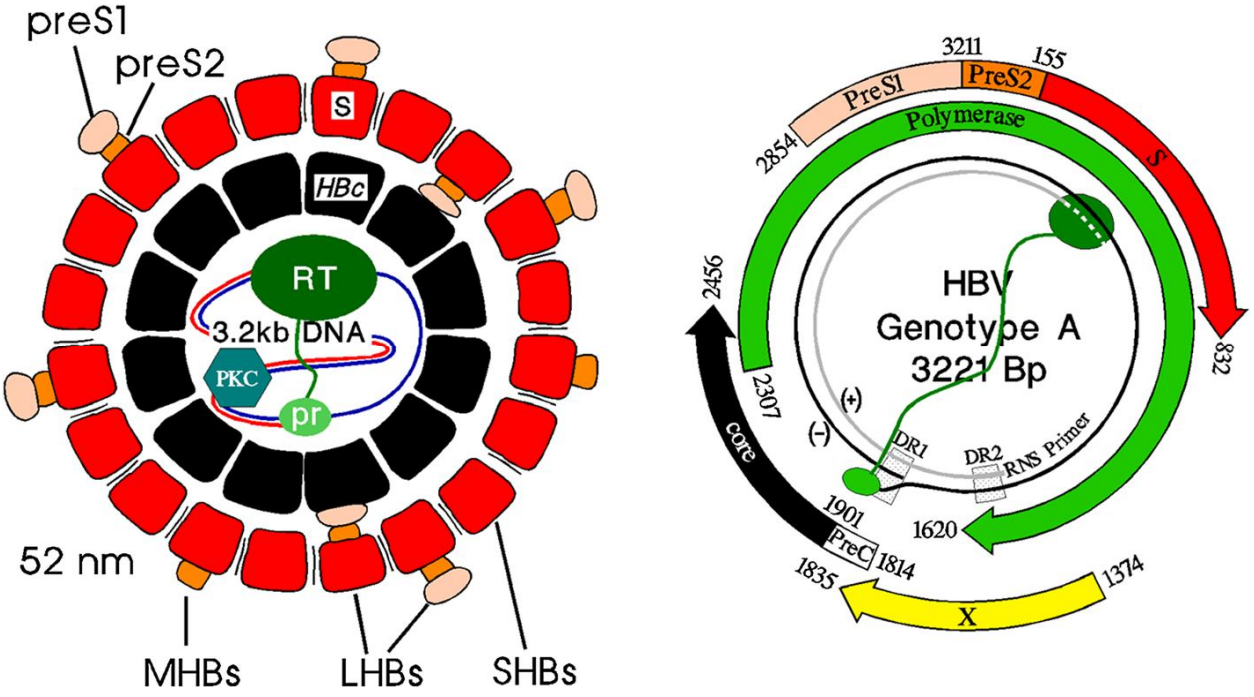


Fig. 2.1: **TheStructural components of HBV (**left**) and open reading frames (OR) for encoding proteins in the covalently closed form of HBV DNA**(right).**(**National ReferenceCenter for Hepatitis B and D,2013).

2.4. Evolution

Attempts have been made to relate these observations to HBV evolution. Much of our knowledge about human hepatitis has relied heavily upon information derived from infection (natural and experimental) of non-human primates during the last 50 years. However, there seem to be some difficulties due to the relationship between the human genotypes A–E and G (and even more divergent genotype F) to each other and to other primate-associated genotypes. The origin of HBV in humans is as confusing as that of the hepadna viruses from other primates. Different theories have been proposed by investigators on HBV origins based on the hypothesis that the numbers of nucleotide and amino-acid substitutions over time, the molecular clocks, are indicators of viral evolution (Mizokami and Orito., 1999). A primate origin for HBV infection was proposed by MacDonald. This theory based on the finding of variants in chimpanzees (MacDonald*et al.*, 2000) woolly monkeys (Lanford*et al.*, 1998) and orangutans (Warren *et al.*, 1999), suggested that these viruses co-evolved with their primate hosts over periods of 10–35 million years. This hypothesis has been supported by the observations that areas of high HBV prevalence in humans are those in which contact with, and cross-species transmission from primates are most likely (South America, Sub Saharan Africa and Southeast Asia). Indeed, certain HBV genotypes are specific to these three areas (F, E, B/C, respectively). Moreover, the mixture of HBV genotypes found outside these areas, such as Europe and North America, may have resulted from much more recent epidemic spread (Simmonds, 2001). In contrast, a recent emergence hypothesis for HBV infection indicated that the current wide distribution of HBV in Apes must have arisen through several cross-species or subspecies transmission in the relatively recent past (Starkman *et al.*,2003). Based on observation of Norder *et al*. that most of the dendrograms obtained from Gibbon and Chimpanzee strains represented early lineages, assumptions were

made that these viruses were indigenous to their respective hosts and not recent acquisitions from man (genotype F). Therefore, they suggested that either genotype F represents an early cross-species transfer from a non-Ape primate to man, or that a hepadna virus of a common ancestor to man and Apes gave rise to two viral lineages (Norder *et al.*, 1996). Thus, they proposed that the evolutionary history of HBV corresponds to the spread of anatomically modern humans as they migrated from Africa 100 000 years ago (Norder *et al.*, 1994). and different genotypes infecting humans evolved since this dispersal. However, this hypothesis does not explain the origin of the various non-human primate viruses which are interspersed among the human genotypes in the phylogenetic tree. The phylogenetic tree of the various primate HBV variants in now day reflects the phylogeny of the host species, as would be expected for co-speciation (Simmonds , 2001). For example the presence of genotype F in native American populations is inconsistent with the presence of genotype B and C in their genetically nearest relatives, the Mongoloid Northeast Asian. Indeed, there is little relationship between HBV genotype distribution with any of the other human population groups (Southeast Asian, Caucasians, and African population). Alternatively, the HBV genotypes may have evolved later than, and independent of, human migration (Gunther *et al.*, 1999). According to the finding that HBV showed a nucleotide substitution rate of 2.1 · 10)5 substitutions per site per year over a mean observation period of 22 years, Orito proposed that the human genotypes of HBV would have originated from a common ancestor approximately 3000 years ago (Orito*et al.*,1989). In their study, they showed that three major clusters of HBV (birds, mammals and humans) diverged from their common origin in the same order as that of host evolution. They concluded that the evolution of the hepadna virus family was independent of host-species divergence and for HBV in humans this has taken place much more recently than has divergence of humans. Alternatively, a New World origin for

HBV was proposed by Bollyky. who suggested that HBV originated from the Americas and spread into the Old World over the last 400 years after contact from Europeans during colonization; a genotype F origin. Further, they considered the possibility that if the virus originally entered the Americas from Asia, this may have required a higher rate of nucleotide substitution as it adapted to this naive human population (Bollyky and Holmes ., 1999). However, the main problem for this hypothesis is the observation of the widespread distribution of HBV in Old World primate species. A remarkable example is a shared genotype of HBV infecting West African chimpanzees (Takahashi*et al.*, 2000), which showed approximately 11% divergence from the human genotypes A–E. This finding was based on analysis of mutations in the C-terminus region of the core protein (which is well conserved among hepadna viruses) between human genotypes E/F and the chimpanzee one. Interestingly, HBV-E/F and the non-human primate hepadna viruses had a common motif within 20 nucleotides upstream from the stop codon for the core gene, whereas, HBV-A/B/C/D genotypes contained a different motif at this site. It has been revealed that sequences in wild-born Old World primates from Africa and Southeast Asia were unrelated to five human HBV strains (A–E); the conclusion is that the virus was not acquired from humans, and all the Old World non-human primate HBVs were on a common ancestral branch (Robertson , 2001).

This finding together with the observation that the closest relative of the woolly monkey HBV is genotype F, led to the speculation that chimpanzees have their own hepadna virus, which resembles the human hepadna virus (genotype F). At present, the problems associated with each of these hypotheses for the origin of HBV prevent a definitive conclusion. Resolution of these issues requires more extensive sequence analysis of HBV in poorly sampled areas as well as combined human and primate studies together with utilizing models of DNA substitution which better describe the process of viral evolution.

2.5. Genome

The viral genome consists partially of double-stranded circular DNA , 3200bp in length. Different HBV isolates share 90-98 % nucleotide sequence homology. The full-length DNA minus strand (L or long strand) is complementary to all HBV mRNAs; the positive strand (S or short strand) is variable and between 50% and 80% of unit length.

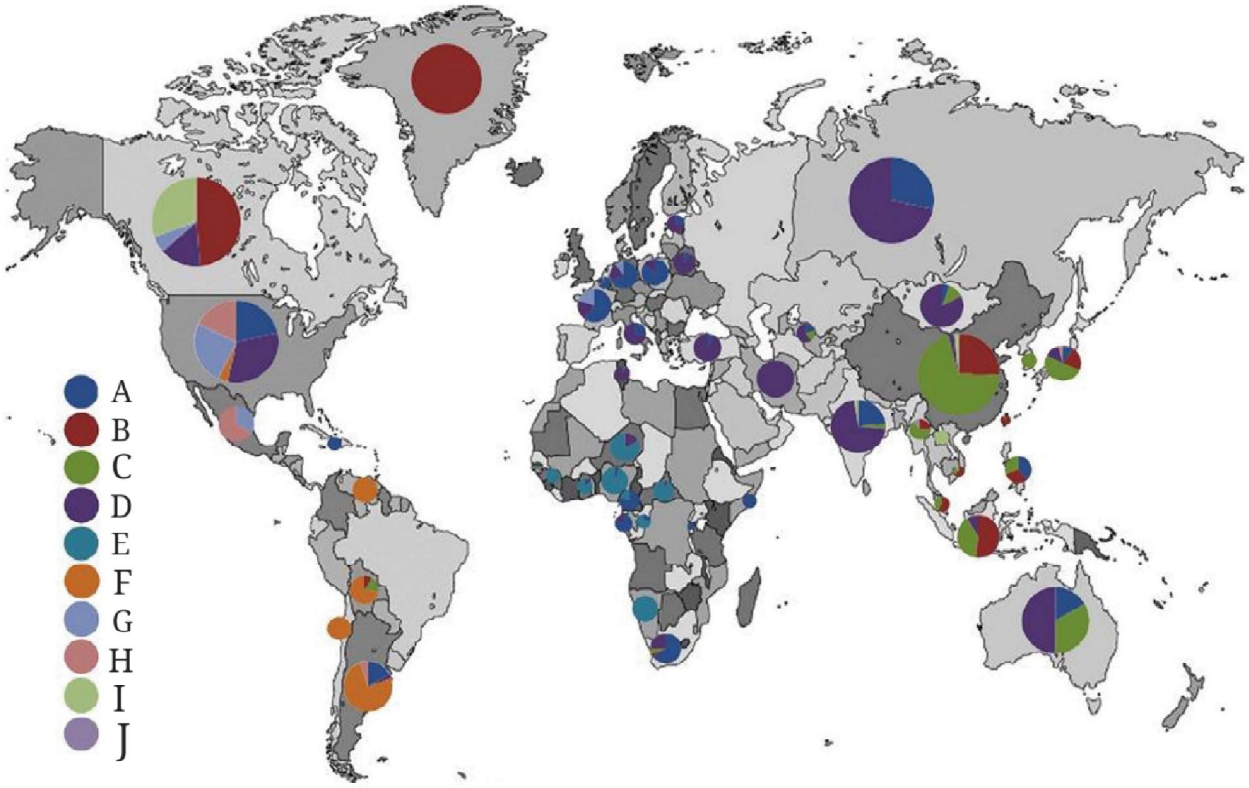
There are four open reading frames that encode seven polypeptides. These include structural proteins of the virion surface and core, a small transcriptional transactivator (X), and a large polymerase (P) protein that include DNA polymerase, reverse transcriptase, and RNase H activities. The S gene has three in-frame initiation codons and encodes the major HBsAg, as well as polypeptide containing in addition pre-S2 or pre-S1and pre-S2 sequences. The C gene has two in-frame initiation codons and encodes HBcAg plus the HBe protein, which is processed to produce soluble HBeAg.The particles containing HBsAg are antigenically complex. Each contain group-specific antigen, a, in addition to two pairs of mutually exclusive subdeterminanats, d/y and w/r. Thus, four phenotypes of HBsAg have been observed: adw, ayw, adr, and ayr.**(**Jawetz *et al,* 2010).

2.5.1. Genotypes

HBV is differentiated into many genotypes, according to genome sequence. To date, eight well-known genotypes (A-H) of the HBV genome have been defined. Moreover, two new genotypes, I and J, have also been identified. Some HBV genotypes are further classified as sub-genotypes. HBV sequence is characterized by > 8% nucleotidedifferences for genotype, and 4%-8% nucleotide differences for sub-genotype. Over 30 related sub-genotypesbelonging to HBV genotypes have

been determined to date, but the mechanisms of different pathogenic characteristics of HBV genotypes are not known for certain. Many studies have reported that different genotypes and sub-genotypes show different geographical distribution, and are related to disease progression, clinical progression, response to antiviral treatment, and prognosis. A-D and F genotypes are divided into various sub-genotypes; no sub-genotypes have been defined for E, G and H genotypes.

