Molecular and Serological Detection of Hepatitis B Virus in Risk Groups and Blood donors in Khartoum state and Determination of Co-infection with Hepatitis C Virus.

BY

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بسم الله الرحمن الرحيم

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صدق الله العظيم

سورة الرحمن الآيات (1 -4)

Dedication

To my Father ...

To my Mother . . .

To my brother, Zubeir ..

To my sisters, Ibtesaam ..
Nagat ..
El-haam ..

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Abstract

This study was carried out in Khartoum State to determine Hepatitis B virus DNA (HBV DNA) by PCR, and to correlate it with serological markers, in samples positive for HBsAg, and to determine sero-prevalence of HBV in Khartoum state using HBsAg ELISA. And to determine sero-prevalence of HCV in HBV patients (co-infection with hepatitis C virus)

Blood sample were randamlly collected from 280 blood donors, 20 multi-transfused individuals, 100 haemodialysis patients and 80 jaundiced patients.

Polymerase chain reaction technique (PCR) was used to detect HBV-DNA using oligonucleotide primers for surface antigen genome region specific (ss genome region specific) and serologic tests for HBV were performed retrospectively.

Eleven (68.7%) out of 16 blood donors were positive for HBV-DNA, two (100%) out of 20 multi-transfused patients were positive, 23 (100%) out of 100 haemodialysis patients were positive and 9 (100%) out of 80 jaundiced patients were positive for HBV-DNA.

More over, the PCR detected two (20%) positive HBV DNA out of 10 samples collected from blood donors that were negative for HBsAg by ICT and ELISA tests.

This result suggests that, detection of HBV DNA by amplification technique serves as an important supplementary tool besides serology in a number of clinical settings, especially in determining low levels of viraemia in patients with non-replicative HBV disease and chronic hepatitis, and also in a few patients with past HBV infection who could be asymptomatic carriers of HBV infection.

Immuno-chromatography technique (ICT), direct sandwich ELISA test were used to detect HBsAg and indirect ELISA to detect HCV-anti bodies in samples positive for HBsAg.

Fifteen (5.3%) out of 100 blood donors were positive for HBsAg by ICT, whereas sixteen (5, 7%) out of 100 blood donors were positive by HBsAg ELISA test. In multi-transfused patients only two (10%) out of 20 patients were found positive by both techniques. In haemodialysis patients twenty three (23%) out of 100 patients were found positive by both techniques. In jaundiced patients nine (11.2%) out of 80 patients were found positive by both techniques.

In various group studied out of 480 samples. ELISA test was able to detect the presence of HBsAg in 50 (10.4%) individuals. but by using ICT test 49 (10.2%) HBsAg positive individuals were detected. However, there was a very high agreement between ICT and ELISA and no statistical significant difference was obtained by the application of Stata NT/98 and both assays were sensitive, specific and reliable

Also the HCV-antibodies were detected in samples positive for HBsAg as co-infection in three (6%) out of 50 HBsAg positive. using indirect HCV-ELISA test using recombinant antigens. and ICT test.

out of 16 HBsAg positive blood donors. One (6.6%) was positive for HCV-antibodies by ICT, whereas two (12.5%) HBsAg positive blood donors were positive by HCV-ELISA test. In haemodialysis group only one (4.3%) out of 23 patients was found positive by both techniques,

multi-transfused and jaundiced patients were found negative for HCV-antibodies by both techniques.

In this study haemodialysis machine (46%) sharing needles (14%), sexual practice (12%), blood transfused (66%) and probably surgical instruments (30%) represent risk factors for transmission of HBV infection. Tattooing and intravenous drug users seem to have no role in predisposition to the disease in this study.

In this study only 4 (8%) patients have background about how HBV is transmitted and how it is prevented. According to the data obtained from the questionnaire this is probably due to the lack of professional and public education. In this study it has been

found that infection with hepatitis virus may be predisposed by more than one risk factors.

بسم الله الرحمن الرحيم

ملخص الأطروحة

أجريت هذه الدراسة في ولاية الخرطوم لتحديد الحامض النووي لفيروس التهاب الكبد الوبائي من نوع (ب).وايضاً مدى انتشار الإنتيجنات لهذا الفيروس في مجموعات مختلفة وايضاً لتحديد مدى انتشار الأجسام المضادة لفيروس التهاب الكبد الوبائي من نوع (ج) في المرضى المصابين بفيروس التهاب الكبد الوبائي من نوع (ب) لمعرفة مدى أنتشار الإصابة المزدوجة بفيروسات التهاب الكبد الوبائي من نوع (ب) و(ج).

تم جمع عينات من الدم عشوائياً من مجموعات مختلفة تشمل:

280عينة دم من الأشخاص المتبرعين بالدم و 100 عينة دم من مرضى الغسيل الدموي و 20 عينة من أشخاص تم لهم نقل دم أكثر من مرة و 80 عينة دم من مرضى اليرقان.

أجرى الاختبار لتحديد الحامض النووي بواسطة تفاعل البلمرة المتسلسل (PCR) بعد استخلاص الحمض النووى منقوص الأوكسجين (*DNA*) للفيروس عن طريق الكلوروفورم والفينول. تفاعل البلمرة المتسلسل المستخدم فى هذ ه الدراسة ادخل كوسيلة حديثة لتشخيص مرض التهاب الكبد الوبائي من نوع (ب). ولتحديد الأنتيجين السطحي لفيروس التهاب الكبد الوبائي من نوع (ج) استعمل تقنية الكروماتوغرافي الممنع والأليزا المباشرة (الساندوتش) وأيضاً استعمل تقنية الكروماتوغرافي الممنع والأليزا غير المباشر لتحديد الإجسام المضادة لفيروس التهاب الكبد الوبائي من نوع (ج).

بعد إجراء الاختبارات وجد أن أحدى عشرة (68.7%) من مجموعة المتبرعين وأثنين (100%) من مجموعة الأشخاص الذين تم لهم نقل دم أكثر من مرة وثلاث وعشرون (100%) من مجموعة مرضى الغسل الدموي وتسع (100%) من مجموعة مرضى اليرقات موجبة للحمض النووي لفيروس التهاب الكبد الوبائي من نوع (ب) باستخدام تفاعل البلمرة المتسلسل(PCR).

في المجموعات التي تمت دراستها خمسون عينة موجبة للأنتيجين السطحي لفيروس التهاب الكبد الوبائي من نوع (ب) استطاع أختبار تفاعل البلمرة المتسلسل(PCR) فى تحديد خمسة واربعون (90%) عينة موجبة للحمض النووي للفيروس، ايضاً وجد أن ثلاث وعشرون عينة (5.3%) من مجموعة المتبرعين للدم موجبة للأنتيجين السطحي للفيروس باستعمال تقنية الكروماتوغرافي الممنع وست عشرة (5.6%) موجبة من نفس المجموعة باستخدام الأليزا الساندوتش المباشر.اما في

مجموعة الأشخاص الذين تم لهم نقل دم أكثر من مرة وجدت عينتان (15%) موجبة باستخدام التقنيين أما في مجموعة مرضى الغسيل الدموي وجدت ثلاث وعشرون عينة (23%) موجبة باستخدام التقنيين أما في مجموعة مرضى اليرقات وجدت تسع عينات (11.2%) موجبة باستخدام التقنيتين.

في المجموعات المختلفة التي تمت دراستها (480) عينة تم الاكتشاف عن خمسون (10.4%) عينة موجبة كالأنتيجين السطحي بواسطة ، أختبار الأليزا الساندوتش المباشر وتسع واربعون (10.2%) عينة موجبة بواسطة تقنية الكروماتوغرافي الممنع.وجد هذا الفرق غير معنوي عند تحليل البيانات أحصائياً باستخدام برنامج ((Kappa statistic) Stata NT/98) وبالتالي فإن كلا الأختبارين يمكن الإعتماد عليهما في كشف الأنتيجين السطح لفيروس التهاب الكبد الوبائي (ب).

وايضاً تم الكشف عن الأجسام المضادة بفيروس التهاب الكبد الوبائي من نوع (ج) في العينات الموجبة كالأنتيجين السطحي بفيروس التهاب الكبد الوبائي من نوع (ب) لتحديد مدى أنتشار الأصابة المزدوجة بفيروس (ب) بواسطة أختبار الكروماتوغرافي الممنع والأليزا غير المباشر وجدت أن عينة واحدة (6.6%) من مجموعة المتبرعين بالدم باستعمال تقنية الكروماتوغرافي الممنع. وعينات

(12.5%) موجبة من نفس المجموعة باستخدام الأليزا غير المباشرة. أما في مجموعة مرضى الغسيل الدموي وجدت عينة واحدة (4.3%) موجبة باستخدام التقنيتين. بينما لم يتم الكشف عن الأجسام المضادة لفيروس التهاب الكبد الوبائي من (ج) في مجموعة الأشخاص الذين تم لهم نقل دم أكثر من مرة ومجموعة مرضى اليرقات.

في هذه الدراسة وجد أن ماكينة الغسيل الدموي (46%) ونقل الدم (66%) واستعمال الأدوات الملوثة (14%) وممارسة الجنس (12%) وأدوات الجراحة (30%) . ربما تمثل عوامل للخطورة في نقل المرض ، بينما الوشم والإبر المخدرة ربما لم يلعبا دوراً في أنتشار المرض في المجموعات المختلفة التي تمت دراستها في السودان.

في هذه الدراسة يوجد أربعة مرضى (8%) فقط من مجموعة الخمسين مريضاً لديهم خلفية عن كيفية أنتقال مرض التهاب الكبد الوبائي من نوع (ب) والوقاية منه.

CHAPTER ONE

INTRODUCTION

Hepatitis B (HB) is a serious and common infectious disease of the liver (Chisari and Ferrari,1997; Ganem and Schneider, 2001; Hollinger and Liang, 2001; Mahoney and Kane, 1999; Kane,1998). Hepatitis B has also been called type B hepatitis, serum hepatitis, homologous serum jaundice (Mahoney and Kane, 1999; Robinson, 1995)

Hepatitis B is caused by hepatitis B virus (HBV) an enveloped virus containing a partially double stranded, circular DNA genome, and classified within the family Hepadnaviridae (Chisari and Ferrari, 1997; Mahoney and Kane, 1999; Robinson, 1994; Robinson, 1995). Of the many viral causes of human hepatitis few are of greater global importance than hepatitis B virus (HBV) (Ganem and Schneider, 2001; Hollinger and Liang, 2001; Mahoney and Kane, 1999; Robinson, 1995).

The severe pathological consequences of persistent HB infections include the development of chronic hepatic insufficiency, cirrhosis, and hepatocelluar carcinoma (HCC). In addition, HBV carriers can transmit the disease for many years (Chisari and Ferrari, 1997; Mahoney and Kane, 1999; Robinson, 1994; Robinson, 1995).

Globally, HBV causes 60 - 80% of the world's primary liver cancers. More than 2 000 million people alive today have been infected with HBV at some time in their lives, of these about 350 million remain infected chronically and become carriers of the virus (Chisari and Ferrari, 1997; Hollinger and Liang, 2001; Mahoney and Kane, 1999; World Health Organization, 2001). Every year there are over 4 million acute clinical cases of HBV, and about 25% of carriers, 1 million people a year, die from chronic active hepatitis, cirrhosis or primary liver cancer (World Health Organization, 2001)

HBV is transmitted through percutaneous or parenteral contact with infected blood, Body fluids, and by sexual intercourse (Ganem and Schneider, 2001; Gitlin, 1997; Hollinger and Liang, 2001; Mahoney and Kane, 1999). Sexual intercourse with multiple

partners or with persons who have multiple partners can be dangerous. Hepatitis B is the only sexually transmitted infection for which there is a protective vaccine (Mahoney and Kane, 1999). The most important mode of HBV transmission globally is perinatal, from the mother to her newborn baby (Hollinger and Liang, 2001). Another important mode of HBV transmission is from child to child during early life resulting from blood contact (Gitlin, 1997).

The world can be divided into three areas where the prevalence of chronic HBV infection is high (>8%), intermediate (2 – 8%), and low (<2%) (Mahoney and Kane., 1999).

The lack of convenient culture system for HBV implies the use of molecular biology to assess viremia. Amplification of viral nucleic acid by the polymerase chain reaction (PCR) provides a highly sensitive and antigen- antibody-independent method to detect ongoing viral infection (Choo *et a.*,1989; Garson *et a.*,1990; Inchaups *et al*, 1991; Okamoto *et a.*, 1990). PCR is useful in diagnosis of virus infection when viral antigens (Ags) or virus specific Antibodies (Abs) can not be detected and when the presence of viral nucleic acid may be the only evidence of infection (Bell *et al*, 1997).Hepatitis B DNA is one of the first things that can be detected in the blood stream after initial infection. It can be detected as soon as 1 week after infection (Pawlotskey, 2003; Servoss, 2004).

Several studies were published on sero-prevalence of HB in the Sudan (Hymas *et al*,1989; McCarthy *et al*, 1989; Elshafie, 1992; Khalil *et al*, 1996; Fahal *et al*,1997; and Suliaman *et al*,1997).)