Genotype A is widespread in sub-Saharan Africa, Northern Europe, and Western Africa; genotypes B and C are common in Asia; genotype C is primarily observed in Southeast Asia; genotype D is dominant in Africa, Europe, Mediterranean countries, and India; genotype G is reported in France, Germany, and the United States; and genotype H is commonly encountered in Central and South America. Genotype I has recently been reported in Vietnam and Laos. The newest HBV genotype, genotype J, has been identified in the Ryukyu Islands in Japan. Geographic distribution of HBV genotypes may be related to route of exposure. For example, genotypes B and C are more common in high-endemic regions of perinatal or vertical exposure, which plays an important role in viral transmission. Other genotypes are primarily observed in regions of horizontal exposure (Sunbul, 2014). Therefore, genotyping provides an epidemiological clue in the investigation of acquisition, because this lies in the geographical distribution of HBV. Figure 2.2 shows genotype distribution across the world.



Figer 2.2**Distribution of hepatitis B virus genotypes worldwide.** Permission for Figure has been granted by the Publisher, from (Shi *et al.*, 2013).

2.6. Life cycle

. Attachment to liver-specific receptors (heparansulfate proteoglycan and NTCP, leads to endocytosis of HBV and release of HBV core particles. These are transported to the nucleus and arrested at the nuclear pore complex where the HBV genome is released to the nucleus. In the nucleus, the viral DNA is “repaired” to the covalently closed circular (ccc) DNA and complexed with nucleosomes (not shown). In interaction with transcription factors (not shown), the ccc DNA is transcribed to the pregenomic and subgenomic mRNAs. The mRNAs are transported, mainly without splicing, to the cytoplasm. The two subgenomic mRNAs for the three HBs proteins are translated at the endoplasmic reticulum, assemble to subviral HBsAg particles and are secreted via the Golgi apparatus. In

parallel, the pregenomic mRNA is translated in the cytosol to the HBV core protein and the viral polymerase, whereby the three components assemble to the immature core particle. The HBV genomes mature within the core particles via reverse transcription of the pregenomic mRNA to DNA. The mature core particles can migrate again to the nuclear pore complex or are enveloped by the surface proteins and secreted via the multivesicular bodies (MVB). (National Reference Center for Hepatitis B and D, 2013).

2.7.Stability

It is difficult to assess the stability of HBV due to the lack of suitable laboratory culture system (Greenwood *et al.,* 1996).

Indirect evidence has been obtained from the study of recipients of blood products treated in various ways and chimpanzee inoculation experiments. Thus it was established that:-Heating to 600 C for 10 hours inactivates the virus also Treatment with hypochlorite. Or 2% glutaraldehyde for 10 min will inactivate the virus.

Studies based on the survival of HBs Ag show that it is much more resistant to destruction (Greenwood *et al.,* 1996).

2.8.Incidence/Epidemiology

Viral hepatitis caused 1.34 million deaths in 2015, Globally, in 2015, an estimated 257 million

people were living with chronic HBV infection ,The epidemic caused by HBV affects mostly

the WHO African Region and the Western Pacifi c Region. Access to affordable hepatitis testing is limited. Few people with viral hepatitis have been diagnosed (9% of HBV-infected persons, 22 million) Among those diagnosed, treatment has

reached only a small fraction. In 2015, 8% of those diagnosed with HBV infection or 1.7 million persons were on treatment.

Among the 36.7 million persons living with HIV in 2015, an estimated 2.7 million had chronic HBV infection Liver diseases are a major cause of morbidity and mortality among those living with HIV and co-infected with viral hepatitis. These people should be diagnosed and provided with appropriate and effective treatment for both HIV and hepatitis as a priority(WHO, 2017).

2.9. HBVin Sudan

Sudan is classifi ed among countries with a high hepatitis B surface antigen (HBsAg) endemicity of more than 8%. Exposure to HBV infection ranges from 47% to 78% with a hepatitis B surface antigen (HBsAg) seroprevalence ranging from as low as 6.8% in central Sudan to as high as 26% in southern Sudan. Identifi ed risk factors for HBV infection in Sudan include living in southern Sudan, parenteral antischistosomal therapy, sexual promiscuity, and scarifi cation which is a common ritual in southern Sudan. There was no association with schistosomal infection or blood transfusion, These rates are comparable to some African countries where seroprevalence of HBsAg was reported at rates of 15.6% in Burundi, 14% in Central African Republic, and 10% in Uganda. Lower rates were however found in other countries such as Tanzania (4.4%), Nigeria (4.98%), and Ethiopia (7%). Seroprevalence of HBsAg among asymptomatic blood donors ranged from 12.3% in southern Sudan to 17.5% in central Sudan.

These studies were carried out in the eighties and nineties when screening of blood and blood products was only done in a few blood banks in the capital, Khartoum. In 2002, a national program for screening blood and blood products for HBV and HCV infection was introduced throughout the whole country.

A high seroprevalence of HBsAg was detected in patients with liver cirrhosis ranging from 31%–61% and similar carrier rates of 43%–60% were found in patients with HCC, indicating that HBV infection is perhaps the commonest risk factor for developing HCC(Mudawi, 2008).

2.10. Transmission of HBV

The hepatitis B virus can survive outside the body for at least 7 days. During this time, the virus can still cause infection if it enters the body of a person who is not protected by the vaccine. The incubation period of the hepatitis B virus is 75 days on average, but can vary from 30 to 180 days. The virus may be detected within 30 to 60 days after infection and can persist and develop into chronic hepatitis B. In highly endemic areas, hepatitis B is most commonly spread from mother to child at birth (perinatal transmission), or through horizontal transmission (exposure to infected blood), especially from an infected child to an uninfected child during the first 5 years of life. The development of chronic infection is very common in infants infected from their mothers or before the age of 5 years. Hepatitis B is also spread by percutaneous or mucosal exposure to infected blood and various body fluids, as well as through saliva, menstrual, vaginal, and seminal fluids. Sexual transmission of hepatitis B may occur, particularly in unvaccinated men who have sex with men and heterosexual persons with multiple sex partners or contact with sex workers. Infection in adulthood leads to chronic hepatitis in less than 5% of cases. Transmission of the virus may also occur through the reuse of needles and syringes either in health-care settings or among persons who inject drugs. In addition, infection can occur during medical, surgical and dental procedures, through tattooing, or through the use of razors and similar objects that are contaminated with infected blood.(WHO,2016).

2.11. Pathogenesis and Immunity

The adaptive immune response is thought to be responsible for viral clearance and disease pathogenesis during hepatitis B virus infection. It is generally acknowledged that the humoral antibody response contributes to the clearance of circulating virus particles and the prevention of viral spread within the host while the cellular immune response eliminates infected cells. The T cell response to the hepatitis B virus (HBV) is vigorous, polyclonal and multispecific in acutely infected patients who successfully clear the virus and relatively weak and narrowly focussed in chronically infected patients, suggesting that clearance of HBV is T cell dependent. The pathogenetic and antiviral potential of the cytotoxic T lymphocyte (CTL) response to HBV has been proven by the induction of a severe necroinflammatory liver disease following the adoptive transfer of HBsAg specific CTL into HBV transgenic mice. Remarkably, the CTLs also purge HBV replicative intermediates from the liver by secreting type 1 inflammatory cytokines thereby limiting virus spread to uninfected cells and reducing the degree of immunopathology required to terminate the infection. Persistent HBV infection is characterized by a weak adaptive immune response, thought to be due to inefficient CD4+ T cell priming early in the infection and subsequent development of a quantitatively and qualitatively ineffective CD8+ T cell response. Other factors that could contribute to viral persistence are immunological tolerance, mutational epitope inactivation, T cell receptor antagonism, incomplete down-regulation of viral replication and infection of immunologically privileged tissues. However, these pathways become apparent only in the setting of an ineffective immune response which is, therefore, the fundamental underlying cause. Persistent infection is characterized by chronic liver cell injury, regeneration, inflammation, widespread DNA damage, and insertional deregulation of cellular growth control

genes which, collectively, lead to cirrhosis of the liver and hepatocellular carcinoma. (Chisari *et al.*,2010).

2.12. Clinical picture

HBV infection leads to a wide spectrum of liver disease ranging from acute hepatitis (including fulminant hepatic failure) to chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC).

The diagnosis of HBV infection and its associated disease is based on a constellation of clinical, biochemical, histological, and serologic findings. (Hollinger *et al.*, 2001).

2.12.1 Acute hepatitis

The incubation period is 1-6 months in the acute phase of hepatitis B infection. Anicteric hepatitis is the predominant form of expression for this disease. The majority of the patients are asymptomatic, but patients with anicteric hepatitis have a greater tendency to develop chronic hepatitis. Patients with symptomatology have the same symptoms as patients who develop icteric hepatitis.

Icteric hepatitis is associated with a prodromal period, during which a serum sickness –like syndrome can occur. The symptomatology is more constitutional and includes the following:

- Anorexia
- Nausea
- Vomiting
- Low-grade fever
- Myalgia

- Fatigability
- Disordered gustatory acuity and smell sensations (aversion to food and cigarettes)
- Right upper quadrant and epigastric pain (intermittent, mild to moderate)

Patients with fulminant and subfulminant hepatitis may present with the following:

- Hepatic encephalopathy
- Somnolence
- Disturbances in sleep pattern
- Mental confusion
- Coma
- Ascites
- Gastrointestinal (GI) bleeding
- Coagulopathy (Nikolaos ,2016).

2.12.2. Chronichepatitis B infection

Between 350 million and 400 million persons worldwide are chronically infected with hepatitis B virus (HBV). (Lavanchy , 2004) The two primary adverse outcomes of chronic infection are hepatocellular carcinoma (HCC) and cirrhosis, either of which can lead to a liver-related death.

2.12.2.1.Phases Of Chronic hepatitis B infection

Table 2.1. Phases of Chronic Hepatitis B Infection

Immune Tolerant Phase
• Occurs primarily after perinatal infection from HBsAg/HBeAg-positive mother
• ALT levels are normal
• HBV DNA _ 200,000 IU/mL (_1 million copies), often above 107-8 IU/mL
• Liver biopsy is normal or shows only minimal inflammation with no or minimal fibrosis
• Occurs most frequently in HBV genotype C infection
Immune Active (Clearance) Phase
• HBeAg-positive chronic hepatitis B
• Elevated ALT levels
• HBV DNA _ 20,000 IU/Ml
• Anti-HBe–positive chronic hepatitis B
• Elevated ALT
• HBV DNA _ 2000 IU/mL
• Hepatic inflammation with or without fibrosis on biopsy often present in both HBeAg-positive and HBeAg-negative immune active phase
Inactive Phase
• Anti-HBe
• ALT levels normal
• HBV DNA _ 2000 IU/mL
• Hepatic inflammation minimal or absent
• Hepatic fibrosis may improve over time
• HBsAg clearance may eventually occur

http://webdev.med.upenn.edu/contribute/gastro/documents/HepatologyHBVnaturalhistory2009.pdf

2.12.3. HBV and hepatocellular carcinoma (HCC)

The risk of hepatocellular carcinoma (HCC) increases with increasing level of hepatitis B virus (HBV) in serum (viral load). However, it is unclear whether

genetic characteristics of HBV, including HBV genotype and specific genetic mutations, contribute to the risk of HCC. We examined the HCC risk associated with HBV genotypes and common variants in the precore and basal core promoter (BCP) regions.HBV genotype C and specific alleles of BCP and precore were associated with risk of HCC. These associations were independent of serum HBV DNA level (Yang *et al.*,2008).

2.12.4. Persistence of HBV

Only a small portion of patients can undergo HBsAg seroclearance with antiviral therapy; this response has been associated with several host and viral factors. Unfortunately, viral rebound often occurs following discontinuation of antiviral therapy. Accumulating evidence has proven that the persistence of covalently closed circular DNA (cccDNA) is the major barrier to eradicating chronic HBV infection.8 In addition, persistent cccDNA can occasionally be detected even in the liver of patients with resolved hepatitis B infections. More importantly, the persistent cccDNA appears to be replication competent at least in a significant portion of these patients, as evidenced by the fact that HBV reactivation occurs when they receive intensive immunosuppressive agents, such as chemotherapy (Hung-Chih and Jia-Horng, 2014).

2.13. Laboratory Diagnosis

2.13.1. Collection, transport and storage of sample

HBV antigen or antibodies are stable at room temperature for several hour to days, can bestored at -20 ˚C to – 70˚C for many years. Repeat freezing and thawing can lead todegradation of HBV serologic markers .furthermore ,use of hemolyzed sample should beavoided as interferes with detection signals .plasma is separated

from blood collected incontainers with EDTA or citrate dextrose as the anticoagulant (Horvart andTegtmeier,2011).

2.13.2.Diagnosis

Diagnosis of hepatitis is made by:

2.13.2.1.Biochemical assessment of liver function. Initial laboratory evaluation should include:

Total and direct bilirubin, ALT, AST, alkaline phosphatase, prothrombin time, total

protein, albumin, serum globulin, complete blood count, and coagulation studies. (Hollinger *et al*., 2001).

2.13.2.2.Diagnosis is confirmed by demonstration of specific Antigens:

Three clinical useful antigen systems have been identified for hepatitis B:

Hepatitis B surface antigen **(HBsAg)**

Hepatitis Bcore antigen**(HBcAg)**

Hepatitis B e antigen **(HBeAg)**

HBsAg can be detected in the serum from several weeks before onset of symptoms tomonths after onset. HBsAg is present in serum during acute infections and persists inchronic infections. The presence of HBsAg indicates that the person is potentiallyinfectious (Mahoney and Kane, 1999).

Acute hepatitis patients who maintain a constant serum HBsAg concentration, or whoseserum HBeAg persists 8 to 10 weeks after symptoms have resolved, are likely to becomecarriers and at risk of developing chronic liver. The presence of HBeAg is associated with relatively high infectivity and severity of disease.

Antibodies : Three clinical useful antibody systems have been identified for hepatitisB**:**

Antibody to HBsAg **(anti-HBs)** Anti-HBs replaces HBsAg as the acute HBV infection isresolving. Anti-HBs generally persists for a lifetime in over 80% of patients and indicatesimmunity. (Lavanchy,2004).

Antibody **(anti-HBc IgM and anti-HBc IgG)** is the first antibody to appear.

Demonstration of anti-HBc in serum indicates HBV infection, current or past. IgM anti-HBc is present in high titre during acute infection and usually disappears within 6months, although it can persist in some cases of chronic hepatitis. This test may thereforereliably diagnose acute HBV infection. IgG anti-HBc generally remains detectable for alifetime (Robinson ,1995).

Antibody to HBeAg **(anti-HBe)** Anti-HBe appears after anti-HBc and its presence correlates to a decreased infectivity. Anti-HBe replaces HBeAg the resolution of the disease (Mahoney and Kane, 1999) .

2.13.2.3.Tests specific for complete virus particles or DNA:

DNA polymerase-containing virions, and for hepatitis Delta antigen (HDAg) and hepatitis Delta virus (HDV) RNA inliver and serum are available only in research laboratories (Hollinger *et al*., 2001)

2.13.2.4.Table 2; Interpretation of the Hepatitis B Panel Tests Results Interpretation

Hepatitis B Virus Serological and Virological Markers

• **HBsAg** HBV infection, both acute and chronic
• **HBeAg** High-level HBV replication and infectivity; marker for treatment response
• **HBV DNA** Level of HBV replication; primary virologic marker for treatment response
• **Anti-HBc** (**IgM**) Acute HBV infection; could be seen in flare of chronic hepatitis B
• **Anti-HBc** (**IgG**) Recovered or chronic HBV infection
• **Anti-HBs** Recovered HBV infection or marker of HBV vaccination; immunity to HBV infection (titer can be measured to assess vaccine efficacy).
• **Anti-HBe** Low-level HBV replication and infectivity; marker for treatment response
• **Anti-HBc** (**IgG**) and **anti-HBs** Past HBV infection; could lose anti-HBs
• **Anti-HBc** (**IgG**) and **HBsAg** Chronic HBV infection
• **Anti-HBc** (**IgG**) and/or anti-HBs and HBV DNA (PCR) Latent or occult HBV infection

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2809016/table/T1/?report=objectonl

2.14. Prevention

The Advisory Committee on Immunization Practices (ACIP) recommends screening pregnant women (including women previously vaccinated or previously tested) for HBsAg during the first prenatal visit of each pregnancy.

Unvaccinated HBsAg-negative pregnant women at continuing risk for HBV exposure should initiate vaccination during pregnancy. Women at risk include

those who are household contacts or sex partners of HBsAg-positive persons, injection drug users, have endstage renal disease, HIV infection, chronic liver disease, diabetes, or other factors. Women who are not screened prenatally, or who continue to be at risk for HBV infection, should be screened or rescreened at presentation for delivery (Noele, 2014).

2.15. Vaccination

Hepatitis B vaccine is produced by recombinant DNA technology, most commonly in yeast.The complete vaccination series consists of three doses of vaccine; the first two doses are usually given 1 month apart, with the third dose 1–12 months later. The WHO recommended schedule for hepatitis B immunization of children consists of a dose within 24 hours of birth followed by a second and third dose of hepatitis B containing vaccines at intervals of at least 4 weeks. A complete series of immunization provides protection for at least 25 years and, according to current scientific evidence, probably for life. Boosters are not recommended for routine immunization programs. Because of the prolonged incubation period of hepatitis B, some protection will be afforded to most travelers following the second dose given before travel however; the final dose should always be give.

A combination vaccine that provides protection against both hepatitis A and hepatitis B should be considered for travelers who may be exposed to both organisms. This inactivated vaccine is administered as follows: day 0; 1 month; 6 months. A rapid schedule of day 0, 1 month and 2 months with an additional dose at 12 months, and a very rapid schedule of day 0, day 7 and day 21 with a booster dose at 12 months, have been proposed by the vaccine manufacturer and approved by national regulatory authorities in some countries. Recommended for Hepatitis B vaccine should be considered for all non-immune individuals travelling to

countries or areas with moderate to high risk of infection. It can be administered to infants from birth (Global Health Report ,2017).

2.16. Treatment

Currently, seven drugs are available: IFN-α, pegylated interferon, lamivudine, adefovir dipivoxil, entecavir, telbivudine and tenofovir. The choice of the drugs should always take into consideration the clinical features of patients, the antiviral efficacy of each drug, the risk of developing resistance, the long-term safety profile, the method of administration and the cost of therapy. Ideal candidates for treatment are hepatitis B e antigen-positive patients with a prolonged phase of immune clearance and hepatitis B e antigen-negative patients with elevated levels of serum HBV DNA, abnormal alanine aminotransferase and histologic evidence of moderate or severe liver necroinflammation and/or fibrosis. NUCs suppress viral replication by inhibiting HBV viral polymerase, whereas interferon therapy works by enhancing the host immune response. The two main treatment strategies are finite therapy with interferon or NUC therapy (for those who maintain an SVR off treatment), or long-term therapy with one or more NUCs, for those with cirrhosis or who do not maintain an SVR sustained virologic response(Di Marco and Craxi, 2009).

CHAPTER THREE
MATERIALS AND METHODS

CHAPTER TREE

Materials and Methods

3.1. Study design

This study was descriptive, cross-sectional and analytical based study.

3.2. Study population

Population with different ages, were the source of samples.

3.3. Study area

The study was conducted at Almanagil province, Sharafat village.

3.4. Sampling

Sampling Technique: Simple random sample

Sample size: A total of ninety two subjects (n=92) were enrolled in this study.

3.5. Selection criteria

3.5.1. Inclusion criteria

Population live at Sharafat countryside Almanagil.

3.5.2Exclusion criteria

Children below 5 years were excluded.

3.6. Ethical consideration

Each individual invited to participate in the study was informed about the purpose of Study before data collection. The study was approval by Ethical committee, College of Medical Laboratory Science, Sudan University of Science and Technology

3.7 Data collection

The data was collected by structured questionnaire(Appendix).

3.7.1. Interview :

An interview with questionnaire to obtain the clinical data was used for each participantin this study.

3.7.2 Blood samples collection

The area of blood collected was disinfected by (70%) Alcohol. Venous blood (3ml) were taken from each participant by standard procedures and put in plain containers then left to clot for 15 minutes and then centrifuged at 1500 rpm for 5 minutes. The serum was separated and kept at -20°C ˚until used.

3.7.3 Methods

3.7.3.1 Detection of HBsAg ByICT

The ICT tests were produced from ACON laboratories. San Diego, USA (Appendix) and supplied as follows:

a) Test devices contain anti-HBsAg particles coated on the membrane.

b) Disposable specimen dropper.

Materials required but not provided

a) Specimen collection containers.

b) Pipette and disposable tips.

c) Centrifuge

d) Timer

Storage and stability

The kits were stored at room temperature or refrigerated (2–30°C). The test deviceswere kept in the sealed pouch until used. The expiry date was observed.

ICT test procedure:

Specimens (serum) were allowed to reach room temperature (20–25°C)before running the test. Frozen specimens were completely thawed and mixed well priorto testing. The test devices were removed from the sealed pouch and placed on clean andlevel surface and used immediately and the test was performed within one hour. Then 3 fulldrops of serum (approx. 100 µl) were transferred into specimen well (S)

of the testdevice. Results were read after 10 minutes as indicated by the appearance of the red lines.

Interpretation of results:

a) Positive test: Two distinct redlines appear, one line should be in the control region© and another line should be in the test region (T).

b) Negative test: When one redline appears in the control test region (T).

c) Invalid test: If no line appeared on the positive control region (C).

3.7.3.2 Detection of HBsAg by Sandwich Enzyme linked Immune-Sorbent Assay (ELISA)

Principle:

The test is an enzyme-immunoassay based on a sandwish principle .polystyrene microtiter strip wells have been coated with monoclonal anti-HBs(antibody toHBsAg). Patient serum sample is added to microwells. During incubation, the specific immune-complex formed in case of presence of HBsAg in the sample, is captured on the solid phase. After washing to remove serum proteins, second antibody conjugated to the enzyme HRB and directed against a different epitope of HBsAg is adde to the wells. During the second incubation step, these HRB conjugated antibodies will be bound to any anti-HBs-HBsAg complexes previously formed during the first incubation, and the unbound HRB conjugate is then removed by washing. After washing to remove unbound HRB conjugate, chromogen solutions containing TMB and urea peroxidase are added to the wells in presence of antibody-antigen-antibody HRB sandwish immune-complex, the colourless chromogens are hydrolyzed by the bound HRBconjugate to a blue coloured product. The blue colour turns yallow after stopping the reaction using stop solution. The colour intensity can be measured and it is proportional to the

amount of antigen captured in the wells and to its maount in the sample respectively . Wells contain samples negative for HBsAg remain colourless.

Procedure

Step1-Reagent preparation

The reagent and sample were allowed to reach room temperature(18-30 °C for 15-30 minutes.

The stock wash buffer had been diluted **1**to**20** with distilled water.

Step2- Numbering of wells

The strip needed were set in strip holder and sufficient number of wells includingone blank(A1) two negative control(B1,C1)and one well as positivecontrol(D1) were numbered.

Step 3-Adding sample diluent:

Twenty ul of sample Diluent was added to each well except the blank and mix by taping the plate gently

Step 4 adding sample

hundred ul of positive control, negative control and specimen were added intotheir respective wells except blank.

Step 5 Incubation

The plate was covered with the plate cover and incubated for 60 minutes at 37°C

Step 6 Added HRP Conjugate:

Then 50ml of HRP-conjugate were also added to each well except to blank well and mixed by tapping the plate gently.

Step 7- Incubation

The plate was covered with the plate cover and incubated for 30 minutes at 37°C.

Step 8- washing

After the end of the incubation the plate cover was removed ,each well was washed 5 times with diluted washing buffer each time the well were allowed to soak for 30-60 second. After the washing the plate was turn down onto blotting paper to remove any remainders.

Step9- Coloring

Fifty ul of chromogen A and 50ul of chromogen B were added into each well including blank and mixed by tapping plate. The plate was Incubated at 37°C for15 minutes, avoiding light. Blue color was developed in positive control and HBsAg positive sample wells.

Step10- stopping the reaction

Fifty ul of stop solution were added into each wells and mixed gently intensive yellow color was developed in positive control and HBsAg positive sample wells.

STEP11 –Measuring the Absorbance

The absorbance was read at 450nm using the ELISA reader.

calculation of the result

The result were calculated by relating each specimen absorbance(A) value to cutoff value(C.O) of the plate.

Calculation of cut-off value

(C.O)=NC×2.1 NC (the mean absorbance value to two negative controls).

Quality control range

The A value of blank well which contains only chromogen and stop solution should be less than 0.080 at 450nm The A value of positive control must be more than or equal 0.800 at 450nm The A value of negative control must be less than 0.100 at 450 nm.

Interpretation of the results

Negative Results

Sample giving A value less than cut-off value are negative for this assay which indicate that no HBsAg antibodies have been detected with this HBsAg ELISA kits therefore the patient is probably not infected with hepatitis B virus.

Positive Results:

Samples giving A value greater than or equal to cut-off value are considered initially reactive which indicate that HBV surface antigen has probably been detected with this HBsAg ELISA kit.

Borderline

Sample with A value to cut-off ratio between 0.9 and 1.00 are considered borderline samples And retest is recommended. repeatedly positive sample can be considered positive for HBsAg

Data Analysis

Collected data were analysis by a computer system using statistic package for social sciences(SPSS) program using the chi-square test statistical significance was set at (p. values<0.05).

3.7.3.3 Detection of HBV DNA by Nested PCR

Methods

DNA extraction by phenol chloroform method

procedure

Five hundred μL of 5% sodium dodecyl sulfat (SDS) was added on Eppindrof tube, then 250 μL of patient's serum and 20 μL of proteinase K was added and incubated at 56°C for one hour then the sample was left at 95°C for 10 minutes to deactivate proteinase K then 500 μL of phenol: chloroform: isoamylalcohol (25:24:1) were added and centrifuged at12000 rpm for 10 minutes.

The upper layer was transferred into new eppendrof tube.The Three steps were repeated twice then 25 µL of 5% sodium chloride were added and mixed. 1 ml of absolute ethanol was then added and well mixed. Then Incubated at -20°C overnight.

The mixure was centrifuged at 14000 rpm for 15 minutes and the supernatant was discarded and the tube was dried . 200 µL 70% ethanol were then added and shaked up until the pellet disappeared and centrifuged at 14000 rpm for 15 minutes. The supernatant was discarded and the tubes were inverted opened for two hours. 150µL of WFI were added and DNA preserved at- 20°C .

Amplification

The first-round PCR primers (outer primer pairs) and the second-round PCR primers (innerprimer pairs) were designed on the Hepatitis B virus strain gmc5 nonfunctional surface antigen (S) gene.