Objectives:

The aim of this study was to:

1- To study by ELISA the prevalence of HBsAg and risk factors among:

(a) Blood donors patients.

(b) Multi-transfused patients.

(c) Jaundice patients.

(d) Haemodialysis patients

2- To improve laboratory diagnosis of HBV infection in the Sudan through introduction of polymerase chain reaction (PCR).

5- To determine the prevalence of HCV among HBsAg positive patients as co-infection.

CHAPTER TWO
LITERATURE REVIEW

2.1 Definition:

Hepatitis is a systemic disease primarily involving the liver (Jawetz *et al*, 1998). This infection or inflammation is the response of tissue injuries and show swelling, redness, increase in temperature and loss of function (Weir and Steward, 1997). Hepatitis literally means any inflammatory lesion of the liver. In practice the term hepatitis is not used for local lesions, such as an abscess but only when there is diffuse involvement of the liver.

Hepatitis may be acute or chronic infection (Soni *et al*, 1995). Hepatitis viruses produce acute inflammation of the liver, resulting in a clinical illness characterized by fever, gastrointestinal symptoms such as nausea, vomiting and jaundice (Jawetz *et al*, 1998).

2.2 Infectious agents:

Hepatitis can be caused by a variety of organisms and toxins (William *et al*, 2001). Accordingly hepatitis may be classified as infectious and non infectious hepatitis. Non-infectious is caused by alcohol, toxins and chemicals or by drugs, whereas infectious hepatitis is caused by different microbial agents. However, viruses are considered the main causative agents of infectious hepatitis (Jawetz *et al*, 1998).

Most cases of acute viral hepatitis seen in children and adult are caused by one of the following agents: in viral hepatitis type A, B, C, D, E and G as shown in Table 1 and 2. Secondary hepatitis due to other viruses includes yellow fever, Marburg virus, and

Ebola virus. Also acute hepatitis may occur as an unusual complication of infection by a number of non-hepatotropic viruses such as Rubella virus, herpes simplex virus, Epistain-Barr virus and cytomegalovirus virus (Mowat *et al*, 2001).

Table 1: Types of viral hepatitis (Albert *et al*, 1991).

Agent (Hepatitis virus)	Preferred terminology	Equivalent terminology
Hepatitis A	HAV	Infectious hepatitis Epidemic jaundice Short incubation hepatitis
Hepatitis B	HBV	Serum or transfusion hepatitis Long incubation hepatitis Homologous serum jaundice
Hepatitis C	HCV	Parenterally transmitted non- A, Non-B hepatitis
Hepatitis D	HDV	Delta hepatitis
Hepatitis E	HEV	Enterically transmitted non-A, non-B hepatitis

2.3 Viral hepatitis:

2.3.1 Hepatitis type A (HAV):

It is caused by a picorna virus genus of the hepatovirus (Minor, 1991) with a linear, single-stranded, positive-sense RNA genome, with a size of 7.5 kb and is non enveloped virus (William *et al*, 2001). There is no antigenic cross-reactivity with HBV or with other hepatitis viruses (Jawetz *et al*., 1998). Virion RNA acts as mRNA and is translated into a polyprotein, which is then cleaved to individual proteins (Murphy *et al*, 1999). The virus is transmitted by oro-feacal route, or ingesting contaminated food or water(Snydman *et al*, 1998), and have short incubation period ranging between 20 to 50 days, therefore the disease is named short incubation viral hepatitis (Hollinger and

Ticehurst, 1996). Formation-inactivated whole viruses vaccine is available (William *et al*, 2001).

2.3.2 Hepatitis type D (HDV):

HDV or "delta agent" is found in nature only as a result of co-infection with HBV (William *et al*, 2001). HDV is a defective virus, which requires HBV to serve as a helper virus; it is often associated with severe form of hepatitis in HBs Ag positive patients (Jawetz *et a*., 1998). HDV has a circular, single stranded negative-sense RNA genome (William *et al*, 2001). It is transmitted by blood and blood products (Table 2) and it does not appear to be transmitted sexually (William *et al*, 2001).

No loss of activity occurred following treatment with ethylene diaminetetra-acetic acid, but partial or complete loss of activity was detected after treatment with alkali, thiocyanate, trichloroacetic acid and proteolytic enzyme (Jawetz *et al*, 1998). Infection acquired at the same time with HBV is called co-infection but if acquire at any time after HBV infection is called super-infection which is much more severe and becomes chronic in as many 80% of cases (Hoofinagle, 1989)

2.3.3 Hepatitis type E (HEV):

Hepatitis type E is caused by a virus that belong to the family Caliciviridae, it has been identified as non -A, non B agent. It is different from the other hepatitis virus it is transmitted enterically and occurs in epidemic form in developing countries (Jawetz *et al*, 1998). It virion is non enveloped, with icosahedral symmetry. The genome is composed of a single molecule of linear positive-sense, single stranded RNA, and it replicate in the cytoplasm (Murphy *et al*, 1999). HEV is a major cause of enterically transmitted, water-borne hepatitis in developing countries. No antiviral treatment or vaccine is currently available (William *et al*, 2001).

2.3.4 Hepatitis type G (HGV):

Hepatitis type G caused by a new member of the family flaviviridae, transmitted by blood and blood products (Karayiannis *et al*, 1997). It is associated with morbidity and mortality among pregnant women. Hepatitis G virus has been isolated but has not been well characterized (Jawetz *et al*, 1998).

2.3.5 Hepatitis C Virus (HCV).

HCV was discovered in 1989 by investigators at Chiron, Inc. Portions of the HCV genome were isolated by screening cDNA expression libraries made from RNA and DNA from chimpanzees infected with serum from a patient with post-transfusion non-A, non-B hepatitis. [Prior to the discovery of HCV, hepatitis following blood transfusion that was not caused by hepatitis A or hepatitis B was referred to as non-A, non-B hepatitis]. To identify portions of the genome that encoded viral proteins, the libraries were screened with antibodies from patients who had non-A, non-B hepatitis. These investigators went on to show that the virus they identified was responsible for the vast majority of cases of non-A, non-B hepatitis. They called the new virus hepatitis C virus (HCV). Subsequently, the complete genomes of various HCV isolates were cloned and sequenced by several groups.

HCV is a positive, single-stranded RNA virus in the Flaviviridae family. The genome is approximately 10,000 nucleotides and encodes a single polyprotein of about 3,000 amino acids. The polyprotein is processed by host cell and viral proteases into three major structural proteins and several non-structural proteins necessary for viral replication. Several different genotypes of HCV with slightly different genomic sequences have since been identified that correlate with differences in response to treatment with interferon alpha (World health organization 2002).

Approximately 170,000,000 peoples worldwide are infected with HCV. The virus is transmitted primarily by blood and blood products. The majority of infected individuals has either received blood transfusion prior to 1990 (when screening of the blood supply for HCV was implemented) or has used intravenous drugs. Sexual transmission between monogamous couples is rare but HCV infection is more common in sexually promiscuous individuals. Perinatal transmission from mother to fetus or infant is also relatively low but possible (less than 10%). Many individuals infected with HCV have no obvious risk factors. Most of these persons have probably been inadvertently exposed to contaminated blood or blood products. (http://www.hon.ch/Library/Theme/HepB/ virology.html).

2.3.6 Hepatitis type B (HBV):

HBV is the cause of serum hepatitis (Jawetz *et al,* 1998) and is a leading cause of chronic hepatitis, cirrhosis and hepatocelular carcinoma (HCC) (William *et al,* 2001). It is classified as hepadnavirus, genus orthohepadna virus (Jawetz *et a.,* 1998). HBV virion DNA is a relaxed circular, partially duplex species of 3.2 kb, have an envelope (Knipe *et al,* 2001).HBV infection results from parenteral exposure to virus-containing blood or blood products or from sexual contact with an infected host (Table 2). HBV is transmitted mainly by blood and known as serum hepatitis or long-incubation hepatitis (Hersh *et al,* 1971), but breast milk does not appear to play a role in transmission (Beasley *et al,* 1975).

Table 2: Characteristics of hepatitis viruses (Jawetz *et al*, 1998).

Virus	Hepatitis A	Hepatitis B	Hepatitis C	Hepatitis D	Hepatitis E
Family	Picorna-viridae	Hepadna-viridae	Flaviviridae	Unclassified	Calicivirida e
Genus	Hepatovirus	Ortho-hepadanavirus	Unnamed	Delta virus	Unnamed
Virion	27 nm, icosahedral	42 nm spherical	30 – 60 nm spherical	35 nm spherical	30 – 32 m icosahedral
Envelope	No	Yes (HBsAg)	Yes	Yes (HBsAg)	No
Genome	ssRNA	dsDNA	ssRNA	SsRNA	ssRNA
Genome size	7.5 kb	3.2 kb	9.5 kb	1.7 kb	7.6 kb
Stability	Heat- and acid-stable	Acid-sensitive	Ether-sensitive, acid-sensitive	Acid-sensitive	Heat-stable
Trans-mission	Fecal-oral	Parenteral	Parenteral	Parenteral	Fecal-oral
Prevalence	High	High	Moderate	Low, regional	Regional
Fulminant disease	Rare	Rare	Rare	Frequent	In pregnancy
Chronic disease	Never	Often	Often	Often	Never
Oncogenic	No	Yes	Yes	?	No

Hepatitis G virus has been isolated but has not been characterized enough to be included here.

Infectious HBV is present in all body fluids of an infected individual, therefore, blood, semen, saliva, serve as source of infection (William *et al*, 2001). Some infected patients never recover completely and are called carriers. Treatment is not very effective, so prevention is crucial Recombinant vaccines provide protection in 90 – 95% of healthy persons; it can give safety to infants, children and adult in three doses (Mulley *et al*, 1982).

2.4 Structure and biology of hepatitis B virus:

The hepatitis B virus, a hepadnavirus, is a 42 nm partially double stranded DNA virus, composed of a 27 nm nucleocapsid core (HBcAg), surrounded by an outer lipoprotein coat (also called envelope) containing the surface antigen (HBsAg).

Three particulate structures are encountered in the sera of the infected people. The most predominant form is that of a small spherical particle 22 nm in diameter with a second filament form 22 nm in diameter in lesser concentration than the spherical. Both consist of an envelope and carry hepatitis B surface antigen (HBsAg) they are not infectious by themselves. A third type is a complex spherical large form with a diameter of 42 nm it is termed Dane particle and consists of an outer envelope containing (HBsAg) and an inner 27 nm nucleocapsid core.

The Dane particle is thought to be the complete infectious virus of hepatitis B. The inner particle contains hepatitis B core antigen (HBcAg) within which double stranded DNA is found with DNA polymerase as well and protein Kinase. In addition hepatitis B `e` antigen (HBeAg) is associated with (HBsAg) has `a` variable epitope and different subtypes sharing `a` common epitope called a which is linked with two mutually exclusive determinant "d" or "γ" and "w" or "r" giving the four main subtypes "adw", "ayw", "adr", "aγr". The different subtypes are useful as epidemiological markers of HBV and are not markers of virulence or chronicity (Bancroft *et a,* 1972; Mosely *et a,* 1972). HBsAg is transcribed to produce three mRNAs; S, M and L. Each mRNA produces a protein contains the major S protein. The M mRNA and the L mRNA produce (pre S1+S) and (pre S2+S) respectively. HBcAg unlike HBsAg is found mostly in the nucleus of the

infected hepatocyte and generally is not found to be circulating. It must be extracelular at some time during infection to be detected by the immune system.

HBeAg exists in a soluble, non-particulate form in contrast to HBsAg and HBcAg. Since the Dane particle is thought to be the infectious virion, the presence of e antigen is a marker of the complete virus and its infectivity. The persistence of HBeAg beyond 8 weeks after the acute hepatitis B may be indicative for the occurrence of chronic hepatitis with more aggressive hepatic lesion than the presence of anti HBe. HbeAg usually found in sera is reactive for HBsAg (Neurath and Kent, 1988; Lutwick, 1991).

2.5 Classification of HBV:

The family of hepadnaviruses (Hepadnaviridae) comprises members recovered from a variety of animal species, including the woodchuck hepatitis virus (WHV), the ground squirrel hepatitis virus (GSHV), and the duck HBV. Common features of all these viruses are enveloped virions containing 3 to 3.3 kb of relaxed circular, partially duplex DNA and virion-associated DNA- dependent polymerases that can repair the gap in the virion DNA template and have reverse transcriptase activities. Hepadnaviruses show narrow host ranges, growing only in species close to the natural host, like gibbons, African green monkeys, rhesus monkeys, and wooly monkeys (Hollinger and Liang, 2001; Robinson, 1994; Robinson, 1995).

Mammalian hepadnaviruses fail to propagate in cell culture (Mahoney and Kane, 1999; Robinson, 1994; Robinson, 1995). Intracellular HBV is non-cytopathic and causes little or no damage to the cell (Chisari and Ferrari, 1997; Ganem and Schneider, 2001; Hollinger and Liang, 2001; Mahoney and Kane, 1999).

2.6 The hepatitis B virus Replication:

The HBV virion binds to a receptor at the surface of the hepatocyte (Ganem and Schneider, 2001). Viral nucleocapsids enter the cell and reach the nucleus, where the viral genome is delivered (Chisari *et al,* 1997; Ganem and Schneider, 2001; Mahoney and Kane, 1999).

In the nucleus, second-strand DNA synthesis is completed and the gaps in both strands are repaired to yield a covalently closed circular (ccc) supercoiled DNA molecule that serves as a template for transcription of four viral RNAs that are 3.5, 2.4, 2.1 and 0.7 kb long (Chisari *et al,* 1997; Ganem and Schneider, 2001; Mahoney and Kane, 1999; Robinson, 1995).

These transcripts are polyadenylated and transported to the cytoplasm, where they are translated into the viral nucleocapsid and precore antigen (C, pre-C), polymerase (P), envelope L (large), M (medium, S (small), and transcriptional transactivating proteins (X) (Chisari *et al,* 1997; Ganem and Schneider, 2001; Mahoney and Kane, 1999; Robinson, 1995). The envelope proteins insert themselves as integral membrane proteins into the lipid membrane of the endoplasmic reticulum (ER).

The 3.5 kb species, spanning the entire genome and termed pregenomic RNA (pgRNA), is packaged together with HBV polymerase and a protein kinase into core particles where it serves as a template for reverse transcription of negative-strand DNA. The RNA to DNA conversion takes place inside the particles. The new, mature, viral nucleocapsids can then follow two different intracellular pathways, one of which leads to the formation and secretion of new virions, whereas the other leads to amplification of the viral genome inside the cell nucleus(Ganem and Schneider, 2001; Mahoney and Kane, 1999).

The precore polypeptide is transported into the ER lumen, where its amino- and carboxy-termini are trimmed and the resultant protein is secreted as precore antigen. The X protein contributes to the efficiency of HBV replication by interacting with different transcription factors, and is capable of stimulating both cell proliferation and cell death (Ganem and Schneider, 2001; Mahoney and Kane, 1999).

2.7 Genome Structure and Proteins of HBV:

HBV virion DNA is a relaxed circular, partially duplex molecule of 3.2 kb, whose circularity is maintained by 5'cohesive ends (Hollinger and Liang, 2001; Robinson, 1995

). The positions of the 5' ends of both strands map to the regions of short (11 nucleotides) direct repeats (DRs) in viral DNA. The 5' end of the minus strand DNA maps within the repeat termed DR, while plus strand DNA begins with DR2. These repeats are involved in priming the synthesis of their respective DNA strands (Chisari *et al,* 1997).

2.8 Pathogenesis and pathology:

Although the biological incubation period of HBV is short, the clinical incubation period is long. During the clinical incubation of HBV, the infected individual despite being HBsAg positive, remains well without biochemical or histologic evidence of liver disease. This observation suggests that the HBV by itself is not cytopathogenic for the hepatocyte (Lutwick, 1991). HBV infection contracted early in life may lead to chronic hepatitis, then to cirrhosis, and finally to HCC, usually after a period of 30 to 50 years. Once infected with HBV, males are more likely to remain persistently infected than women, who are more likely to be infected transiently and to develop anti-HBs.

Damage to the liver cells is attributed to the host immunologic response due to viral antigens. The core and surface antigens induce both B and T cells. The damage could result from both antibody dependent cytotoxicity and T cell cytotoxic action but action of cytotoxic T, cells specific for HBcAg is the major factor (Dudley *et al*, 1972). It is possible that in man HBV is not carcinogenic by a direct viral mechanism. Instead, the role of HBV may be to cause chronic liver cell damage with associated host responses of inflammation and liver regeneration that continues for many years. This pathological process, especially when leading to cirrhosis, may be carcinogenic without involving a direct oncogenic action of the virus.

The expression of HBV proteins and the release of virions precedes biochemical evidence of liver disease. Moreover, large quantities of surface antigen can persist in liver cells of many apparently healthy persons who are carriers. HBV is therefore not directly cytopathic (Chisari and Ferrari, 1997).

2.9 Endemicity of hepatitis B virus:

There are no seasonal preferences for primary HBV infections (Hollinger and Liang, 2001; Robinson, 1994).

Hepatitis B is highly endemic in all of Africa, some parts of South America, Alaska, northern Canada and parts of Greenland, Eastern Europe, the eastern Mediterranean area, Southeast Asia, China and the Pacific Islands, except Australia, New Zealand and Japan. In most of these areas, 5 to 15% of the population are chronically infected carriers of HBV, and in some areas may also carry HDV, which may lead to severe liver damage (Mahoney and Kane, 1999).

2.10 Epidemiology of hepatitis B virus:

The hepatitis B virus is a ubiquitous virus with a global distribution (Hollinger and Liang, 2001; Robinson, 1994). Hepatitis B is one of the 4 world's most common and serious infectious diseases. It is estimated that more than one third of the world's population has been infected with the hepatitis B virus. About 5% of the populations are chronic carriers of HBV, and nearly 25% of all carriers develop serious liver diseases such as chronic hepatitis, cirrhosis, and primary hepatocullular carcinoma. HBV infection causes more than one million deaths every year (Hollinger and Liang, 2001; Mahoney and Kane, 1999; Robinson, 1994).

The HBsAg carrier rate varies from 0.1 to 20% in different populations around the word. The incidence of the HBsAg carrier state in populations is related most importantly to the incidence and age of primary infection (Mahoney and Kane, 1999).

In low-risk areas of the world, the highest incidence of the disease is seen in teenagers and young adults. Deposit the low incidence of disease seen in the general population, certain groups who are sexually promiscuous or who have frequent contact with blood or blood products have a high rate of HBV infection. Nevertheless, the availability of an effective vaccine, optimized blood donor screening, and better sterilization procedures for blood derivatives have lowered substantially the infection risk (Hollinger and Liang, 2001).

In endemic areas of Africa and Asia, different epidemiological patterns are seen. In these regions, most infections occur in infants and children as a result of maternal-neonatal transmission or close childhood contact, although percutaneous exposure with

contaminated needles or following unsafe injections is always a possibility in these countries (Hollinger and Liang, 2001; Mahoney and Kane, 1999).

The chronic liver disease and HCC associated with HBV infections are among the most important human health problems in high-prevalence regions (World Health Organization, 2002).

2.11 Transmission:

Currently, there are four recognized modes of transmission From mother to child at birth (perinatal), by contact with an infected person (horizontal), by sexual contact and by parenteral (blood-to-blood) exposure to blood or other infected fluids (Hollinger and Liang, 2001; World Health Organization, 2002).

There is considerable variation between areas, countries and continents as to the age at which most transmission takes place. There can be carriers with or without hepatitis (Robinson, 1995). There is no convincing evidence that airborne infections occur and faeces are not a source of infection, since the virus is inactivated by enzymes of the intestinal mucosa or derived from the bacterial flora. HBV is not transmitted by contaminated food or water, insects or other vectors (Hollinger and Liang, 2001; Robinson, 1995).

HBsAg has been found in all body secretions and excretions. However, only blood, vaginal and menstrual fluids, and semen have been shown to be infectious (Hollinger and Liang, 2001; Mahoney and Kane, 1999; Robinson, 1994; Robinson, 1995).

Transmission occurs by percutaneous and permucosal exposure to infective body fluids. Percutaneous exposures that have resulted in HBV transmission include transfusion of unscreened blood or blood products, sharing unsterilized injection needles for intra venous drug use, haemodialysis, acupuncture, tattooing and injuries from contaminated sharp instruments sustained by hospital personnel. Sexual and perinatal HBV transmission usually result from mucous membrane exposures to infectious blood and body fluids. Perinatal transmission is common in hyper endemic areas of Southeast

Asia and the Far East, especially when HBsAg carrier mothers are also HBeAg positive (Hollinger and Liang, 2001, Mahoney and Kane, 1999, Robinson, 1995).

Infection may also be transmitted between household contacts and between sexual partners, either homosexual or heterosexual, and in oddler-aged children in groups with high HBsAg carrier rates (Hollinger and Liang, 2001; Mahoney and Kane, 1999).

HBV is stable on environmental surfaces for at least 7 days, and indirect inoculation of HBV can occur via inanimate objects like toothbrushes, bay bottles, toys, razors, eating utensils, hospital equipment and other objects by contact with mucous membranes or open skin breaks. Infectious HBV can be present in blood without detectable HBsAg, so that the failure to detect antigen does not exclude the presence of infectious virus (Robinson, 1994).

The source of infection cannot be identified in about 35% of cases.

The natural reservoir for HBV is man, closely related hepadnaviruses have been found in woodchucks and ducks, but they are not infectious for humans (Ganem and Schneider, 2001). The reuse of the same, unsterilized needle and syringe for vaccination of many different children accounts for many unnecessary HBV infections. (Robinson, 1994)

2.11.1The role of non-human primates in the transmission of HBV:

The only non-human primates that can develop productive HBV infection are the great apes (e.g. chimpanzees and gorillas), Chimpanzees have served as the model for the study of HBV infection for over 20 years (Holliger and Liang, 2001) though chimpanzees may be infected in nature, there is no evidence that they are important sources for transmission for human infection, because transmission from infected individuals requires specific patterns and intimate contact (Robinson, 1995).

Gibbons are susceptible to HBV and have been infected successfully experimentally and also naturally by contact in captivity (Thornton *et al*, 2001).

2.12 Hepatitis B virus DNA and hepatocellular carcinoma:

More than 85% of hepatocullular tumours examined harbor integrated HBV DNA, often multiple copies per cell. The viral DNA integrants are usually highly rearranged, with deletions, inversions, and sequence reiterations all commonly observed. Most of these rearrangements ablate viral gene expression, but the integrations alter the host DNA (Ganem and Schneider, 2001; Robinson, 1994; Zuckerman, 1996).

There is no similarity in the pattern of integration between different tumours, and variation is seen both in the integration site(s) and in the number of copies or partial copies of the viral genome. The molecular mechanisms by which hepadnaviruses predispose to malignancy are still unknown (Zuckerman, 1996).

2.13 HBV mutants:

Naturally occurring envelope, precore, core, and polymerase variants have been described (Gitlin, 1997; Hollinger and Liang, 2001; Mahoney and Kane, 1999). Envelope antigenic variants may have a selective advantage over wild type under immune selection pressure, as observed in some cases after hepatitis B IG (HBIG) treatment of HBV vaccination, an epidemiological shift has not been observed yet (World Health Organization, 2002). A number of precore mutations preventing HBeAg synthesis have been identified in HBeAg negative carriers. The most frequent variant has a G to A point mutation at nucleotide 83.

HBV is far more heterogenous than is generally thought. The HBV genome seems not to be characterized by a single representative genomic molecule, but by a pool of genomes which differ both in structure and function. The public health importance of mutant hepatitis B viruses is currently under debate. Further studies and a strict surveillance to detect the emergence of these viruses are crucial for a correct evaluation of the effectiveness of current immunization strategies (Mahoney and Kane, 1999 ;Zuckerman, 1996; Zuckerman, 2000).

2.14 Antigenicity of hepatitis B virus:

All three coat proteins of HBV contain HBsAg, which is highly immunogenic and induces anti-HBs (humoral immunity). Structural viral proteins induce specific T-lymphocytes, capable of eliminating HBV-infected cells (cytotoxic T-cells; cellular immunity), (Chisari and Ferrari, 1997; Hollinger and Liang, 2001). HBsAg is heterogeneous antigenically, with a common antigen designated a, and two pairs of mutually exclusive antigens, d, and y, and w (including several sub determinants) and r, resulting in 4 major subtype adw, ayw, adr and ayr. (Mahoney and Kane, 1999; Robinson, 1994; Robinson, 1995).

The core antigen shares its sequences with the e antigen (HBeAg), identified as a soluble antigen, but no cross reactivity between the two proteins is observed (Robinson, 1994; Robinson, 1995).

Viral oligopeptides of 8-15 amino acids are loaded on host cell MHC-class I molecules and are transported to the surface of the cell. HBV-specific T-lymphocytes can then detect infected cells and destroy them. This cell deletion triggered by inflammation cells may result in acute hepatitis. When the infection is self limited, immunity results. If HBV is not eliminated, a delicate balance between viral replication and immunodefence prevails which may lead to chronic hepatitis and liver cirrhosis. In chronically infected cells the HBV DNA may integrate into the host cell DNA. As a long term consequence, integration may lead to hepatocellular carcinoma (Hollinger and Liang, 2001; Mahoney and Kane, 1999; Zuckerman, 1996).

2.15 Stability of hepatitis B virus:

The stability of HBV does not always coincide with that of HBsAg (Hollinger and Liang, 2001). Exposure to ether, acid (pH 2.4 for at least 6 h), and heat (98ºC for 1min; 60ºfor 10 h) does not destroy immunogenicity or antigenicity. However, inactivation may be incomplete under these conditions if the concentration of virus is excessively high. Antigenicity and probably infectivty are destroyed after exposure of HBsAg to 0.25% sodium hypochlorite for 3min (Hollinger and Liang, 2001).