PCR was carried out in a tube containing 25µL ofthe reaction mixture made up of 0.2µL of the outer primer(1ul F and 1ul R), Maxime PCR PreMix Kit cotain(2.5U of i-Taq DNA polymerase,2.5Mm each of the fourdeoxynucleotides, 1x of reaction buffer, 1x of Gell loading buffer),5 µL of DNA Template was added, Then 13 µL of distilled water. The sampleswere denatured for two min at 95°C followed by30 cycles each consisted of 30 s at 95°C, annealing for 30 sat 57°C, and extention one min at 72°C in an Eppendrofthermal cycler . 5 µL aliquot of the first-round PCR productwas added to a tube containing the second of inner primer , each of thedeoxynucleotides, i-Taq DNApolymerase, PCR buffer and DW as in the first reaction. The samples were amplified as above .

Detection of PCR product

Ten microliter of the reaction product was electrophoresed on a 2% agarose gel, stained by ethidium bromide, and evaluated under UV light.

Negative and positive controls were also treatedas samples.

Table 3.1: Sequence of the oligonucleotide primers and expected PCR product size

Primer direction	5---3 sequence	Product size
Outer F	TTATCGCTGGATGTGTCTGC	405
Outer R	TACAGACTTGGCCCCCAATA	
Nested F	GTTGCCCGTTTGTCCTCTAA	250
Nested R	AAGCCCTACGAACCACTGAA	

3.8. Data collection and analysis

Processing and analysis of data were carried out by means of the statistical package for the social sciences (SPSS). A descriptive statistic frequency was used to assess the risk, Cross tabulation (chi-squire) was used to compare the variable with positive result.

CHAPTER FOUR

RESULTS

RESULTS

Out of 92 samples, 6 (6.5%) out of these where found positive by ICT, 30(32.6) were found positive for HBsAg by ELISA. The PCR confirmed that 30 positive cases obtained by ICT and ELISA.

4.1 ICT results:

Ninety two serum samples were collected from people at Sharafat village.6(6.5%) samples were found positive for HBsAg respectively when screened by ICT.

4.2 ELISA results:

The previous test samples when tested by ELISA using HBsAgsandwich ELISA, 30 (32.6%) samples were foundpositive for HBsAg.

4.3 Distribution between risk factors:

Gender: 16 (17.4%) of cases were male and 14 (15.2%) were female.(table 4.1 and figure 4.1).

Age: The highest results (8.7%) were distributed at age group (5-15) years and there were no infections between age group (56-65).

Marital status: 18 (19.6%) of cases were married and 12 (13%) were not married.

Blood transfusion: No infections noticed among transfused people.

Surgical history: 9 (9.8%) of cases had history of surgical operation and 21 (22.8%) had no.

4.4 Comparison between ICT and ELISA tests of HBsAg:

Out of 30 positive tests by ICT and ELISA, there were 24 positive samples that were found negative by ICT test.

4.5 Nested PCR Results: (Detection of HBV-DNA):

Thirty serum samples with HBsAg positive screened by ICT and ELISA were investigated to detect HBV-DNA using the nested PCR. HBV-DNA was detected in 30 samples of HBsAg positive by ELISA .

Table4.1 Frequency of HBV in males and females

HBV			positive	negative	Total
Sex	Male	Count	16	39	55
		% of Total	17.4%	42.4%	59.8%
	Femal	Count	14	23	37
		% of Total	15.2%	25.0%	40.2%
Total		Count	30	62	92
		% of Total	32.6%	67.4%	100.0 %

P(0.380) Insignificant level

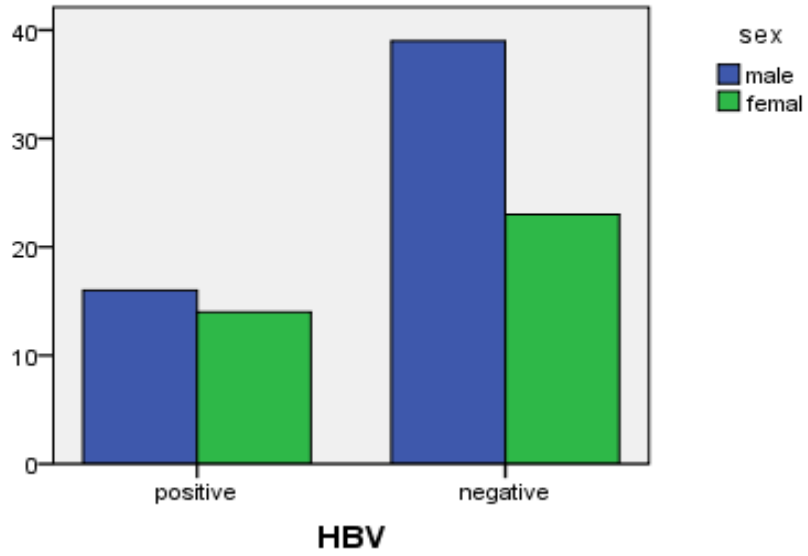


Fig 4.1Frequency of HBV in male and female.

(Table 4.2) The highest percentage of population infected by HBV was found in the age 5-15 years and no hepatitis infection was found in the age 56-65 years.

Table 4.2 frequency of HBV among age groups

Age groups		Positive ELISA	Negative ELISA	Total
5-15	Count	8	9	17
	% of Total	8.7%	9.8%	18.5%
16-25	Count	7	10	17
	% of Total	7.6%	10.9%	18.5%
26-35	Count	4	23	27
	% of Total	4.3%	25.0%	29.3%
36-45	Count	7	9	16
	% of Total	7.6%	9.8%	17.4%
46-55	Count	4	10	14
	% of Total	4.3%	10.9%	15.2%
56-65	Count	0	1	1
	% of Total	.0%	1.1%	1.1%
Total	Count	30	62	92
	% of total	32.6%	67.4%	100%

P(0.185)

The distribution of HBV infection on married and non married is shown in (Table 4.3) and (Fig 4.2).

Table 4.3 Frequency of HBV on married and non married

HBV			positive	Negative	Total
Marid	yes	Count	18	47	
Total	no	% of Total	19.6%	51.1%	65
		Count	12	15	70.7%
	no Count	% of Total	13.0%	16.3%	27
		30	62	92	29.3%
% of Total			32.6%	67.4%	100%

P(0.146)

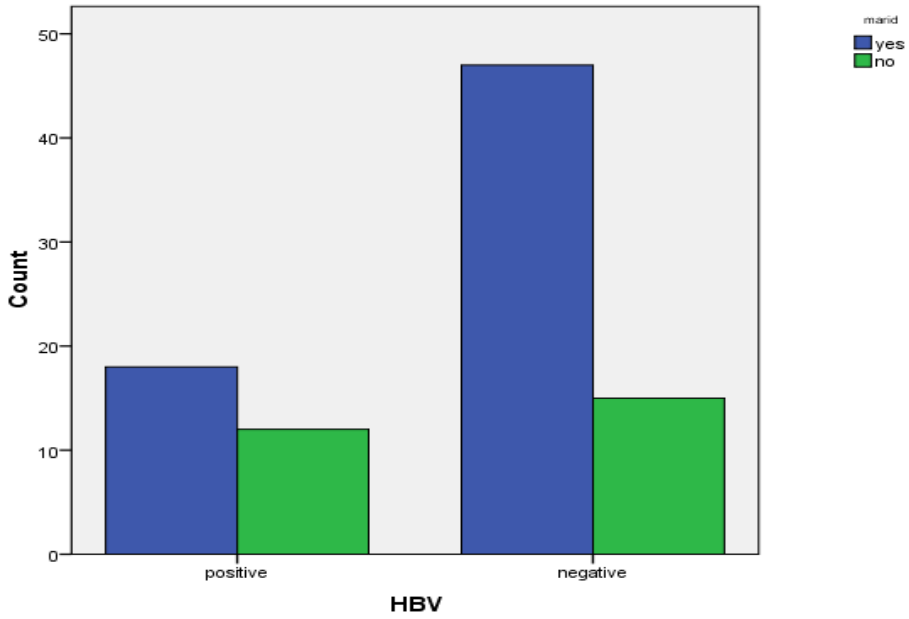


Fig 4.2 Frequency of HBV among married people

(Table 4.4), (Figure 4.3) showed the effect of surgery on the prevalence of HBV.

Distribution of HBV among who's had a surgical history

HBV			positive	negative	Total
surgical	yes	Count	9	13	22
		% of Total	9.8%	14.1%	23.9%
	no	Count	21	49	70
		% of Total	22.8%	53.3%	76.1%
Total		Count	30	62	92
		% of Total	32.6%	67.4%	100.0 %

P(0.242)

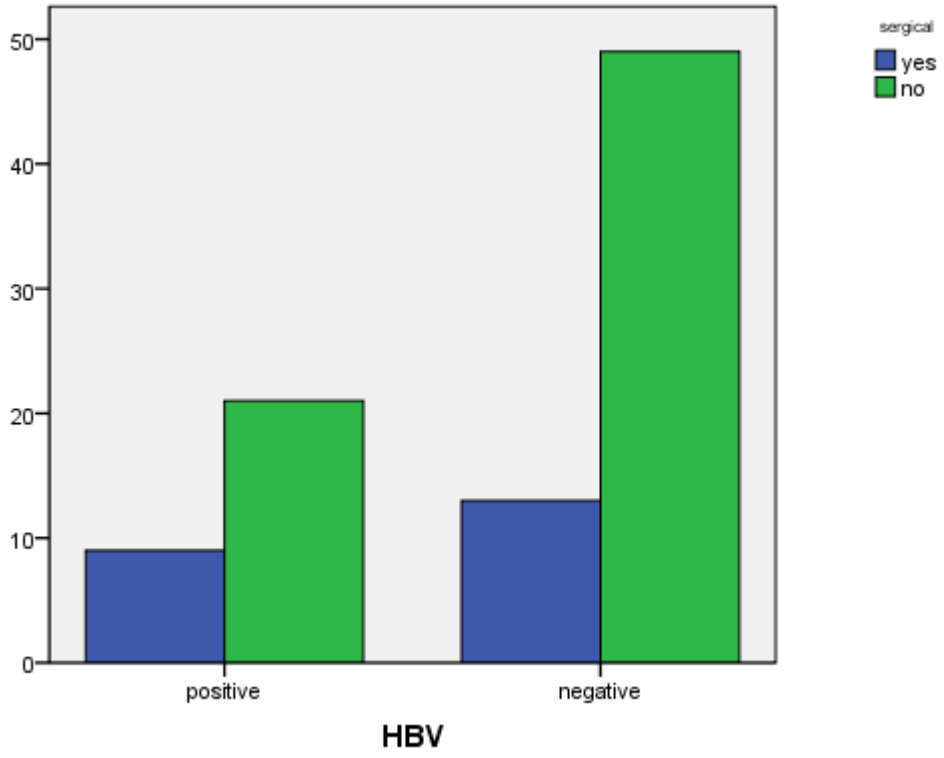


Figure4.3 Frequency of HBV among who's had surgical process

Table 4.5, Figure 4.4 showed no affect of transfusion on the prevalence of HBV

Table 4.5 Effect of blood transfusion on HBV infection

HBV			positive	negative	Total
Transfusion	yes	Count	0	5	5
		% of Total	.0%	5.4%	5.4%
	no	Count	30	57	87
		% of Total	32.6%	62.0%	94.6%
Total		Count	30	62	92
		% of Total	32.6%	67.4%	100.0 %

P(0.110)>0.05

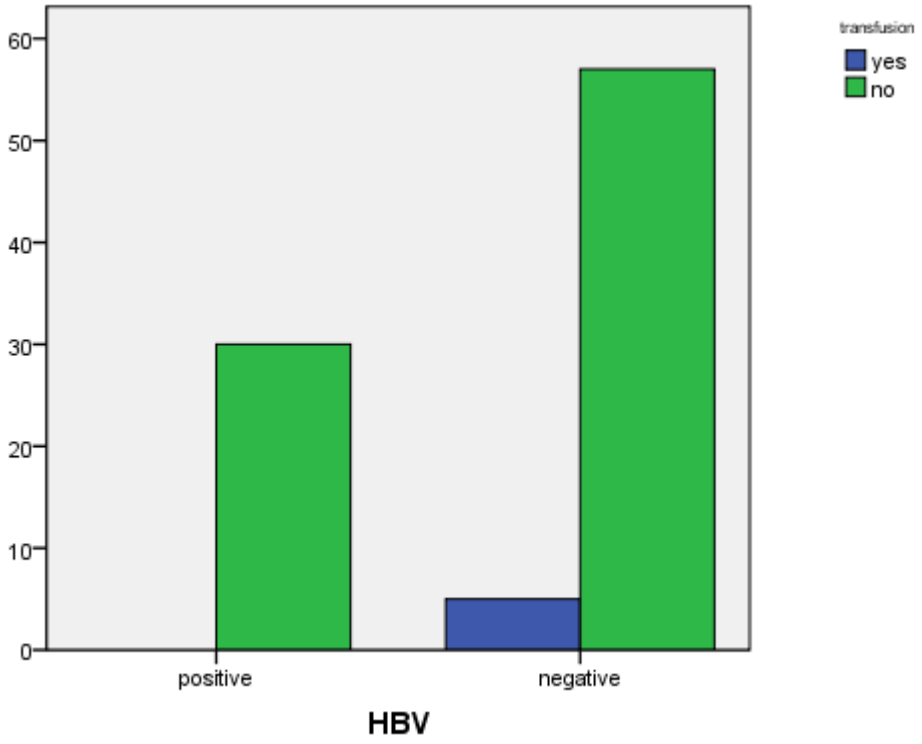


Fig 4.4 Effect of blood transfusion on HBV infection

Table 4.6, Fig 4.5 show a comparison between ICT result and ELISA result and there was a great difference between them.

Table 4.6 Comparison between ICT and ELISA

			+ve ELISA	-ve ELISA	Total
ict	Positive	Count	6	0	6
		% of Total	6.5%	.0%	6.5%
	Negative	Count	24	62	86
		% of Total	26.1%	67.4%	93.5%
Total		Count	30	62	92
		% of Total	32.6%	67.4%	100.0 %

P (0.001)

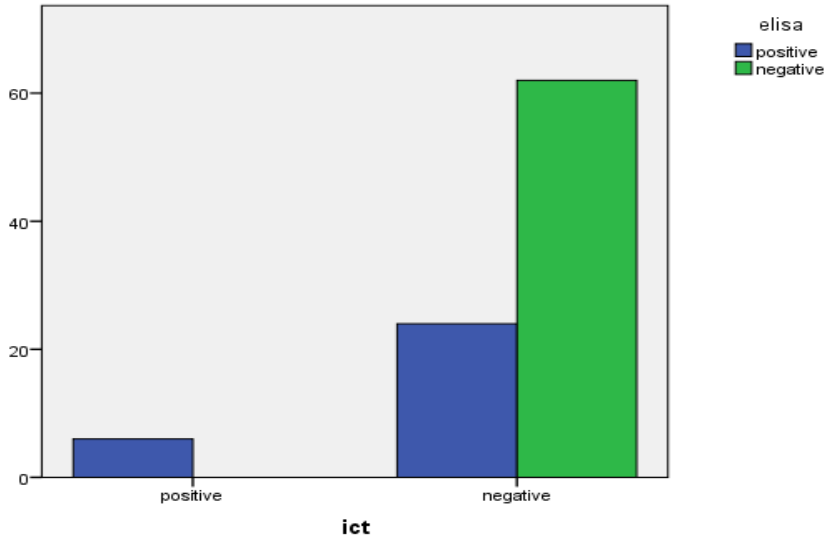


Fig 4.5 Comparison between ICT and ELISA

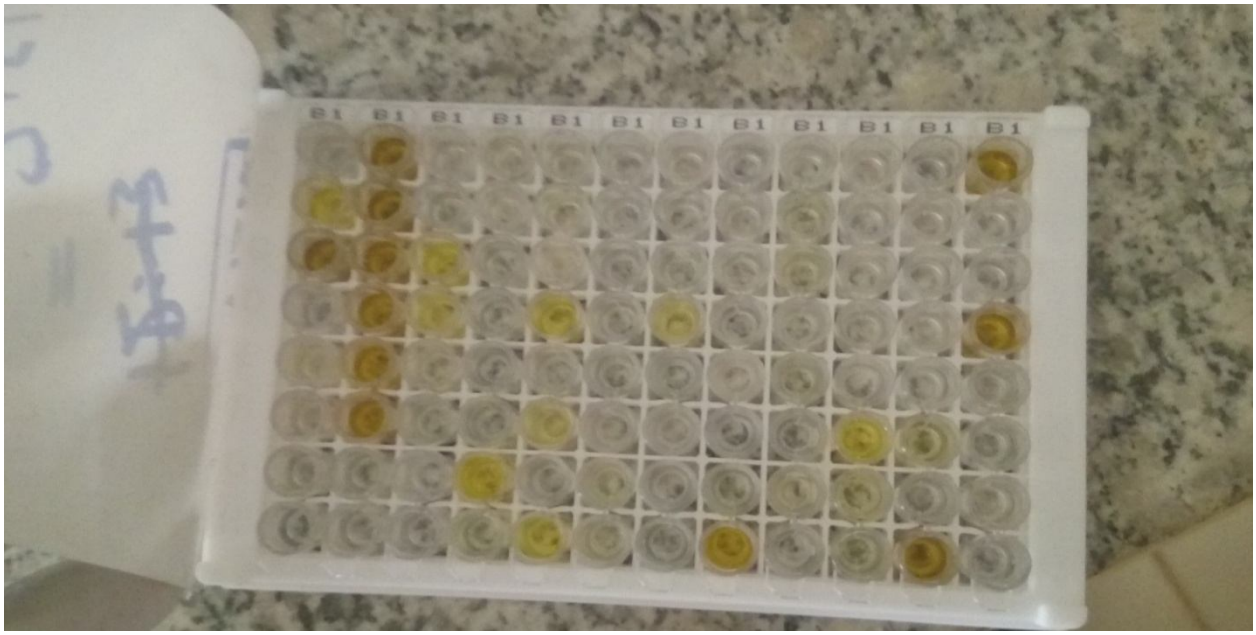


Fig. 4.6: Detection of HBsAg by ELISA test

Fig.4.7: Ethidium bromide stained agarose gel (2%) electrophoresis of H**BV** nested PCR products with a band of expected size 250 bp, carried out on DNA samples extracted from serum, Lane 5: 50 bp ladder, with fragment size indicated along the Center of the figure, from the left 3 positive control, 4 negativecontrol,5 ladder, 6-7-8 serum samples showed Positive results at 250 bp.

CHAPTER FIVE

DISCUSION

CHAPTER FIVE

DISCUSION

5.1 DISCUSION

Hepatitis B virus remains a major problem in developing countries. The HB viruses (HBV) express antigens such as HBsAg (surface antigen) on its surface, provoking both cell-mediated and humoral responses (Gill and Beeching, 2004).

This study is focused on prevalence of HBV on Almanagil province at Algazeira State in Sudan by molecular and serological diagnosis. The number of 92 serum samples were collected randomly. Out of 92 samples 6 (6.5%) were found positive for HBsAg, when tested by ICT, while 30 samples (32.6%) were found positive for HBsAg. The intensity of the red colour in the test region (T) in ICT mayvary depending on the concentration of HBsAg present in the sample. Therefore, anyshade of red color that develops in the test region was considered positive., (Elsheik *et al.*,2007) reported seroprevalence of 5.6% among antenatal care attendants in central Sudan.

When we compared the ELISA and ICT test the ELISA test detected 24 more positive sample that were negative by ICT test. Previous study by (Maaweya, 2002) showed a very high agreement between ICT assay and ELISA test 99.79 % and no statistical significant difference was obtained and both assay were sensitive, specific and reliable tests. The low prevalence of HBV infection when testd by ICT compare to ELISA indicate that ICT test is less sensitive, the test could be used for screening but it should be confirmed by ELISA test. In this study HBV infection was found high among young age group (8.7%), which indicate congenital transmission of HBV from mother to fetus among HBsAg positive patients

(Ayoola *et al*.,1981). The present study is in support of this assumption and 26.6% of the positive HBsAg within the age of less than 15 years.

Five persons of cases had history of blood transfusion but all of them show negative result for HBsAg. Screening of blood donors and blood products for HBsAg has been shown to be highly effective in preventing transmission and post-transfusion of hepatitis B virus and eliminate the risk of infection (Zou, 2009).In the present study PCR was covered out 30 positive samples tested for HBsAg by ELISA. (Mas *et al,* 1990) reported that Hepatitis B virusDNA was found in only two patients out of 29 patients.

Generally, results of this study were considerably higher than that reported at Central Sudan(6.8%) (Mudawi, 2008) .

The prevalence of HBV was higher among males(17.4%) than females (15.2) similar resuls were reported by Baig (2009). It was higher in age (5-15) and no infection at age (56-65). Also it higher in the married sample (19.6%) than the unmarried (13%) similar results were reported by Amini *et* al (1994). And higher for those had no surgical operation (22.8%) than those with history of surgical (8.9%). The result of blood transfusion disagree with the study of Yuen *et al* (2011) that showed 8.2% of HBV infection came through blood transfusion.

CONCLUSION

5.2 CONCLUSION

1. High incidence of HBsAg (32.6%) was found in randomly selected sample from Sharafat village at Almanagil and increased among young ages which indicate congenital transmission of disease .
2. In this study we detected the HBV DNA to confirm HBsAg-ELISA positive samples .
3. ICT is less reliable as ELISA for screening for HBsAg.
4. There was a very low agreement between ICT and ELISA and significant difference was observed by

Recommendations

5.3 Recommendations:

1. ELISA should be introduced along side ICT test.
2. Any person should be vaccinated .
3. Blood donors must be tested for HBsAg before transfusion to recipient.
4. Further studies with large number of participants and more advanced techniques.
5. Public awareness of HBV must be increased.

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Appendix

Sudan University of Science and Technology

College Of Graduate Studies

Questionnaire

Serological and Molecular detection of Hepatitis B visus in Elmanagil District in Algazeira State.

Name: …….. Serial No: ……

Gender: …….. Age: …….……

Marital Status:

Married ( ) Single ( )

Previous blood transfusions:

Yes ( ) No ( )

History of Surgical operation:

Yes ( ) No ( )

Symptoms of jaundice:

Yes ( ) No ( )

APPENDIX

Fig. 4.7: Detection of HBsAg by Immune-chromatography Test (ICT).

Fig. 10: Ethidium bromide stained agarose gel (2%) electrophoresis of H**BV** nested PCR products with a band of expected size 250 bp, carried out on DNA samples extracted from serum, Lane(5) M: 100 bp ladder, with fragment size indicated along the Center of the figure, from 1-8 serum samples showed Positive.

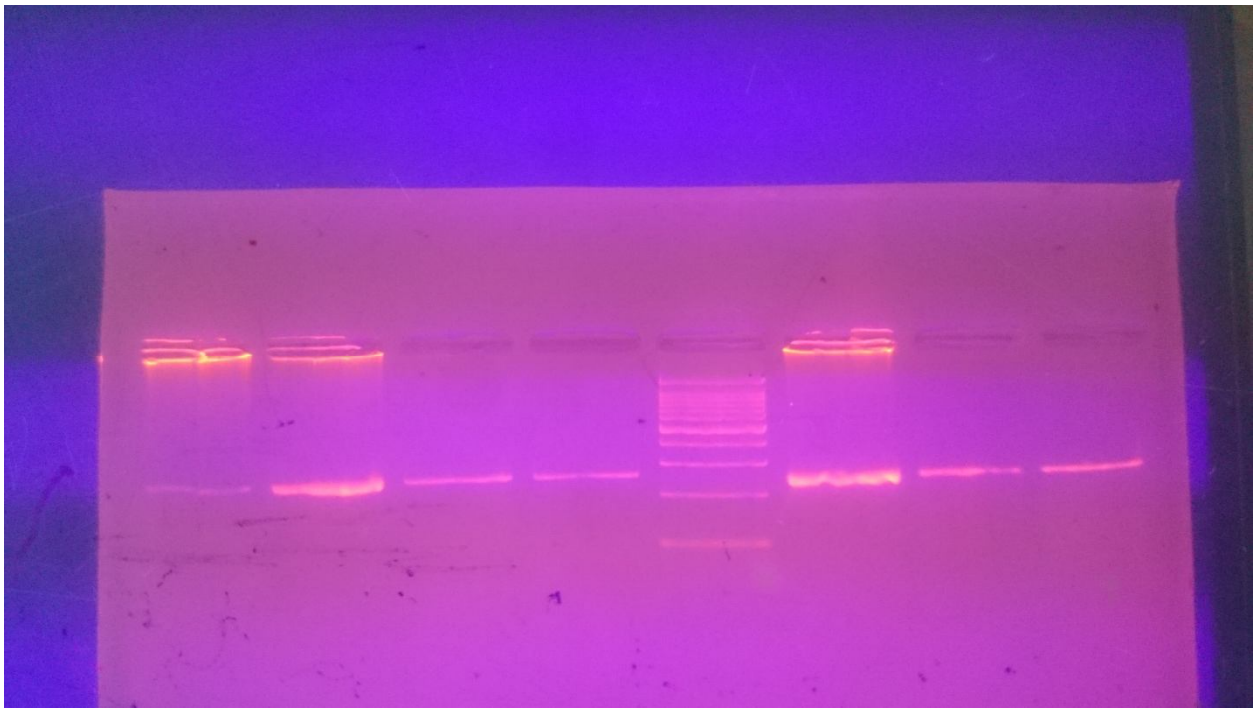

Fig. 11: Ethidium bromide stained agarose gel (2%) electrophoresis of H**BV** nested PCR products with a band of expected size 250 bp, carried out on DNA samples extracted from serum, Lane M: 100 bp ladder, with fragment size indicated along the Center of the figure, from 1-7 serum samples showed Positive.

Maxime PCR PreMix Series

ISO 9001/14001 Certified Company

Maxime PCR PreMix Kit (*i*-Taq)

for 20μl rxn / 50μl rxn

Cat. No. 25025(for 20μl rxn, 96 tubes) Cat. **No. 25026**(for 20μl rxn, 480 tubes)
Cat. No. 25035(for 50μl rxn, 96 tubes)

DESCRIPTION

iNtRON's *Maxime* PCR PreMix Kit has not only various kinds of PreMix Kit according to experience purpose, but also a 2X Master mix solution.
Maxime PCR PreMix Kit (*i*-Taq) is the product what is mixed every component; *i*-Taq™ DNA Polymerase, dNTP mixture, reaction buffer, and so on- in one tube for 1 rxn PCR. This is the product that can get the best result with the most convenience system. The first reason is that it has every components for PCR, so we can do PCR just add a template DNA, primer set, and D.W. The second reason is that it has Gel loading buffer to do electrophoresis, so we can do gel loading without any treatment. In addition, each batches are checked by a thorough Q.C., so its reappearance is high. It is suitable for various sample's expenence by fast and simple using method.