Infectivity is lost after autoclaving at 121ºC for 20 min or dry heat treatment at 160ºC for 1 h (Hollinger and Liang, 2001; Robinson, 1995).

HBV is inactivated by exposure to sodium hypochlorite (500 mg free chlorine per litter) for 10 min, 2% aqueous glutaraldehyde at room temperature for 5 min, heat treatment at 98ºC for 2 min, sporicidin (pH 7.9), formaldehyde at 18.5 g/l (5% formalin in water), 70% isopropyl alcohol, 80% ethyl alcohol at 11ºC for 2 min, wescodyne diluted 1:213, or combined β-propriolactone and UV irradiation (Hollinger and Liang, 2001; World Health Organization, 1998). HBV retains infectivity when stored at 30ºC to 32ºC for at least 6 months and when frozen at-15ºC for 15 years. HBV present in blood can withstand drying on a surface for at least a week (Hollinger and Liang, 2001; Robinson, 1995).

2.16 Persistent of Hepatitis B Infection :

Persistent or chronic HBV infection is among the most common persistent viral infections in humans. More than 350 million people in the world today are estimated to be persistently infected with HBV. A large fraction of these are in eastern Asia and sub-Saharan Africa, where the associated complications of chronic liver disease and liver cancer are the most important health problems (Robinson, 1995).

A small number of long-established chronic carriers apparently terminate their active infection and become HBsAg-negative (about 2% per year). Survivors of fulminant hepatitis rarely become infected persistently, and HBsAg carriers frequently have no history of recognized acute hepatitis (World Health Organization, 2002).

The infecting dose of virus and the age of the person infected are important factors that correlate with the severity of actual or chronic hepatitis B (Mahoney and Kane, 1999; Robinson, 1995).

Primary HBV infection may be associated with little or no liver disease or with acute hepatitis of severity ranging from mild to fulminant (Robinson, 1995). HBV

infection is transient in about 90% of adults and 10% of newborn, and persistent in the remainder (Mahoney and Kane, 1999).

2.17 Clinical features:

The clinical incubation period of HBV varies between 2-6 months with a mean of approximately 3 months. The onset is insidious. The early symptoms of acute stage include fatigue and malaise with or without less common serum sickness type illness. Fever is less common. Acute hepatic manifestation of HBV ranges from asymptotic to severe icteric disease. HBV infection is an iceteric in 50% of cases or more. The jaundice when present is preceded by nausea, vomiting, anorexia and mild fever. These symptoms and the jaundice fade in the second or third weeks of illness. A small percentage (0.2 – 0.5%) of icteric hepatitis B cases may develop acute fulminate hepatitis which carries a high fatality rate (70 – 90%). A significant subacute hepatic necrosis may develop to post-necrotic cirrhosis (Lutwick, 1991). HBV infection may shows extra-hepatic manifestations including skin rash, polyarteritis and glomerulonephritis.

Although most cases of acute HBV recover within 4 months, the typical HBV infection is characterized by the development of persistent surface antigenaemia (Nordenfelt, 1990; Lutwick, 1991).

The determinant factors for developing persistent infection is not yet clear. Epidemiological studies showed that age, sex and immunologic status of the infected person are important factors (Nordenfelt *et al*, 1982; Hoofinagle and Alter, 1984; Peutherer, 1992). Neonates exposed to HBV infection at the time of birth and children less than 5 years are more amenable to develop persistent infection than adults. Women are less predisposed to develop into chronic asymptotic carriers than men. Finally immuocompromised individuals are more amenable to develop persistent infection. In prospective studies conducted in dialysis units it was found that the renal dialysis patients develop into chronic carriers more than controls. Down's syndrome also makes the individuals more susceptible to the persistent infection (Nordenfelt *et al*, 1982; Hoofinagle and Alter, 1984).

2.17.1 Clinical features of acute hepatitis B infection :

The acute form of the disease often resolves spontaneously after a 4-8 week illness. Most patients recover without significant consequences and without recurrence. However, a favorable prognosis is not certain, especially in the elderly who can develop fulminating, fatal cases of acute hepatic necrosis. Young children rarely develop acute clinical disease, but many of those infected before the age of seven will become chronic carriers (Chisari and Ferrari, 1997; Hollinger and Liang, 2001; Mahoney and Kane, 1999; Robinson, 1994; Robinson, 1995).

2.17.2 Clinical features of chronic hepatitis B :

Although most adult patients recover completely from an acute episode of hepatitis B, in a significant proportion, 5 to 10%, the virus persists in the body. This figure is much higher in children 70 to 90% of infants infected in their first few years of life become chronic carriers of HBV (Mahoney and Kane., 1999; Robinson., 1995).

Chronic hepatitis can cause serious destructive disease of the liver and it contribute greatly to the worldwide burden of the disease (Mahoney and Kane, 1999). Chronic hepatitis generally develops over many years during which individual patients will pass through a number of disease states (World Health Organization, 2002)

Chronic hepatitis B is a prolonged (> 6 months) infection with persistent serum levels of HBsAg and lgG anti-HBsAg antibody response. HBV DNA and HBeAg are often detectable at high concentrations, but may disappear if viral replication ceases or if mutations occur that prevent the synthesis of the viral precore protein precursor of HBeAg. The associated inflammatory liver disease is variable in severity. It is always much milder than in acute hepatitis B, but it can be last for decades and proceed to cirrhosis, and it is associated with a 100-fold increase in the risk of developing a hepatocellular carcinoma (Hollinger and Liang, 2001; Robinson, 1995)

2.18 HBV and hepatocellular carcinoma (HCC):

A number of HBV patients with chronic hepatitis will develop hepatocellular carcinoma. Persons at increased risk of developing HCC include adult male and chronic hepatitis B patients with cirrhosis who contracted hepatitis B in early childhood. Only about 5% of patients with cirrhosis develop HCC patients have underlying cirrhosis (Mahoney and Kane, 1999; Hollinger and Liang, 2001; Robinson, 1995).

The incidence of HCC varies with geography, race, age, and sex. HCC is responsible for 90% of the primary malignant tumours of the liver observed in adults. Worldwide, it is the seventh most frequent cancer in males and ninth most common in females. Liver cancer is the causes of more than 500 000 deaths annually through the world, with a male: female ratio of 4:1. The frequency of HCC follows the same general geographic distribution patterns as that of persistent HBV infection. The age distribution of patients with clinically recognized tumours suggest that these tumours appear after a mean duration of about 35 years of HBV infection (Hollinger and Liang, 2001; Robinson, 1995).

HBV causes 60-80% of the world's primary liver cancer, and primary liver cancer is one of the three most common causes of cancer deaths in males in east and south-east Asia, the pacific basin, and sub-Saharan Africa. Primary liver cancer is the eight most common cancers in the world, up to 80% of liver cancers are due to HBV (Robinson, 1995). When HCC presents clinically, the disease is fatal (World Health Organization, 2002).

2.19 Co-infection or super infection with HDV:

Hepatitis Delta virus (HDV) is a defective virus that is only infectious in the presence of active HBV infection. HDV infection occurs as either co-infection with HBV or super infection of an HBV carrier. Co-infection usually resolves. Super infection, however, causes frequently chronic HDV infection and chronic active hepatitis. Both types of infections may causes fulminant hepatitis and routes of transmission are similar to those of HBV (Centers for Disease control and prevention, 1991; Robinson, 1995).

Preventing acute and chronic HBV infection of susceptible persons by vaccination will also prevent HDV infection (Centers for Disease control and prevention, 1991; Mahoney and Kane, 1999).

2.20 Immunological response to HBV infection:

The humoral immune response to HBV becomes detectable some weeks after the appearance of HBsAg. The first antibody detected is anti-HBc antibody. Although anti-HBsAg may be produced during the surface antigenemic period it is usually undetectable. Anti-HBc antibody continues to rise during acute infection to high titers in early convalescence. These antibodies are initially IgM and then IgG. HBeAg becomes detectable late in the incubation period and remains detectable for a variable time during acute disease. After HBeAg is cleared from the serum, anti-HBe becomes detectable either immediately or within several weeks. Following HBsAg is present but high levels of anti-HBc (including IgM anti-HBc) are found (Lutwick, 1991).

In some cases anti-HBs antibody titers continue to rise following recovery from acute hepatitis B for 6-12 months and in contrast to anti-HBc, the individuals with the highest titers of anti-HBs exhibit the shortest periods of HBsAg positivity. In convalescence the three antibodies can be found for sometime (Lutwick, 1991; Jawetz *et al*, 1998).

Anti-HBe was first. Anti-HBs is protective and usually signifies immunity against future infection. Anti-HBc persists for years after the acute infection and it's not protective experimentally. The persistence of anti-HBc
antibody titers at high levels indicates continuing production of HBcAg in patients who do not clear HBsAg. HBeAg may also continue to circulate in these patients. HBeAg may eventually be replaced by anti-HBe in chronic carriers some chronic HBsAg carriers seem to have a specific B lymphocyte defect of anti-HBs production and some defects in regulatory T cell (Lutwick, 1991; Jawetz *et al*, 1998).

2.21 Prevalence of hepatitis B virus:

HBV occurs worldwide (Figure1) (Mahoney and Kane., 1999; Robinson., 1995). The highest rates of HBsAg carrier rates are found in developing countries with primitive or limited medical facilities (Mahoney and Kane, 1999). In areas of Africa and Asia, widespread infection may occur in infancy and childhood. The overall HBsAg carrier rates may be 10 to 15%.

In North America infection is most common in young adults. In the USA and Canada, serological evidence of previous infection varies depending on age and socioeconomic class. Overall, 5% of the adult USA population has anti-HBc, and 0.5% are HBsAg positive (World Health Organization, 2002).

In developed countries, exposure to HBV may be common in certain high risk groups. Adults infected with HBV usually acquire acute hepatitis B and recover, but 5 to 10% develop the chronic carrier state.

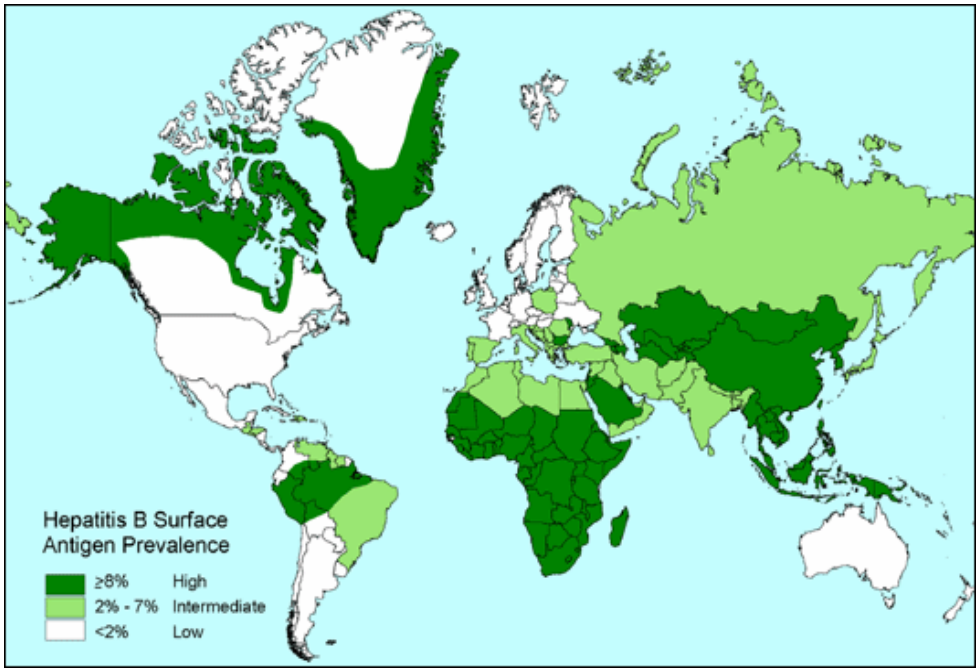

Fig. 1: Geographic distribution of Hepatitis B prevalence (Centers for disease Control and Prevention).

Infected children rarely develop acute disease, but 25 to 90% become chronic carriers. About 25% of carriers will die from cirrhosis or primary liver cancer as adults (Centers for Disease control and prevention, 1991; 1991; Mahoney and Kane, 1999).

In the past, recipients of blood and blood products were at high risk (for HBV infection), over the last 25 years, testing blood donations for HBsAg has become a universal requirement. Testing procedures have made major progress in sensitivity in the last 15 – 20 years. However 19% of countries reported that they were not testing all blood donations for HBsAg (WHO Global Database on Blood Safety, unpublished data). In the many countries where pre-transfusion screening of blood donations for HBsAg is carried out systematically, the residual risk of HBV transmission is minimal. Moreover, plasma derived medicinal products (including anti-haemophilic factors) undergo additional viral inactivation and removal procedures resulting in greatly reduced or no transmission of HBV by these products.

However, the risk is still present in many developing countries. Contaminated and inadequately sterilized syringes and needles have resulted in outbreaks of hepatitis B among patients in clinics and physicians' offices. Occasionally, outbreaks have been traced to tattoo priors and acupuncturists. Rarely, transmission to patients from HBsAg positive health care workers has been documented (Viral Hepatitis Prevention Board, 1998).

2.21.1 Hepatitis B in the Sudan:

Now, there are few data regarding the presence of HBV was widely prevalent in the Sudan. Hyams *et al.* (1989) reported seropositivity of 18.7% for HBsAg and 63.9% for HBV markers from two villages of Gezira region. A sero-survey for HBV antibodies among sexually active heterosexuals in Port Sudan conducted by McCarthy *et al.* (1989) showed HBsAg in 14% of the entire study population and anti-HBs, Anti-HBc in additional 49% and 27% of sera respectively.

Hyams *et al.* (1991) reported a prevalence of 10% HBV in Sudanese children in one of the public pediatric clinics. A study from Gezira area in blood donors and technical staff showed a prevalence of 17.3% and 12% in each group respectively (El Shaffie, 1992). McCarthy *et al.* (1994) in a survey for HBV and HIV in Juba found HBsAg in 20% of the population, anti-HBc in 67% and HCV in 3% only. In a study on

gastrointestinal tract cancer, HBsAg was detected in 18% of the patients and 12% of the normal subjects (Fahal *et al.* 1995).

In a survey on hepatitis A and B virus in subjects consisted of 25 control hospitalized patients, 21 volunteer blood donors, 23 patients with hepatosplenic schistosomiasis, 13 patients with liver cirrhosis and 6 patients with hepatocellular carcinoma, HBsAg was positive in 4, 24, 22, 31 and 67% respectively, and the antibodies for HBV in undiluted sera were 60, 57, 65, 77 and 83% respectively (Khalil *et al,* 1996). Suliaman *et al.* (1997) found a prevalence of 27.7% of HBV among haemodialysis patients from Khartoum Kidney Dialysis Center.

Some of these studies suggested that the principal mode of transmission in the Sudan is the parenteral mode of transmission (Khalil, 1996; Hyams *et al,* 1989; McCarty *et al,* 1989; Suliaman *et al,* (1997).

The prevalence of HBV among male Sudanese screened for employment in Gulf countries was 10% (Elkhidir,personal communication).

2.22 Vaccines of HBV:

Hepatitis B is a vaccine-preventable disease, but although global control of hepatitis B is achievable, it has not been attained yet (Centers for Disease Control and Prevention, 1998; VanDamme *et al,* 1997). In fact, a large pool of carriers and the burden of their disease remains, so that efforts must necessarily continue to treat the various stages of disease (World Health Organization, 2002)**.**

HB vaccine is the first and currently the only vaccine against a major human cancer. Vaccination is the most effective tool in preventing the transmission of HBV and HDV. Vaccines are composed of the surface antigen of HBV (HBsAg), and are produced by two different methods plasma derived or recombinant DNA. When administered properly, hepatitis B vaccine induces protection in about 95% of recipients (Centers for Disease Control and Prevention, 1998).

Systematic hepatitis B vaccination of newborns renders the screening of pregnant women for HBsAg status before delivery superfluous (Mahoney and Lane, 1999). The

primary objective of hepatitis B immunization is to prevent chronic HBV infections which result in chronic liver disease later in life. By preventing chronic HBV infections, the major reservoir for transmission of new infections is also reduced (World Health Organization, 2002).

2.23 Diagnosis of HBV:

Diagnosis of hepatitis is confirmed by demonstration in sera of specific antigens and/or antibodies. Three clinically useful antigen-antibody systems have been identified for hepatitis B. Hepatitis B surface antigen (HBsAg) and antibody to HBsAg (anti-HBs). Antibody (anti-HBc IgM and anti-HBc IgG) to hepatitis B core antigen (HBcAg). Hepatitis B e antigen (HBeAg) and antibody to HBeAg (anti-HBe) .

2.24 Immunodiagnostic tests:

Enzyme immunoassays referred to as enzyme linked immuno-sorbent assay (ELISA), have revolutionized diagnostic virology. Assay can be designed to detect viral antigen (A) or antibodies (Abs). The exquisite sensitivity of the method enables less than 1 ng of viral Ag per ml to be detected. A wide variety of different assays procedures are used including: direct, indirect, sandwich and competitive assay.

ELISA was first utilized in hepatitis research to detect HBs-Ag and anti-HBs Ab using sandwich method principle employed in solid-phase radioimmunoassay (RIA) procedure that predated ELISA. In this technique the enzyme labeled Ab is substituted for radionuclide labeled preparation, its presence being reflected by hydrolysis of the subsequent added enzyme substrate. The colour produced can be quantitated spectrophotomerically (Feuch *et al,* 1995).

HBsAg can be detected in the serum from several weeks before onset of symptoms to months after onset. HBsAg is present in serum during acute infections and persists in chronic infections. The presence of HBsAg indicates that the person is potential infectious (Hollinger and Liane, 2001; Mahoney and Kane, 1999; Robinson, 1995).

Very early in the incubation period, pre-S1 and pre-S2 antigens are present. They are never detected in the absence of HBsAg. Hepatitis B virions, HBV DNA, DNA polymerase, and HBsAg are then also detected. The presence of HBeAg is associated with relatively high infectivity and severity of disease (Hollinger and Liane, 2001; Robinson, 1995).

Anti-HBc 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 titer during acute infection and usually disappears within 6 months. Although it can persist in some cases of chronic hepatitis. This test may therefore reliably diagnose acute HBV infection. IgG anti-HBc generally remains detectable for a lifetime. Anti-HBe appears after anti-HBc and its presence correlates to a decreased infectivity. Anti-HBe replaces HBeAg in the resolution of the disease. Anti-HBs replace HBsAg as the acute HBV infection is resolving. Anti-HBs generally persists for a lifetime in over 80% of patients and indicates immunity (Hollinger and Liane, 2001; Mahoney and Kane, 1999; Robinson, 1995).

Acute hepatitis patients who maintain a constant serum HBsAg concentration, or whose serum HBeAg persists 8 to 10 weeks after symptoms have resolved, are likely to become carriers and at risk of developing chronic liver disease. Complication in the diagnosis of hepatitis B is the rare identification of cases in which viral mutations change the antigens so they are not detectable (Hollinger and Liane, 2001).

Hepatitis B DNA is one of the first things that can be detected in the blood stream after initial infection. It can be detected as soon as 1 week after infection using sensitive tests. It is believed that the level of HBV DNA may indicate how fast the virus is replicating. The test for HBV DNA is performed using PCR technique (http://www.hon.ch/Library/Theme/HepB/ virology.html).

Immunofluorescence studies, in situ hybridization, immunohisto-chemistry, and thin-section electron microscopy are used to examine pathological specimens for the presence of HBV-associated antigens or particles, providing information about the relationship between HBV DNA replication and HBV gene expression within the

hepatocyte, HBsAg localizes in the cytoplasm, and HBcAg is seen in the nucleus and/or the cytoplasm. Detection of complete virions in the liver is uncommon (Hollinger and Liane, 2001).

DNA hybridization techniques and PCR assays have shown that almost all HBsAg/HBeAg-positive patients have detectable HBV DNA in their serum, whereas only about 65% of the HBsAg/anti-HBe reactive patients are positive. All patients who recover from acute hepatitis B are negative for HBV DNA. Some patients infected chronically who have lost their HBsAg remain HBV DNA positive (Hollinger and Liane, 2001; Robinson, 1995)

2.24.1 HBV serological markers in hepatitis patients:

The three standard blood tests for hepatitis B can determine if a person is currently infected with HBV, has recovered, is a chronic carrier, or is susceptible to HBV infection (Hollinger and Liane, 2001; Mahoney and Kane, 1999; Robinson, 1995).

During HBV infection, the serological markers vary depending on whether the infection is acute or chronic (Gitlin, 1997; Mahoney and Kane, 1999; Robinson, 1995).

2.25 Immunochromatography technique (ICT):

The HBV ICT or one step test device (serum/plasma) is a rapid immunoassay for the qualitative detection the presence of antigens to HBV (Choo., 1989). ICT are modeled after the home pregnancy test used by women in many countries. They differ from usual systems for measuring antigen-antibody reactions in that the specimen from the human subject is made to flow through a filter after which it is immobilized in a membrane at the a site where antigen (or antibody) in the specimen comes into contact with the test antibody (or antigen) already present in the membrane. All controls are included in the membrane as well, and results are seen as colored bands or lines, as one of the test reagents is conjugated to colloidal gold or chromogenic substance (Murphy *et al*, 1999).

2.26 Polymerase chain reaction (PCR):

The polymerase chain reaction (PCR) is an *in vitro* method for enzymatic synthesis of specific DNA sequence using two oligonculeotide primers, usually of about 20 residues, that hybridize to opposite strands and flank the region of interest of the target DNA, the primer pairs are sometimes referred to as forward and reverse primer. Computer programmes are used for the design of optimal primer sets and to predict the parameters (time/temp.) for the reactions. Depending on the degree of conservation of the putative target sequence, primers may be chosen to be strain, genus or family specific (Bukh *et al*, 1993).

PCR is useful in diagnosis of virus infection when viral antigens (Ags) or virus specific antibodies (Abs) can not be detected and when the presence of viral nucleic acid may be the only evidence of infection. This particularly true for latent virus infections, non cultivable virus, or difficult to cultivate and for virus that grow without a visible cytopathic effect and for zoonotic virus PCR is preferred to minimize the risk of exposure for laboratory personnel (Bell *et al*, 1997). The presence of HBV DNA in peripheral blood (serum or plasma) is a reliable marker of active HBV replication (Chu and Hussain, 2002)

HBV levels are detectable by 30 days after infection, generally reach a peak at the time of clinical acute hepatitis, gradually decrease, and disappear when the infection resolves spontaneously. Patients with chronic HBV fail to clear the virus and remain HBsAg positive. Such cases may be classified as replicative chronic HBV (high HBVDNA levels, hepatitis Be antigen, [HBeAg] positive) or non replicative chronic HBV (low or un-detectable HBV DNA levels, hepatitis Be antigen negative) (Pawlotskey, 2003; Servoss, 2004).

Patients with replicative chronic HBV have more serious liver disease and are more infectious than patients with non replicative disease (Chu and Hussain, 2002) In cases of acute viral hepatitis with equivocal HBsAg test results, testing for HBV DNA in serum may be a useful adjunct in the diagnosis of acute HBV infection, since HBV DNA can be

detected approximately 21 days before HBsAg appears in the serum (Pawlotskey, 2003; Servoss, 2004).

Testing for HBV DNA levels in serum are used to determine the status of chronic HBV infection, by differentiating between replicative (high HBV DNA levels) and non replicative (low HBV levels) states. Reactivation of inactive chronic HBV infection (HBeAg-negative state) may occur with or without reappearance of HBeAg in serum. In patients with HBV infection, detection of HBV DNA is the only reliable confirmation of active HBV replication (Chu and Hussain, 2002)

2.27 Other laboratory and clinical tests:

The diagnosis of liver disease depends up on a combination of history, physical examination, laboratory testing and sometime radiological studies and biopsy.

2.27.1 Ultra sound scan of liver:

It is a routine test that does not show how much damage there is to the liver but it gives an indication of the shape and size of the liver (Mowat *et al*, 2001).

2.27.2 Liver function tests:

These are routine tests performed in the investigation of a liver disease, these tests are non-specific and in most cases are not true indications of liver dysfunction. They are only suggestive liver disease (Mowat *et al*, 2001).

2.27.3 Aspartate aminotransferase (AST):

The enzymes AST previously known as glutamate oxaloacetate transaminase GOT, is concerned with amino acid metabolism. Large amounts of AST are present in the liver, kidneys, cardiac muscle. Small amount of the enzyme are present in the brain, pancreas, and lungs. In district laboratories the measurement of AST activity is mainly performed to investigate liver disease and myocardial infraction (Cheesbrough, 1998).

2.27.4 Alanine aminotransferase (ALT):

The enzyme ALT formerly known as glutamate pyruvate transaminase (GPT), is concerned with amino acid metabolism. ALT is found principally in the liver with only small amounts being present in other organs When there is liver cell damage the serum or plasma levels of enzymes are raised (Cheesbrough, 1998).

2.27.5 Albumin:

Albumin is produced entirely in the liver and constitutes about 60% of total serum protein. It is important in regulating the flow of water between the plasma and tissue fluid. When the concentration of albumin is significantly reduced, the plasma osmotic pressure is insufficient to draw water from the ties spaces back into the plasma. This leads to a build-up of fluid in the tissues, culminating in oedema.

Albumin also has important binding and transport function. It binds and inactivates substances including calcium, fatty acid, hormones and magnesium. When albumin levels are reduced, toxic effects can develop from an increase in unbound substances. Low serum albumin concentration indicates poor liver function (Cheesbrough, 1998).