STORAGE

Store at -20℃, under this condition, it is stable for at least a year.

CHARACTERISTICS

- High efficiency of the amplification
- Ready to use: only template and primers are needed
- Stable for over 1 year at -20℃
- Time-saving and cost-effective

CONTENTS

- *Maxime* PCR PreMix (*i*-Taq, for 20μl rxn) 96 (480) tubes
- *Maxime* PCR PreMix (*i*-Taq, for 50μl rxn) 96 tubes

Component in	20 μl reaction	50 μl reaction
i-Taq™ DNA Polymerase(5U/μl)	2.5U	5U
dNTPs	2.5mM each	2.5mM each
Reaction Buffer(10x)	1x	1x
Gel Loading buffer	1x	1x

Note : The PCR process is covered by patents issued and applicable in certain countries. iNtRON Biotechnology does not encourage or support the unauthorized or Unlicensed use of the PCR process. Use of this product is recommended for persons That either have a license to perform PCR or are not required to obtain a license.

PROTOCOL

1. Add template DNA and primers into *Maxime* PCR PreMix tubes (*i*-Taq).
   Note 1 : Recommended volume of template and primer : 3μl~8μl
   Appropriate amounts of DNA template samples
   - cDNA : 0.5-10% of first RT reaction volume
   - Plasmid DNA : 10pg-100ng
   - Genomic DNA : 0.1-1ug for single copy

   Note 2 : Appropriate amounts of primers
   - Primer : 5-20pmol/μl each (sense and anti-sense)
2. Add distilled water into the tubes to a total volume of 20μl or 50μl. Do not calculate the dried components

Example **Total 20μl or 50μl reaction volume**

PCR reaction	Add	Add
Template DNA	1 ~ 2μl	2 ~ 4μl
Primer (F : 10pmol/μl)	1μl	2 ~ 2.5μl
Primer (R : 10pmol/μl)	1μl	2 ~ 2.5μl
Distilled Water	16 ~ 17μl	44 ~ 41μl
Total reaction volume	**20 μl**	**50 μl**

Note : This example serves as a guideline for PCR amplification. Optimal reaction conditions such as amount of template DNA and amount of primer, may vary and must be individually determined.

3. Dissolve the blue pellet by pipetting.
   Note : If the mixture lets stand at RT for 1-2min after adding water, the pellet is easily dissolved.
4. (Option) Add mineral oil.
   Note : This step is unnecessary when using a thermal cycler that employs a top heating method(general methods).
5. Perform PCR of samples.
6. Load samples on agarose gel without adding a loading-dye buffer and perform electrophoresis.

SUGGESTED CYCLING PARAMETERS

PCR cycle		Temp.	PCR product size		
			100-500bp	500-1000bp	1Kb-5Kb
Initial denaturation		94℃	2min	2min	2min
30-40 Cycles	Denaturation	94℃	20sec	20sec	20sec
	Annealing	50-65℃	10sec	10sec	20sec
	Extension	65-72℃	20-30sec	40-50sec	1min/Kb
Final extension		72℃	Optional. Normally, 2-5min		

EXPERIMENTAL INFORMATION

- ***Comparison with different company kit***

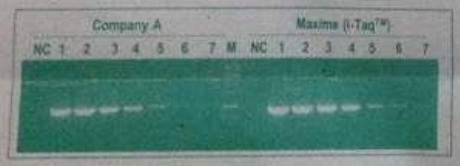

Fig.1. Comparison of *Maxime* PCR PreMix (*i*-Taq) and Company A's PreMix system by amplifying 1 Kb DNA fragment.
After diluting the λDNA as indicates, the PCR reaction was performed with *Maxime* PCR PreMix (*i*-Taq) and company's A product.
Lane M, SiZer-1000 DNA Marker; **lane 1**, undiluted λDNA; **lane 2**, 200 ng λDNA; **lane 3**, 40 ng λDNA; **lane 4**, 8 ng λDNA; **lane 5**, 1.6 ng λDNA; **lane 6**, 320 pg λDNA; **lane 7**, 64 pg λDNA; **lane NC**, Negative control

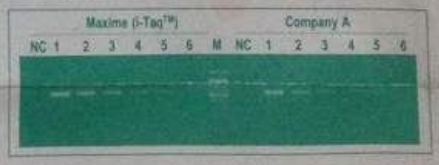

Fig.2. Comparison of *Maxime* PCR PreMix (*i*-Taq) and Company A's PreMix system by amplifying 570 bp DNA fragment (GAPDH).
Total RNA was purified from SNU-1 using easy-BLUE™ Total RNA Extraction Kit (Cat. No. 17061). And then, the first strand of cDNA was synthesized using Power cDNA Synthesis Kit (Cat. No. 25011). After diluting the cDNA mixture as indicates, the RT-PCR reaction was performed.
lane M, SiZer-100 DNA Marker; **lane 1**, undiluted cDNA; **lane 2**, 1/2 diluted cDNA; **lane 3**, 1/4 diluted cDNA; **lane 4**, 1/8 diluted cDNA; **lane 5**, 1/16 diluted cDNA; **lane 6**, 1/32 diluted cDNA; **lane NC**, Negative control

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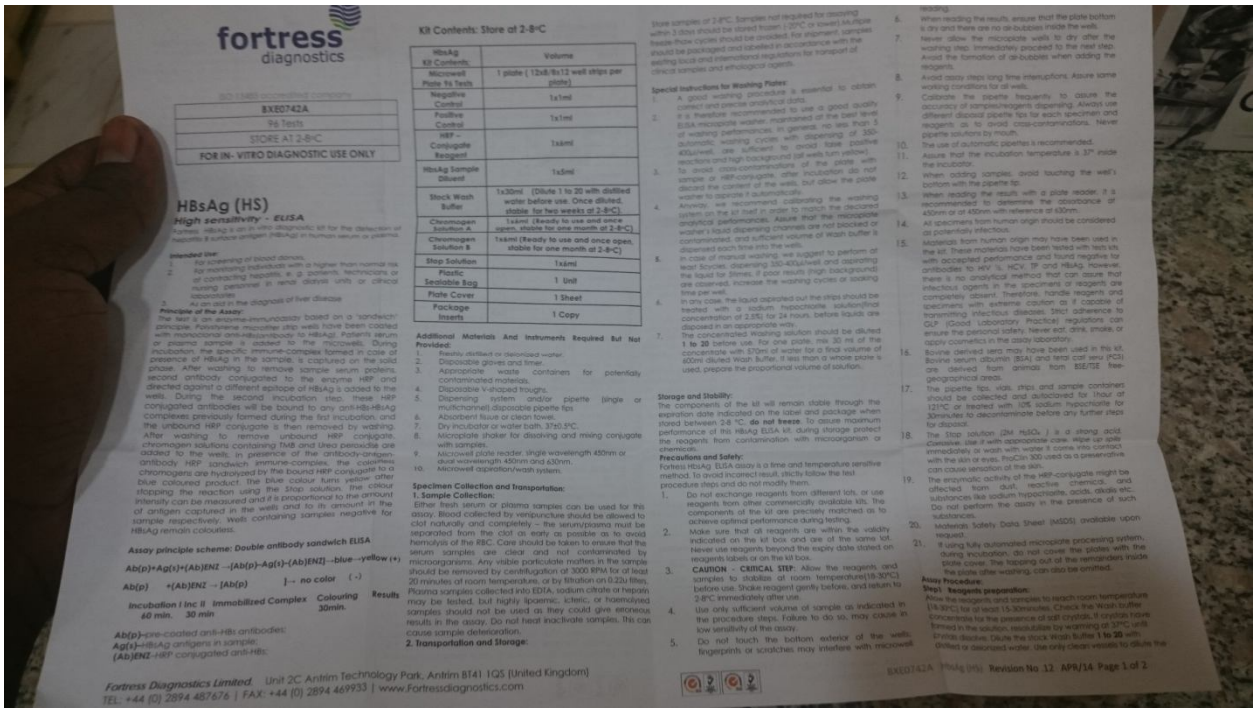


fortress diagnostics

BXE07424
96 Tests
STORE AT 2-8°C
FOR IN- VITRO DIAGNOSTIC USE ONLY

HBsAg (HS)

High sensitivity – ELISA

For the in vitro diagnostic kit for the detection of hepatitis B surface antigen (HBsAg) in human serum or plasma.

Intended Use:
1. For screening of blood donors.
2. For monitoring individuals with a higher than normal risk of contracting hepatitis, e.g. patients, technicians or nursing personnel in renal dialysis units or clinical laboratories.
3. As an aid in the diagnosis of liver disease

Principle of the Assay:
The test is an enzyme-immunoassay based on a 'sandwich' principle. Polystyrene microtiter strip wells have been coated with monoclonal anti-HBs(antibody to HBsAg). Patient's serum or plasma sample is added to the microwells. During incubation, the specific immune-complex formed in case of presence of HBsAg in the sample, is captured on the solid phase. After washing to remove sample serum proteins, second antibody conjugated to the enzyme HRP and directed against a different epitope of HBsAg is added to the wells. During the second incubation step, these HRP conjugated antibodies will be bound to any anti-HBs-HBsAg complexes previously formed during the first incubation. And the unbound HRP conjugate is then removed by washing. After washing to remove unbound HRP conjugate, chromogen solutions containing TMB and Urea peroxide are added to the wells. In presence of the antibody-antigen-antibody HRP sandwich immune-complex, the colourless chromogens are hydrolysed by the bound HRP conjugate to a blue coloured product. The blue colour turns yellow after stopping the reaction using the Stop solution. The colour intensity can be measured and it is proportional to the amount of antigen captured in the wells and to its amount in the sample respectively. Wells containing samples negative for HBsAg remain colourless.

Assay principle scheme: Double antibody sandwich ELISA

Ab(p)+Ag(s)+(Ab)ENZ →[Ab(p)–Ag(s)–(Ab)ENZ] --blue →yellow (+)

Ab(p) +(Ab)ENZ → [Ab(p)]→ no color (-)

Incubation I hr	II Immobilized Complex 60 min.	30 min	Colouring 30min.	Results

Ab(p)–pre-coated anti-HBs antibodies:
Ag(s)–HBsAg antigens in sample;
(Ab)ENZ–HRP conjugated anti-HBs:

Kit Contents: Store at 2-8°C

HBsAg Kit Contents:	Volume
Microwell Plate 96 Tests	1 plate (12x8/8x12 well strips per plate)
Negative Control	1x1ml
Positive Control	1x1ml
HRP – Conjugate Reagent	1x6ml
HbsAg Sample Diluent	1x5ml
Stock Wash Buffer	1x30ml (Dilute 1 to 20 with distilled water before use. Once diluted, stable for two weeks at 2-8°C).
Chromogen Solution A	1x6ml (Ready to use and once open, stable for one month at 2-8°C)
Chromogen Solution B	1x6ml (Ready to use and once open, stable for one month at 2-8°C)
Stop Solution	1x6ml
Plastic Sealable Bag	1 Unit
Plate Cover	1 Sheet
Package Inserts	1 Copy

Additional Materials And Instruments Required But Not Provided:
1. Freshly distilled or deionized water.
2. Disposable gloves and timer.
3. Appropriate waste containers for potentially contaminated materials.
4. Disposable V-shaped troughs.
5. Dispensing system and/or pipette (single or multichannel) disposable pipette tips
6. Absorbent tissue or clean towel.
7. Dry incubator or water bath, 37±0.5°C.
8. Microplate shaker for dissolving and mixing conjugate with samples.
9. Microwell plate reader, single wavelength 450nm or dual wavelength 450nm and 630nm.
10. Microwell aspiration/wash system.