2.27.6 Bilirubin:

Bilirubin is formed from the breakdown of erythrocytes and other haem-containing proteins such as myoglobin and cytochrmes. It is two types, un-conjugated (indirect) bilirubin, it is not soluble in water and can not be excreted in the urine. It is bound to albumin transported in the blood to the liver. This bilirubin is referred to as conjugated (direct) bilirubin and is water-soluble (Cheesbrough, 1998). If the liver is not functioning well there is a decreased removal of the bilirubin from the blood stream (Mowat *et al*, 2001).

2.27.7 Prothrombin time (PT):

Many factors necessary for blood clotting are made in liver. When liver function is severely abnormal, their synthesis and secretion into the blood is decreased. The (PT) is a type of blood clotting test performed in the laboratory and it is prolonged when the blood

concentration of some of the clotting factors made by the liver are low. The (PT) can also be prolonged in case of vitamin K deficiency and by drugs (Mowat *et al*, 2001).

2.27.8 Alkaline phosphatase (AP):

Alkaline phosphatase is an enzyme, or more precisely a family of related enzymes, produced in bile ducts, intestine, kidney, placenta and bone. An elevation in level of serum AP suggests disease of the bile duct (Cheesbrough, 1998).

2.27.9 Gamma-glutamy transpeptidase (GGT):

GGT an enzyme produced in bile ducts that like (AP), may be elevated in the serum of patient with bile ducts disease. Measurement of GGT is an extremely sensitive test, however, and it may be elevated in virtually any liver disease (Mowat *et al*, 2001).

2.28 Treatment:

Currently, there is no treatment available for acute hepatitis B. Symptomatic treatment of nausea, anorexia, vomiting, and other symptoms may be indicated. Treatment of chronic hepatitis B is aimed at eliminating infectivity to prevent transmission and spread of HBV, at halting the progression of liver disease and improving the clinical and histologic picture, and at preventing HCC from developing, by losing markers of HBV replication in serum and liver like HBV DNA, HBeAg, and HBcAg. Normalization of ALT activity, resolution of hepatic inflammation and the improvement of a patients' symptoms usually accompany these virological changes (Hollinger and Liang, 2001; Mahoney and Kane, 1999).

There are two main classes of treatment. Antiviral aimed at suppressing or destroying HBV by interfering with viral replication (Mahoney and Kane, 1999). Immune modulaters aimed at helping the human immune system to mount a defense against the virus.

Currently, chronic hepatitis B is treated with interferon (Gitlin, 1997; Hollinger and Liang, 2001; Mahoney and Kane, 1999; Robinson, 1995). The only approved ones are interferon-α-2a and interferon- α-2b. Interferons display a variety of properties that

include antiviral, immunomodulatory, and anti-proloferative effects. They enhance T-cell helper activity, cause maturation of B lymphocytes, inhibit T-cell suppressors, and enhance HLA type I expression. Patients with acute infection, end stage cirrhosis or other major medical problems should not be treated. Interferon- α-2b produces a long-term, sustained remission of the disease in 35% of those with chronic hepatitis B, with normalization of liver enzymes and loss of the three markers for an active infection (HBeAg, HBV DNA, and HBsAg), Complete elimination of the virus is achieved in some carefully selected patients (Hollinger and Liang, 2001; Mahoney and Kane, 1999; Robinson, 1995; Tassopoulos *et al*, 1997).

Interferon therapy for patients with HBV-related cirrhosis decreases significantly the HCC rate, particularly inpatients with a larger amount of serum HBV DNA. In patients with HBeAg-positive compensated cirrhosis, virological and biochemical remission in following interferon therapy is associated with improved survival. In patients with chronic HBV infection, the clearance of HBeAg after treatment with interferon- α is associated with improved clinical outcomes (Fattovich, 1999; Hollinger and Liang, 2001; Ikeda, 1998; Mahoney and Kane, 1999; Niederau, 1996).

2.29 Prevention and control:

The prevention of chronic HBV infection has become a high priority in the global community (Mahoney and Kane, 1999).

Immunization with hepatitis B vaccine is the most effective means of preventing HBV infection and its consequences (Center for Disease Control and Prevention, 1991; Gitlin, 1997; Hollinger and Liang, 2001; Robinson, 1994; VanDamme and Kane, 1997).

HBIG protects by passive immunization if given shortly before or soon after exposure to HBV. It is also administered in combination with HBV vaccines to newborns of HBsAg positive mothers. The protection is immediate, but of short duration, HBIG is not recommended as a pre-exposure prophylaxis because of high cost, limited availability, and short-term effectiveness (Gitlin, 1997; Hollinger and Liang, 2001).

Preventing HBV transmission during early childhood is important because of the substantial likelihood of chronic HBV infection and chronic liver disease that occurs when children less than 5 years of age become infected. Integrating HB vaccine into childhood vaccination schedules has been shown to interrupt HBV transmission (Center for Disease Control and Prevention, 1991).

In order to avoid unnecessary risks of HBV infection, patients who depend on recurrent transfusion should be vaccinated (World Health Organization, 2002).

Universal precautions should be used when handling human blood and body fluids. Specific precautions include the use of gloves, protective garments, and masks, when handling potentially infectious or contaminated materials. There is no substitute for good personal hygiene, strict surveillance, and appropriate environmental control measures to limit transmission. Autoclaving and the use of ethylene oxide gas are accepted methods for disinfecting metal objects, instruments, or heat-sensitive equipment (Hollinger and Liang, 2001).The expense and difficulty of treating hepatitis B medically and by hepatic transplantation is in contrast with the fact that the infection can be prevented by vaccination. Vaccination campaigns have shown that control of the disease is feasible, even in endemic areas. Some countries incorporate hepatitis B immunoglobulin (HIG) in their vaccination strategies. In endemic areas, procurement of low cost vaccine, education and acceptance, vaccine integration in the expanded program of immunization (EPI), prevention of vertical transmission, antibody escape mutations, protective efficacy, long term immunity and natural boosting are important questions and issues. Since most HBV carriers are unaware of their condition, but pose a significant risk to health care workers and other people exposed to their blood, workers are advised to assume that all patients are potentially infectious, and should practice "universal precautions" (World Health Organization, 2002)

CHAPTER THREE
MATERIALS AND METHODS

3.1 Collection of samples:

A total of 480 blood samples were collected from different group in Khartoum State between January and July 2006. Data on respondent background, ideas, attitudes, and behavior on various issues in form of a questionnaire were collected, these groups include:

1. Blood donors groups: 280 blood samples were collected from blood donors in Khartoum Teaching Hospital.
2. Haemodialysis patient groups: 100 blood samples were collected from haemodialysis patients at Khartoum Center for Kidney Diseases and Bahri Renal Centre (National Centre of Kidney Diseases).
3. Multiple transfused individuals: 20 blood samples were collected from multiple transfused individuals at Ahmed Gasim Hospital & Khartoum Teaching Hospital.
4. Jaundice patients groups: 80 blood samples were collected from jaundice patients visiting the Central National Health Laboratory.

Venous blood samples were collected using vacutainer containers and needles (syringe 5ml). The blood was drawn into the tube without any anticoagulant and the blood was allowed to clot at room temperature for no more than 1 hour. The serum was removed and placed in a sterile capped microfuge tube and the tube was centrifuged at 1000 rpm for 5 min at 4^{o}C. The serum was then removed without disturbing the pellet and the tubes were stored at -20^{o}C till used.

3.2 Immuno chromatography technique (ICT) for detection of hepatitis B surface antigen (HBsAg) in 480 serum samples:

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

3.2.1 Materials provided:

a) Test devices contain anti-HBsAg particles coated on the membrane.
b) Disposable specimen dropper.

3.2.2 Materials required but not provided:

a) Specimen collection containers.
b) Pipette and disposable tips.
c) Centrifuge
d) Timer

3.2.3 Storage and stability:

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

3.2.4 Test procedure:

Specimens (serum) were allowed to reach room temperature (20–25^{o}C) before running the test. Frozen specimens were completely thawed and mixed well prior to testing. The test devices were removed from the sealed pouch and placed on clean and level surface and used immediately and the test is performed within one hour. Then 3 full drops of serum (approx. 100 µl) were transferred into specimen well (S) of the test device. Results were read after 10 minutes as indicated by the appearance of the red lines. (Fig. 4).

3.2.5 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).

3.3 Direct (sandwich) ELISA for detection of HBsAg in 480 serum samples:

The ELISA kits were purchased from Shanghai SHC Kehua biotech Co. LTD, Shanghai, China and supplied as follows:

1. 5 micro titer plate 12 x 8 wells (96 tests) strips per plate coated with monoclonal anti-HBs (mouse) and sealed in an aluminum bag containing a silica gel bag as desiccant.
2. Conjugate: 1 vial of 6-2 ml/horseradish HRP-labeled guinea-pig anti-HBs.
3. Positive control: 1 vial of 1.0 ml contain HBsAg but non-reactive for anti-body to HIV-1 and HIV-2.

4. Negative control: 1 vial of 1.0 ml derived from human blood prepared only from donations which have been tested for HBsAg as well as for anti-body to HIV by reliable method and found to be negative.
5. Washing solution: 1 bottle of 40 ml concentrated, which must be diluted 1:25 before use.
6. Chromogen A: 1 vial of 8.0 ml (contains hydrogen peroxide).
7. Chromogen B: 1 vial of 8.0 ml contains Teteramethyle benzedine (TMB) and Dimethyl sulphoxide.
8. Stopping solution: 1 vial of 7.0 ml contains (2M H_2SO_4).
9. Plate covers: 2 pieces.
10. Instruction manual: 1 copy.

3.3.1 Other materials not included in kit:

1. Distilled or deionized water.
2. Multichannel pipette and micropipettes capable of delivering 20 – 100 and 1000 µl.
3. Disposable pipette tips.
4. An automatic microplate washer.
5. Microplate mixer
6. Incubator with high relative humidity, at 37^{o}C.
7. Timer
8. Microplate reader equipped with a 450 nm and 630 nm filter.
9. Absorbent tissue.

3.3.2 Storage and stability of ELISA kits:

All kits components were stored at 2–8^{o}C. The bag containing the microtiter plate should be brought to room temperature before opening to avoid condensation in wells. Unused strips should be stored between 2–8^{o}C tightly sealed in the plastic bag provided

with the silica gel bag inside. The washing solution once diluted is stored between 2–8^{o}C. The chromogen was stored protected from light.

3.3.3 Test procedure:

3.3.4 Preparation of reagents:

Reagents, test samples, controls, conjugate, diluted wash solution, substrate, aluminum bag containing the microplate and the vial containing TMB were allowed to come to reach room temperature (20–25^{o}C) before running the assay. All liquid reagents were mixed before use. The concentrated washing solution is diluted 1:25 with distilled water. All dilutions were made according to the manufacture instructions.

3.3.5 Assay procedure:

Five wells were reserved for one blank, two negative controls and two positive controls. Fifty µl of positive control was transferred into each of the two wells, 50 µl of negative control was transferred into each of the two wells and remained one blank.

Fifty µl of each sample (serum) was distributed to the designated well. Five µl of conjugate transferred into each wells (excluding the blank well). The plates were covered with a plate sealer, mixed gently and incubated for 1 hour at 37^{o}C. Thereafter, the adhesive plate covers were removed and each well was filled with a proximately 300 µl diluted washing solution. This process was repeated five times. After the last washing the microplates were blotted on absorbent tissue to remove any excess liquid from the wells then 50 µl of chromogen A and 50 µl of chromogen B were transferred into each wells of the microplate (including the blank well). The plates were covered again with a fresh plate sealer and incubated at 37^{o}C for 30 minutes in an incubator. The adhesive plate covers were removed and discarded. Then the reaction was stopped by adding 50 µl of stopping solution to each well (including the blank well) and mixing completely. The spectrophotometer was blanked at 450 nm with blank well and absorbances of each well were read within 10 minutes.

3.3.6 Calculation of the results:

The judgment of results is based on the photometric reading data.

N = The mean absorbance of the negative control
P = The mean absorbance of positive control
S = The absorbance of the test sample.

3.3.7 Calculation of cut-off value:

The cut-off value is 2.1 x N

1F N is less than 0.05 then N= 0.05

1F N is greater than or equal, let N equal its actual value.

3.3.8 Reading of test result:

The presence or absence of HBsAg in samples analyzed was determined by relating the absorbance value of each sample to the cut-off value of the technique.

A test is positive if $S \geq$ cut-off value

A test is negative if $S <$ cut-off value

A test-run is only valid if $N < 0.1$ and $P > 1.0$.

3.3.9 Interpretation of the test:

If the test value of a serum was above the calculated cut-off value the test serum was considered positive and if the test value was lower than the cut-off value, the sample was considered negative for the presence of HBsAg. However, sera having test value equal to the cut-off value, should be retested in duplicates and if one duplicate has a value above the cut-off value the test was considered positive even if the other duplicate value was below the cut-off value.

3.4 Polymerase chain reaction (PCR):

3.4.1 DNA extraction:

Extraction of viral DNA from serum was made according to Tripathy (2003) using the phenol-chloroform extraction method with some modification. o.5 ml of cell lysis solution (10 mm Tris-HCl, pH 7.4, 100 mm NaCl, 10 mm EDTA, 0.5% SDS) and 2% B mercaptoethanol) (Appendix 1) were added to 2 ml eppendorf tube containing 500 µl of serum, then 15 µl of protinase K solution (10 mg/ml) (Appendix 2) were added and the mixture was vortixed for few seconds, incubated at 55^{o}C for 4 hours or at 37^{o}C for overnight. The sample was vortixed after every 15 minutes during incubation. 10 ul of RNase were added and the tube was inverted 25 times, and then incubated at 37^{o}C for 15 minutes. DNA was extracted from the digest in the presence of equal volume of phenol-chloroform-isoamy-alchohol (50:2:48). The extraction last for 30 second with mixing by inverting eppendorf tube followed by centrifugation at 10.000 rpm for 1 min. The tube content will divided into three layers, phenolic phase, interphase (protein) and an aqueous phase. The aqueous phase (which contains the nucleic acids) was removed to another eppendorf tube for further extracted with phenol-chloroform and this step was repeated for three times. The last aqueous phase was replaced to another 1.5 ml eppendorf tube and 3 M Na acetate was added in a volume equivalent to 1:10 of the aqueous phase and equal volume of isopropanol alcohol was added, mixed and stored at -20^{o} overnight. The DNA was pelleted by centrifugation at 13000 rpm for 45 minutes at +4^{o}C. Then the DNA pellet was washed with 300 µl 70% alcohol and centrifuged at 13000 rpm for 15 min at +4^{o}C, and the ethanol was poured off. The tube was then allowed to dry for 10 - 15 minutes. 50 µl of DNA hydration solution was added and the DNA was dissolved by incubating at 65^{o}C for 1 hour and then the DNA was stored at +4^{o}C until used.

3.5 Primer selection and PCR condition:

3.5.1 Reconstitution of primers:

Primers were purchased as freeze dried oligonucleotides (MWG, German) reconstituted with 473 DDW to get a final concentration of 100 pmol/µl and left overnight at +4^{o}C. Working solutions for each primers were prepared so as to contain 10 pmol/µl of oligonucleotides.

The sequences of the oigonucleotide primers used in this study are specific primers for surface antigen genome region specific or ss genome region specific. These primers are expected to give PCR product size of 477 bp (Table 3) (Crirish and Kotwal., 1998).

3.5.2 PCR for hepatitis B virus:

PCR was performed in a final volume of 50 µl including 5 µl of template DNA, 0.5 µl of DNA polymerase, 2 µl **HBV2R** and HBV_S primers, 3 µl of $Mgcl_2$, 5 µl PCR buffer, 2 µl of dNTP and then 30.5 µl of distilled water, were added to complete the volume to 50 µl. After the initial denaturation for 30s at 94°C, the DNA was amplified for 35 cycles of 30s of denaturation at 94°C, 30s of annealing at 56°C and 9 min of extension at 68°C, with a final extension step of 10 min at 68°C. The amplification was performed in a T personal thermocycler (Biometra, German).

Table (3): Sequence of the oligonucleotide primers targets and expected PCR product size (Girish and Kotwal., 1998).

Primer and Direction	Sequence	Product size	Amplification target
HBV 2R	**5. AAG CCC CTA CGR ACC ACT GAA CAA ATG GCA C-3**	**477bp**	**HBS Ag (surface antigen) or ss genome region**
HBV_S	**5- ATA CCA CAG AGT CTA GAC TCG TGG TGG ACT-3**	**-**	**-**

3.5.3 Analysis of PCR products:

3.5.3.1 Gel electrophoresis:

The PCR products (amplicons) were separated electro-phoritically in 1.5% agarose gels (Appendix 3.1) (SIGMA) 10 µl of 100 bp DNA ladder (INVTROGEN) (Appendix 3.4) was loaded in the first slot of the gel. Then 16 µl of the PCR product was mixed with 4 µl of loading Dye (Appendix 3.3) and loaded on the rest wells.

Electrophoresis was performed in a Mini gel electrophoresis (Biometra) using 75 volt for 45 min after the gel was covered with TAE buffer (Appendix 3.2) using standard power Pack P25 (Biometra). DNA bands were visualized using the Bio Doc analyze gel documentation system (Biometra).

3.5.3.2 Interpretation:

Negative results indicated that HBV DNA is not detected in the specimen. Positive results indicated that HBV DNA is detected in the specimen.

3.6 Indirect ELISA for the detection of HCV-antibodies in serum samples positive for HBsAg :

3.6.1 ELISA kits:

The ELISA kits were purchased from Biokit, A.S. Spain and supplied as follows:

1. 5 microtiter plate 12 x 8 wells coated with HCV recombinant antigens.
2. Concentrated conjugate (1-5 ml). Rabbit anti-human IgG antibodies conjugated with peroxidase, contains red dye, preservatives and protein stabilizers. To be diluted 1/51 with the conjugate diluents just before use.
3. Conjugate diluents. Tris buffer containing yellow dye, additives and preservative.
4. Sample diluents. Tris buffer with protein stabilizers and sodium acids as preservative.
5. Concentrated washing solution is phosphate buffer containing 1% tween 20 and 0.01% thimerosal.
6. Substrate solution which is citrate-acetate buffer containing hydrogen peroxide.

7. Chromogen which is 3, 3^1, 5, 5^1-tetramethyl benzidine (TMB) dissolved in dimethyl sulphoxide (DMSO).
8. Positive control which is diluted heat inactivated human serum containing antibodies to HCV. Contains sodium acids as preservative and green dye.
9. Negative control which is dilute human serum negative for anti-HCV. Contains sodium azide as preservative and yellow dye.
10. Stopping solution which is 1 N sulphuric acid.
11. Adhesive seals to cover plate during incubation.
12. Resealable bag for storage of unused wells.

3.6.2 Other materials not included in the kit

1. Distilled or deionized water.
2. Multichannel pipette and micropipettes.
3. Automated wash system.
4. Microplate reader with 450 nm filter
5. Incubator with high relative humidity, at 37^{o}C.

3.7 Storage of ELISA kits:

All kits components were stored at 2–8^{o}C. The bag containing the microtiter plate was brought to room temperature before opening to avoid condensation in the wells. Unused strips were stored between 2–8^{o}C tightly sealed in the plastic bag provided with the silica gel bag inside. The washing solution when diluted was stored between 2–8^{o}C. The diluted conjugate is stable for 15 days at 2–8^{o}C. The chromogen was stored protected from light whereas substrate –TMB solution was prepared according to the manufacture instructions.

3.8 Test procedure:

3.8.1 Preparation of reagents:

Reagents were allowed to reach room temperature (20–25^{o}C) before running the assay. All liquid reagents were mixed before use. The concentrated washing solution was

diluted (1/10) with distilled water. The concentrated conjugate was diluted (1/51) with the conjugate diluent. All dilutions were made according to the manufacture instructions.

3.8.2 Assay procedure:

Six wells were reserved for one blank, three negative controls and two positive controls, each filled with 200 µl of the appropriate serum control. Two hundred µl of sample diluents and 10 µl of each sample were distributed to the designated wells. The plates were covered with adhesive seals, mixed gently and incubated for 1 hour at 37^{o}C. Thereafter the adhesive plate covers were removed and the content was discarded by aspiration and each well was filled approximately with 300 µl diluted washing solution. The process was repeated five times. After the last washing the microplates were blotted on absorbent tissue to remove any excess liquid from the wells. Then 100 µl of diluted conjugate were transferred into each well of the microplates, except the blank. The plates were covered again with the adhesive seals and incubated for 30 minutes at 37^{o}C.

The adhesive plate covers were removed and discarded. The plate's contents were aspirated and washed six times with washing buffer. Then 100 µl of substrate- TMB solution were added to each well. All plates were incubated uncovered for 30 minutes at room temperature. The reaction was stopped by adding 100 µl of stopping solution in the same sequence and time intervals as for the substrate-TMB addition. The spectrophotometer was blanked at 450 nm with the blank well and the absorbencies of each well were read within 30 minutes.

3.8.3 Reading of the result:

For the validity of the assay the accomplishment of the following points were observed.

a) Negative control means (NC): Absorbance of individual negative control values were less than or equal to0.200 OD.

b) Positive control means (PC): It was equal or greater than 0.800 OD.

c) The presence or absence of anti-HCV antibodies in the samples analyzed was determined by relating the absorbance value of each sample to the cut-off value of the technique. This value was the mean value obtained from the negative control plus 0.300 OD.

Cut-off = NC + 0.300 OD

3.9 Interpretation of the test:

If the test value of a serum was above the calculated cut-off value, the test serum was considered positive and if the test value was lower than the cut-off value, the sample was considered negative for the presence of HCV-antibodies. However, sera had test values equal to the cut-off value, these sera were retested in duplicates and if one duplicate had a value above the cut-off value the test was considered positive even if the other duplicate value was below the cut-off value.

3.10 Immuno chromatography technique (ICT) for detection of HCV-antibodies in serum samples positive for HBsAg :

The ICT test kits purchased from ACON laboratories, San Diego, USA and supplied as follows

3.10.1 Materials provided:

a) Test devices

b) Disposable specimen dropper.

C) Buffer

3.10.2 Materials required but not provided:

a) Specimen collection containers.

b) Pipette and disposable tips.

c) Centrifuge

d) Timer

3.10.3 Storage and stability:

The kits were stored at room temperature or refrigerated (2–30^{o}C). The test devices were kept in the sealed pouch until use. The expiry date was observed.

3.10.4 Test procedure:

Specimens (serum) were allowed to reach room temperature (20–25^{o}C) before running the test. Frozen specimens were completely thawed and mixed well prior to testing. Then the test device was allowed to equilibrate at the room temperature. The test devices and buffer were removed from the sealed pouch and placed on clean and level surface and used immediately and the test was performed within one hour. Then 5 µl full of serum were transferred into specimen well (S) of the test device and then two drops of buffer (approximately 80µl) were added carefully to avoid trapping air bubbles in the specimen well. Results were read after 10 minutes as indicated by the appearance of the red lines.

3.10.5 Interpretation of results:

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

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

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

3.11 Statistical Analysis :

Stata NT/ 98 Germany was used for analysis of the data collected.

CHAPTER FOUR
RESULTS

The presence of HBsAg, HBV-DNA in sera of different groups and anti-HCV antibodies detected in HBsAg-positive patients as marker of co-infection in sera of different groups of patients were as follows:

4.1 ICT results:

280, 20, 100 and 80 blood samples were collected from blood donors, multi-transfused, haemodialysis and jaundiced patients and 15 (5.3%), 2 (10%), 23 (23%) and 9 (11.5%) samples were found positive for HBsAg respectively when screened by ICT (Table 4 and Fig. 2,7).

4.2 ELISA results:

In contrast to the previous test samples when tested by ELISA using HBsAg sandwich ELISA we found 16 (5.7%), 2 (10%), 23 (23%) and 9 (11.2%) samples positive for HBsAg when blood donors, multi-transfused, haemodialysis and jaundiced patients were tested respectively (Table 5 and Fig. 3,8,9).

4.3 Comparison between ICT and ELISA tests of HBsAg:

In comparison between ICT and ELISA detected one positive samples that were found negative by ICT test. However, there was a very high agreement between ICT and ELISA and no statistical significant difference was obtained by the application of Stata NT/98 and both assays were sensitive, specific and reliable (Tables 10, 11, and 15).

4.4 PCR Results: (Detection of HBV-DNA):

Fifteen serum samples with HBsAg positive and 10 samples with HBsAg negative screened by ICT and ELISA were investigated to detect HBV-DNA using the PCR. We detect HBV-DNA in 45 (90%) samples of HBsAg positive. HBV-DNA detected in eleven (68.7%), two (100%), 23 (100%) and nine (100%) samples collected from blood donors, multi-transfused, haemodialysis and jaundiced patients, and in two (20%) samples negative for HBsAg collected from blood doors (Tables 8,9,14 and Fig.6,12,13,14,15).

4.5 ELISA results for HCV-antibodies in HBsAg positive samples:

In contrast to the previous test samples when tested by ELISA using recombinant HCV-antigens we found 2 (12.5%) blood donors and one (4.3%) haemodialysis patients were positive for HCV-antibodies when patients were tested respectively (Table 7& Fig. 5,10).

4.6 ICT results of HCV-antibodies in HBsAg positive samples:

. Sixteen, 2, 23 and 9 HBsAg positive samples from blood donors, multi-transfused, haemodiaysis and jaundice patients were tested and one (6.2%) blood donors one (4.3%) haemodialsis patients samples were positive for HCV-antibodies respectively when screened by ICT (Table 6 and Fig. 4,11).