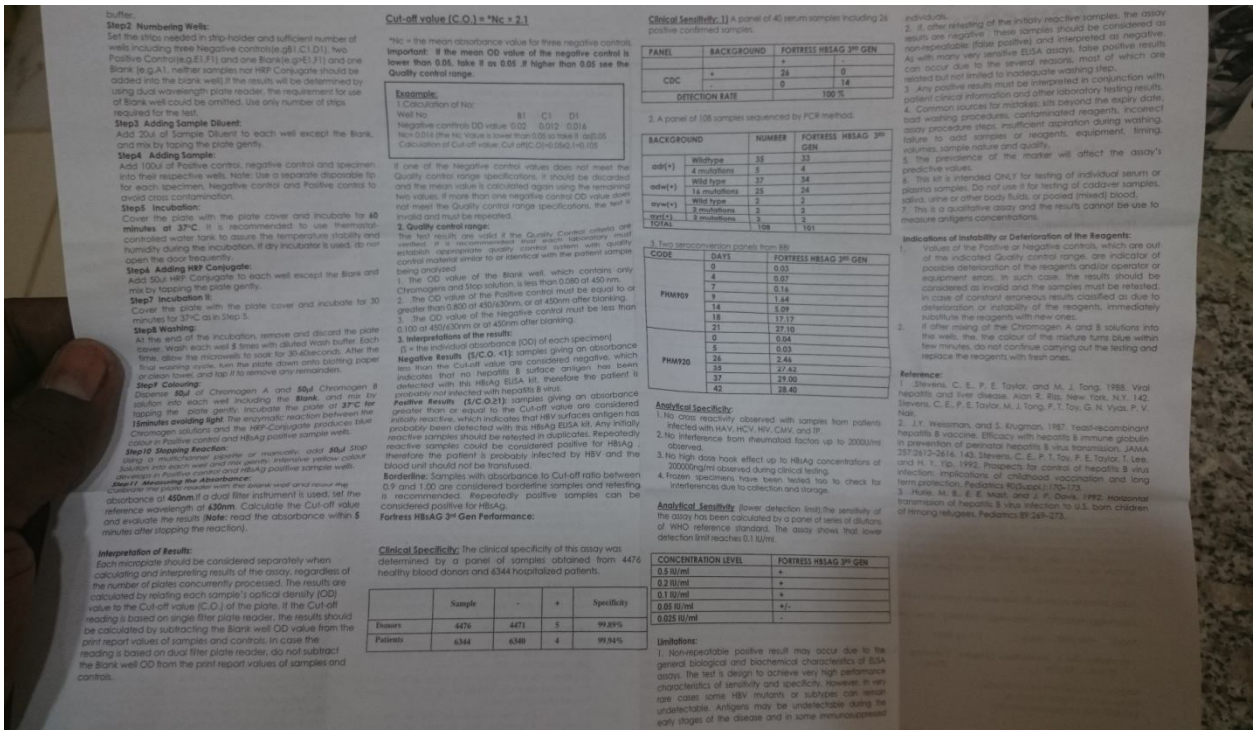
Specimen Collection and Transportation:
1. Sample Collection:
Either fresh serum or plasma samples can be used for this assay. Blood collected by venipuncture should be allowed to clot naturally and completely – the serum/plasma must be separated from the clot as early as possible as to avoid hemolysis of the RBC. Care should be taken to ensure that the serum samples are clear and not contaminated by microorganisms. Any visible particulate matters in the sample should be removed by centrifugation at 3000 RPM for at least 20 minutes at room temperatures, or by filtration on 0.22u filters. Plasma samples collected into EDTA, sodium citrate or heparin may be tested, but highly lipaemic, icteric, or haemolysed samples should not be used as they could give erroneous results in the assay. Do not heat inactivate samples, this can cause sample deterioration.
2. Transportation and Storage:

Store samples at 2-8°C. Samples not required for assaying within 3 days should be stored frozen (-20°C or lower). Multiple freeze-thaw cycles should be avoided. For shipment samples should be packaged and labelled in accordance with the existing local and international regulations for transport of clinical samples and etiological agents.

Special Instructions for Washing Plates:
1. A good washing procedure is essential to obtain consistent and precise analytical data.
2. It is therefore recommended to use a good quality ELISA microplate washer, maintained at the best level of washing performances. In general, no less than 5 automatic washing cycles with dispensing of 350-400µl/well, are sufficient to avoid false positive reactions and high background (all wells turn yellow).
3. To avoid cross-contamination of the plate with sample or HRP-conjugate, after incubation do not discard the content of the wells, but allow the plate washer to aspirate it automatically.
4. Another way to avoid contaminating the washing system on the kit itself in order to match the declared analytical performances. Assure that the microplate washer's liquid dispensing channels are not blocked or contaminated, and sufficient volume of wash buffer is dispensed each time into the wells.
5. In case of manual washing, we suggest to perform at least 5cycles, dispensing 350-400µl/well and aspirating the liquid for 30mes. If poor results (poor precision) are observed, increase the washing cycles or soaking time per well.
6. In any case, the liquid aspirated out the strips should be treated with a sodium hypochlorite solution(final concentration of 2.5%) for 24 hours, before liquids are disposed in an appropriate way.
7. The concentrated Washing solution should be diluted **1 to 20** before use. For one plate, mix 30 ml of the concentrate with 570ml of water for a final volume of 600ml diluted Wash Buffer. If less than a whole plate is used, prepare the proportional volume of solution.

Storage and Stability:
The components of the kit will remain stable through the expiration date indicated on the label and package when stored between 2-8 °C, **do not freeze**. To assure maximum performance of this HBsAg ELISA kit, during storage protect the reagents from contamination with microorganism or chemicals.
Precautions and Safety:
Fortress HBsAg ELISA assay is a time and temperature sensitive method. To avoid incorrect result, strictly follow the test procedure steps and do not modify them.
1. Do not exchange reagents from different lots, or use reagents from other commercially available kits. The components of the kit are precisely matched as to achieve optimal performance during testing.
2. Make sure that all reagents are within the validity indicated on the kit box and are of the same lot. Never use reagents beyond the expiry date stated on reagents labels or on the kit box.
3. **CAUTION - CRITICAL STEP:** Allow the reagents and samples to stabilize at room temperature(18-30°C) before use. Shake reagent gently before, and return to 2-8°C immediately after use.
4. Use only sufficient volume of sample as indicated in the procedure steps. Failure to do so, may cause in low sensitivity of the assay.
5. Do not touch the bottom exterior of the wells, fingerprints or scratches may interfere with microwell reading.
6. When reading the results, ensure that the plate bottom is dry and there are no air-bubbles inside the wells.
7. Never allow the microplate wells to dry after the washing step. Immediately proceed to the next step. Avoid the formation of air-bubbles when adding the reagents.
8. An assay steps long time interruptions. Assure same working conditions for all wells.
9. Calibrate the pipette frequently to assure the accuracy of samples/reagent dispensing. Always use different disposal pipette tips for each specimen and reagents as to avoid cross-contaminations. Never pipette solutions by mouth.
10. The use of automatic pipettes is recommended.
11. Assure that the incubation temperature is 37° inside the incubator.
12. When adding samples, avoid touching the well's bottom with the pipette tip.
13. When reading the results with a plate reader, it is recommended to determine the absorbance of 450nm or at 450nm with reference at 630nm.
14. All specimens from human origin should be considered as potentially infectious.
15. Materials from human origin may have been used in the kit. These materials have been tested with test kits with accepted performance and found negative for antibodies to HIV ½, HCV, TP and HBsAg. However, there is no analytical method that can assure that infectious agents in the specimens or reagents are completely absent. Therefore, handle reagents and specimens with extreme caution as if capable of transmitting infectious diseases. Strict adherence to GLP (Good Laboratory Practice) regulations can ensure the personal safety. Never eat, drink, smoke, or apply cosmetics in the assay laboratory.
16. Bovine derived sera may have been used in this kit. Bovine serum albumin (BSA) and fetal calf sera (FCS) are derived from animals from BSE/TSE free-geographical areas.
17. The pipette tips, vials, strips and sample containers should be collected and autoclaved for 1hour at 121°C or treated with 10% sodium hypochlorite for 30minutes to decontaminate before any further steps for disposal.
18. The Stop solution (2M H_2SO_4) is a strong acid. **Corrosive.** Use it with appropriate care. Wipe up spills immediately or wash with water if come into contact with the skin or eyes. ProClin 300 used as a preservative can cause sensation of the skin.
19. The enzymatic activity of the HRP-conjugate might be affected from dust, reactive chemicals, and substances like sodium hypochlorite, acids, alkalis etc. Do not perform the assay in the presence of such substances.
20. Materials Safety Data Sheet (MSDS) available upon request.
21. If using fully automated microplate processing system, during incubation, do not cover the plates with the plate cover. The fogging out of the remainders inside the plate after washing, can also be emitted.

Assay Procedure:
Step1 Reagents preparation:
Allow the reagents and samples to reach room temperature (18-30°C) for at least 15-30minutes. Check the Wash buffer concentrate for the presence of salt crystals. If crystals have formed in the solution, resolubilize by warming at 37°C until crystals dissolve. Dilute the stock Wash Buffer **1 to 20** with Grifted or deionized water. Use only clean vessels to dilute the



buffer.
Step2 Numbering Wells:
Set the strips needed in strip-holder and sufficient number of wells including three Negative controls(e.g.B1,C1,D1), two Positive Control(e.g.E1,F1) and one Blank(e.g.H1) and one Blank (e.g.A1, neither samples nor HRP Conjugate should be added into the blank well).If the results will be determined by using dual wavelength plate reader, the requirement for use of Blank well could be omitted. Use only number of strips required for the test.
Step3 Adding Sample Diluent:
Add 20ul of Sample Diluent to each well except the Blank, and mix by taping the plate gently.
Step4 Adding Sample:
Add 100ul of Positive control, negative control and specimen into their respective wells. Note: Use a separate disposable tip for each specimen, Negative control and Positive control to avoid cross contamination.
Step5 Incubation:
Cover the plate with the plate cover and incubate for **60 minutes at 37°C**. It is recommended to use thermostat-controlled water bank to assure the temperature stability and humidity during the incubation. If dry incubator is used, do not open the door frequently.
Step6 Adding HRP Conjugate:
Add 50ul HRP Conjugate to each well except the Blank and mix by tapping the plate gently.
Step7 Incubation II:
Cover the plate with the plate cover and incubate for 30 minutes for 37°C as in Step 5.
Step8 Washing:
At the end of the incubation, remove and discard the plate cover. Wash each well 5 times with diluted Wash buffer. Each time, allow the microwells to soak for 30-40seconds. After the final washing cycle, turn the plate down onto blotting paper or clean towel, and tap it to remove any remainders.
Step9 Colouring:
Dispense **50µl** of Chromogen A and **50µl** Chromogen B solution into each well including the **Blank**, and mix by tapping the plate gently. Incubate the plate at **37°C for 15minutes avoiding light**. The enzymatic reaction between the Chromogen solutions and the HRP-Conjugate produces blue colour in Positive control and HBsAg positive sample wells.
Step10 Stopping Reaction:
Using a multichannel pipette or manually, add **50µl** Stop solution into each well and mix gently. Intensive yellow colour develops in Positive control and HBsAg positive sample wells.
Step11 Measuring the Absorbance:
Calibrate the plate reader with the blank well and read the absorbance at **450nm**.If a dual filter instrument is used, set the reference wavelength at **630nm**. Calculate the Cut-off value and evaluate the results (**Note:** read the absorbance within **5 minutes after stopping the reaction**).

Interpretation of Results:
Each microplate should be considered separately when calculating and interpreting results of the assay, regardless of the number of plates concurrently processed. The results are calculated by relating each sample's optical density (OD) value to the Cut-off value (C.O.) of the plate. If the Cut-off reading is based on single filter plate reader, the results should be calculated by subtracting the Blank well OD value from the print report values of samples and controls. In case the reading is based on dual filter plate reader, do not subtract the Blank well OD from the print report values of samples and controls.

Cut-off value (C.O.) = *Nc × 2.1
*Nc = the mean absorbance value for three negative controls.
Important: If the mean OD value of the negative control is lower than 0.05, take it as 0.05 .If higher than 0.05 see the Quality control range.

Exaample:
1.Calculation of Nc:
Well No B1 C1 D1
Negative controls OD value 0.02 0.012 0.016
Nc= 0.016 (the Nc Value is lower than 0.05 so take it as0.05
Calculation of Cut-off value: Cut off(C.O)=0.05x2.1=0.105

If one of the Negative control values does not meet the Quality control range specifications, it should be discarded and the mean value is calculated again using the remaining two values. If more than one negative control OD value does not meet the Quality control range specifications, the test is invalid and must be repeated.
2. Quality control range:
The test results are valid if the Quality Control criteria are verified. It is recommended that each laboratory must establish appropriate quality control system with quality control material similar to or identical with the patient sample being analyzed.
1. The OD value of the Blank well, which contains only Chromogens and Stop solution, is less than 0.080 at 450 nm.
2. The OD value of the Positive control must be equal to or greater than 0.800 at 450/630nm, or at 450nm after blanking.
3. The OD value of the Negative control must be less than 0.100 at 450/630nm or at 450nm after blanking.
3. Interpretations of the results:
(S = the individual absorbance (OD) of each specimen)
Negative Results (S/C.O. <1): samples giving an absorbance less than the Cut-off value are considered negative, which indicates that no hepatitis B surface antigen has been detected with this HBsAg ELISA kit, therefore the patient is probably not infected with hepatitis B virus.
Positive Results (S/C.O.≥1): samples giving an absorbance greater than or equal to the Cut-off value are considered initially reactive, which indicates that HBV surfaces antigen has probably been detected with this HBsAg ELISA kit. Any initially reactive samples could be considered positive for HBsAg ; therefore the patient is probably infected by HBV and the blood unit should not be transfused.
Borderline: Samples with absorbance to Cut-off ratio between 0.9 and 1.00 are considered borderline samples and retesting is recommended. Repeatedly positive samples can be considered positive for HBsAg.
Fortress HBsAG 3rd Gen Performance:

Clinical Specificity: The clinical specificity of this assay was determined by a panel of samples obtained from 4476 healthy blood donors and 6344 hospitalized patients.

	Sample	-	+	Specificity
Donors	4476	4471	5	99.89%
Patients	6344	6340	4	99.94%

Clinical Sensitivity: 1) A panel of 40 serum samples including 26 positive confirmed samples.

PANEL	BACKGROUND	FORTRESS HBSAG 3RD GEN	
		+	-
CDC	+	26	0
		0	14
DETECTION RATE		100 %	

2. A panel of 108 samples sequenced by PCR method.

BACKGROUND		NUMBER	FORTRESS HBSAG 3RD GEN
adr(+)	Wildtype	35	33
	4 mutations	5	4
adw(+)	Wild type	37	34
	16 mutations	25	24
ayw(+)	Wild type	3	3
	3 mutations	2	2
ayr(+)	2 mutations	3	2
TOTAL		109	101

3. Two seroconversion panels from BBI

CODE	DAYS	FORTRESS HBSAG 3RD GEN
PHM909	0	0.03
	4	0.07
	7	0.16
	9	1.44
	14	15.09
	18	17.17
PHM920	21	27.10
	0	0.04
	5	0.03
	26	2.46
	35	27.62
	37	29.00
	42	28.40

Analytical Specificity:
1. No Cross reactivity observed with samples from patients infected with HAV, HCV, HIV, CMV, and TP.
2. No interference from rheumatoid factors up to 2000IU/ml observed.
3. No high dose hook effect up to HBsAg concentrations of 200000ng/ml observed during clinical testing.
4. Frozen specimens have been tested too to check for interferences due to collection and storage.

Analytical Sensitivity (lower detection limit):The sensitivity of the assay has been calculated by a panel of series of dilutions of WHO reference standard. The assay shows that lower detection limit reaches 0.1 IU/ml.

CONCENTRATION LEVEL	FORTRESS HBSAG 3RD GEN
0.5 IU/ml	+
0.2 IU/ml	+
0.1 IU/ml	+
0.05 IU/ml	+/-
0.025 IU/ml	-

Limitations:
1. Non-repeatable positive result may occur due to the general biological and biochemical characteristics of ELISA assays. The test is design to achieve very high performance characteristics of sensitivity and specificity, however, in very rare cases some HBV mutants or subtypes can remain undetectable. Antigens may be undetectable during the early stages of the disease and in some immunosuppressed individuals.
2. If, after retesting of the initially reactive samples, the assay results are negative these samples should be considered as non-repeatable (false positive) and interpreted as negative. As with many very sensitive ELISA assays, false positive results can occur due to the several reasons, most of which are related but not limited to inadequate washing steps.
3. Any positive results must be interpreted in conjunction with patient clinical information and other laboratory testing results.
4. Common sources for mistakes: kits beyond the expiry date, bad washing procedures, contaminated reagents, incorrect assay procedure steps, insufficient aspiration during washing, failure to add samples or reagents, equipment, timing, volumes, sample nature and quality.
5. The prevalence of the marker will affect the assay's predictive value.
6. This kit is intended ONLY for testing of individual serum or plasma samples. Do not use it for testing of cadaver samples, saliva, urine or other body fluids, or pooled (mixed) blood.
7. This is a qualitative assay and the results cannot be use to measure antigens concentrations.

Indications of Instability or Deterioration of the Reagents:
1. Values of the Positive or Negative controls, which are out of the indicated Quality control range, are indicator of possible deterioration of the reagents and/or operator or equipment errors. In such case, the results should be considered as invalid and the samples must be retested. In case of constant erroneous results classified as due to deterioration or instability of the reagents, immediately substitute the reagents with new ones.
2. If after mixing of the Chromogen A and B solutions into the wells, the, the colour of the mixture turns blue within few minutes, do not continue carrying out the testing and replace the reagents with fresh ones.

Reference:

1. Stevens, C. E., P. E. Taylor, and M. J. Tong. 1988. Viral hepatitis and liver disease. Alan R. Riss, New York, N.Y. 142.
Stevens, C. E., P. E. Taylor, M. J. Tong, P. T. Toy, G. N. Vyas, P. V. Nair,
2. J.Y. Wasserman, and S. Krugman. 1987. Yeast-recombinant hepatitis B vaccine. Efficacy with hepatitis B immune globulin in prevention of perinatal hepatitis B virus transmission. JAMA 257:2612-2616. 143. Stevens, C. E., P. T. Toy, P. E. Taylor, T. Lee, and H. Y. Tip. 1992. Prospects for control of hepatitis B virus infection: implications of childhood vaccination and long term protection. Pediatrics 89(Suppl.):1170-173.
3. Hurie, M. B., E. E. Mast, and J. P. Davis. 1992. Horizontal transmission of hepatitis B virus infection to U.S. born children of Hmong refugees. Pediatrics 89:249-273.

ACON®

HBsAg Rapid Test Cassette (Serum/Plasma) Package Insert

REF L031-10231 | English

A rapid test for the qualitative detection of Hepatitis B Surface Antigen (HBsAg) in serum or plasma.
For professional in vitro diagnostic use only.

INTENDED USE

ACON HBsAg Rapid Test Cassette (Serum/Plasma) is a rapid chromatographic immunoassay for the qualitative detection of Hepatitis B Surface Antigen in serum or plasma.

SUMMARY

Viral hepatitis is a systemic disease primarily involving the liver. Most cases of acute viral hepatitis are caused by Hepatitis A virus, Hepatitis B virus (HBV) or Hepatitis C virus. The complex antigen found on the surface of HBV is called HBsAg. Previous designations included the Australia or Au antigen.[1] The presence of HBsAg in serum or plasma is an indication of an active Hepatitis B infection, either acute or chronic. In a typical Hepatitis B infection, HBsAg will be detected 2 to 4 weeks before the ALT level becomes abnormal and 3 to 5 weeks before symptoms or jaundice develop. HBsAg has four principal subtypes: adw, ayw, adr and ayr. Because of antigenic heterogeneity of the determinant, there are 10 major serotypes of Hepatitis B virus.
ACON HBsAg Rapid Test Cassette (Serum/Plasma) is a rapid test to qualitatively detect the presence of HBsAg in serum or plasma specimen. The test utilizes a combination of monoclonal and polyclonal antibodies to selectively detect elevated levels of HBsAg in serum or plasma.

PRINCIPLE

ACON HBsAg Rapid Test Cassette (Serum/Plasma) is a qualitative, lateral flow immunoassay for the detection of HBsAg in serum or plasma. The membrane is pre-coated with anti-HBsAg antibodies on the test line region of the cassette. During testing, the serum or plasma specimen reacts with the particle coated with anti-HBsAg antibody. The mixture migrates upward on the membrane chromatographically by capillary action to react with anti-HBsAg antibodies on the membrane and generate a colored line. The presence of this colored line in the test region indicates a positive result, while its absence indicates a negative result. To serve as a procedural control, a colored line will always appear in the control line region indicating that proper volume of specimen has been added and membrane wicking has occurred.

REAGENTS

The test cassette contains anti-HBsAg particles and anti-HBsAg coated on the membrane.

PRECAUTIONS

- For professional in vitro diagnostic use only. Do not use after expiration date.
- Do not eat, drink or smoke in the area where the specimens or kits are handled.
- Handle all specimens as if they contain infectious agents. Observe established precautions against microbiological hazards throughout testing and follow the standard procedures for proper disposal of specimens.
- Wear protective clothing such as laboratory coats, disposable gloves and eye protection when specimens are being tested.
- Humidity and temperature can adversely affect results.

STORAGE AND STABILITY

- The kit can be stored at room temperature or refrigerated (2-30°C).
- The test cassette is stable through the expiration date printed on the sealed pouch. The test cassette must remain in the sealed pouch until use.
- Do not use the components beyond the expiration date.
- **DO NOT FREEZE.**

SPECIMEN COLLECTION AND PREPARATION

- ACON HBsAg Rapid Test Cassette (Serum/Plasma) can be performed using either serum or plasma.
- Separate the serum or plasma from blood as soon as possible to avoid hemolysis. Only clear, non-hemolyzed specimens can be used.
- Testing should be performed immediately after the specimens have been collected. Do not leave the specimens at room temperature for prolonged periods. Specimens may be stored at 2-8°C for up to 3 days. For long term storage, specimens should be kept below -20°C.
- Bring specimens to room temperature prior to testing. Frozen specimens must be completely thawed and mixed well prior to testing. Specimens should not be frozen and thawed repeatedly.
- If specimens are to be shipped, they should be packed in compliance with federal, state or local regulations for the transportation of etiologic agents.

MATERIALS

Materials Provided

- Test Cassettes
- Package insert
- Specimen droppers

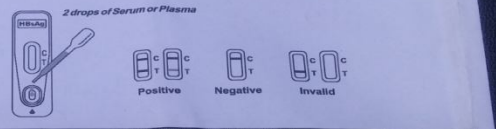
Materials Required But Not Provided

- Specimen collection container
- Centrifuge (for plasma only)
- Timer

DIRECTIONS FOR USE

Allow test cassette, serum or plasma specimen to equilibrate to room temperature (15-30°C) prior to testing.

1. Bring the pouch to room temperature before opening it. Remove the test cassette from the sealed pouch and use it as soon as possible. Best results will be obtained if the assay is performed within one hour.
2. Place the test Cassette on a non-absorbent flat surface. With arrows pointing toward the serum or plasma specimen, add two drops of specimen vertically to the sample pad under the arrows.
3. Start the timer and wait for the red line(s) to appear. The result should be read at 10 minutes. Note: A low HBsAg concentration might result in a weak line appearing in the test region (T) after an extended period of time; therefore, do not interpret the result after 30 minutes.



INTERPRETATION OF RESULTS

(Please refer to the illustration above)
POSITIVE:* Two distinct red lines appear. One line should be in the control region (C) and another line should be in the test region (T).
***NOTE:** The intensity of the red color in the test line region (T) will vary depending on the concentration of HBsAg present in the specimen. Therefore, any shade of red in the test region (T) should be considered positive.
NEGATIVE: One red line appears in the control region (C). No apparent red or pink line appears in the test region (T).
INVALID: Control line fails to appear. Insufficient specimen volume or incorrect procedural techniques are the most likely reasons for control line failure. Review the procedure and repeat the test with a new test cassette. If the problem persists, discontinue using the test kit immediately and contact your local distributor.

QUALITY CONTROL

A procedural control is included in the test. A red line appearing in the control region (C) is the internal procedural control. It confirms sufficient specimen volume and correct procedural technique.
Control standards are not supplied with this kit; however, it is recommended that a positive control (containing 10 ng/mL HBsAg) and a negative control control (containing 0 ng/mL HBsAg) be tested as a good laboratory practice to confirm the test procedure and to verify proper test performance.

LIMITATION

1. ACON HBsAg Rapid Test Cassette (Serum/Plasma) is for in vitro diagnostic use only. This test should be used for the detection of HBsAg in serum or plasma specimen.
2. ACON HBsAg Rapid Test Cassette (Serum/Plasma) will only indicate the presence of HBsAg in the specimen and should not be used as the sole criteria for the diagnosis of Hepatitis B viral infection.
3. As with all diagnostic tests, all results must be considered with other clinical information available to the physician.
4. ACON HBsAg Rapid Test Cassette (Serum/Plasma) cannot detect less than 0.79 ng/mL of HBsAg in specimens. If the test result is negative and clinical symptoms persist, additional follow-up testing using other clinical methods is suggested. A negative result at any time does not preclude the possibility of Hepatitis B infection.

EXPECTED VALUES

ACON HBsAg Rapid Test Cassette (Serum/Plasma) has been compared with a leading commercial HBsAg EIA test. The correlation between these two systems is over 98%.

PERFORMANCE CHARACTERISTICS

Sensitivity

ACON HBsAg Rapid Test Cassette (Serum/Plasma) has been tested with WHO International Standard Sample 12/226 and low titer specimens. The test can detect 0.79 ng/mL of HBsAg in 10 minutes, and 2 ng/mL of WHO International standard in 10 minutes.

Specificity

Antibodies used for ACON HBsAg Rapid Test Cassette (Serum/Plasma) were developed against whole Hepatitis B antigen isolated from Hepatitis B virus. Specificity of ACON HBsAg Rapid Test Cassette (Serum/Plasma) was also tested with Hepatitis A positive specimen, Hepatitis C positive specimen, Hepatitis D positive specimen and Hepatitis E positive specimen. They all yielded negative results.

HBsAg Reference Method

Method		EIA		
HBsAg Test Cassette	Results	Positive	Negative	Total Results
	Positive	184	0	184
	Negative	0	309	309
Total Results		184	309	493

Relative Sensitivity: 100% (98.02%-100%)*
Overall Agreement: 99.6% (99.25%-100%)*
Relative Specificity: 100% (98.81%-100%)*
*95% Confidence Interval

Precision

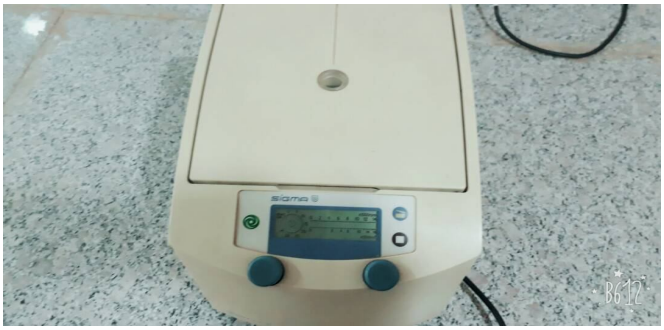
Intra-Assay
Within-run precision has been determined by using 10 replicates of four specimens: a negative, a low positive, a medium positive and a high positive. The negative, low positive, medium positive and high positive values were correctly identified >99% of the time.
Inter-Assay
Between-run precision has been determined by 10 independent assays on the same four specimens: a negative, a low positive, a medium positive and a high positive. Three different lots of the ACON HBsAg Rapid Test Cassette (Serum/Plasma) have been tested using negative, low positive and high positive specimens. The specimens were correctly identified >99% of the time.

BIBLIOGRAPHY

1. Blumberg, B.S. *The Discovery of Australian Antigen and its relation to viral hepatitis.* Vitro. 1971; 7: 223

Index of Symbols

	Manufacturer	Σ	Tests per kit	2°C 30°C	Store between 2-30°C
IVD	For *in vitro* diagnostic use only		Use by	2	Do not reuse
LOT	Lot Number	REF	Catalog #		



Micro centrifuge

PCR Machine (Thermocycler)

Gell electrophore

Microwave