4.7 Risk factors associated with HBsAg positive samples:

Tables 12 and 13 summarized data obtained from the questionnaire and all risk factors that might contribute to HBV infection. All patients who were positive for HBsAg had an age ranging between 17 – 63 years. We found all haemodialysis patients had received blood transfusion and HBV infection in this group was probably blood transfusion mediated disease. Sexual practice (12%) sharing needles (14%) and surgical instruments (30%) represented a risk factor. Intravenous drug users and tattooing had no

role in predisposition to the disease. In the groups studied high incidence of HBsAg (23%) was found in haemodialysis group and probably haemodialysis machine had a role in transmission of the disease.

Table 4: HBsAg positive individuals in various groups screened by ICT test.

Group tested	Number screened	Number of HBsAg positive	Number of HBsAg% positive
Blood donors	**280**	**15**	**5.35%**
Multi-transfused	**20**	**2**	**10%**
Haemodialysis patients	**100**	**23**	**23%**
Jaundice patients	**80**	**9**	**11.5%**
Total	**480**	**49**	**10.2%**

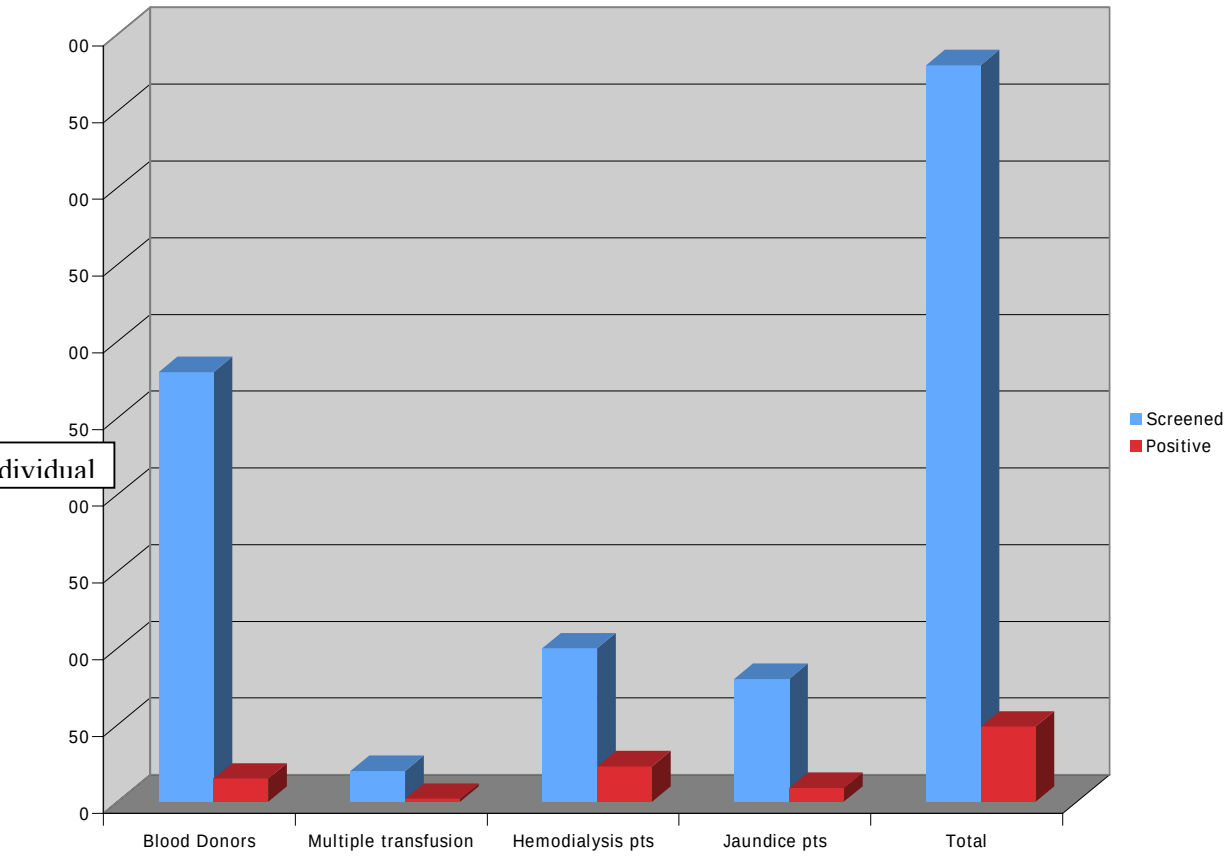

Fig. 2: HBsAg positive individuals in various groups screened by ICT test.

* Pts = Patient.

Table 5: HBsAg positive individuals in various groups screened by ELISA test.

Group tested	Number screened	Number of HBsAg positive	Number of HBsAg% positive
Blood donors	**280**	**16**	**5.7%**
Multi-transfused	**20**	**2**	**10%**
Haemodialysis patients	**100**	**23**	**23%**
Jaundice patients	**80**	**9**	**11.2%**
Total	**480**	**50**	**10.4%**

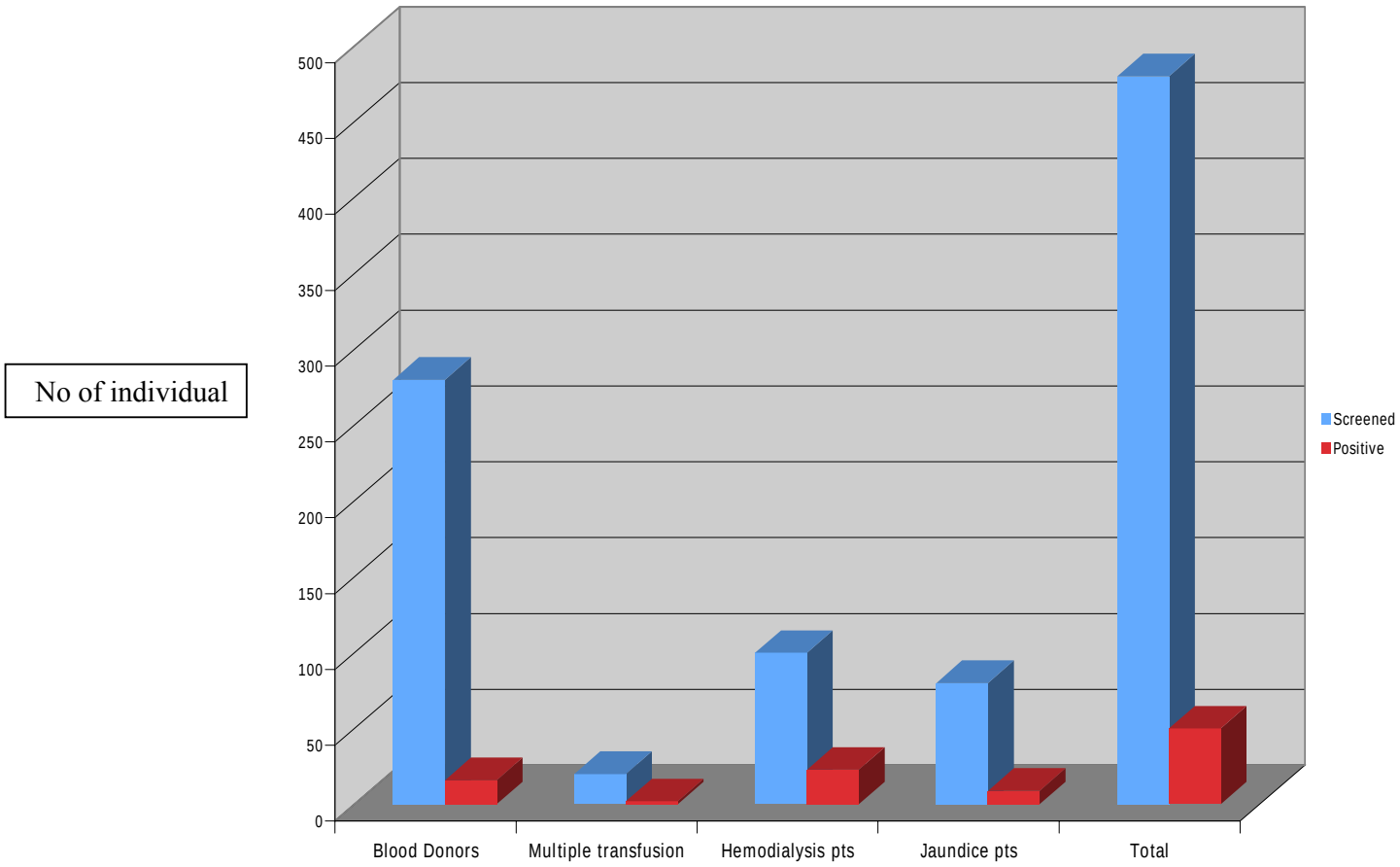

Fig. 3: HBsAg positive individuals in various groups screened by (ELISA) test.

* Pts = Patient.

Table 6: HCV-antibody positive individuals in groups of HBsAg positive screened by ICT test

Group tested	Number screened	Number of HCV-antibodies positive	Number of HCV-antibodies% positive
Blood donors	**16**	**1**	**6.2%**
Multi-transfused	**2**	**-**	**-**
Haemodialysis patients	**23**	**1**	**4.3%**
Jaundice patients	**9**	**-**	**-**
Total	**50**	**2**	**4%**

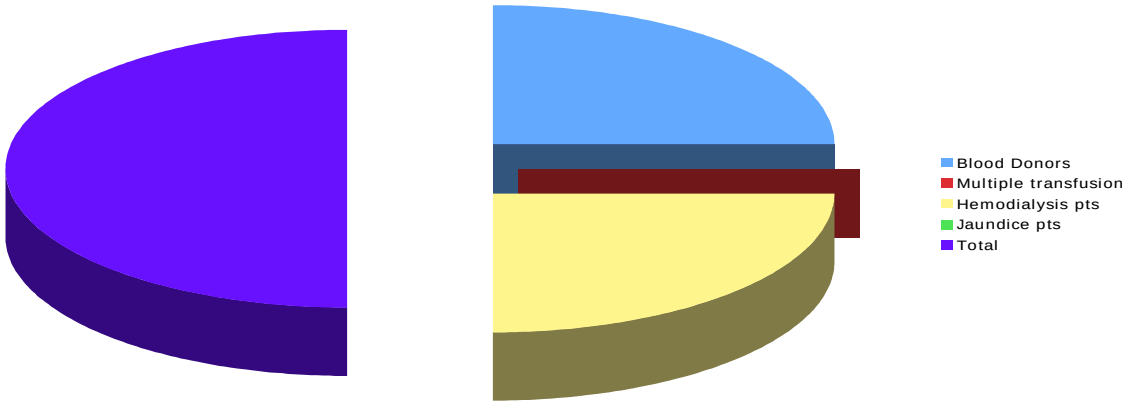

Fig. 4: HCV-antibody positive individuals in groups of HBsAg positive screened by ICT test

Table 7: HCV-antibody positive individuals in groups of HBsAg positive screened by ELISA test

Group tested	Number screened	Number of HCV-antibodies positive	Number of HCV-antibodies% positive
Blood donors	**16**	**2**	**12.5%**
Multi-transfused	**2**	**-**	**-**
Haemodialysis	**23**	**1**	**4.3%**
Jaundice patients	**9**	**-**	**-**
Total	**50**	**3**	**6%**

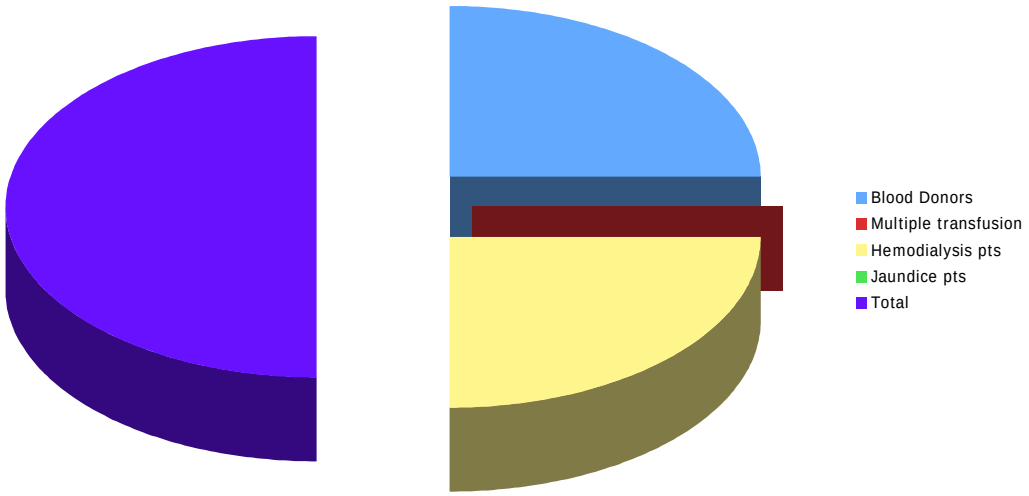

Fig. 5: HCV-antibody positive individuals in groups of HBsAg positive screened by ELISA test

* Pts = Patient.

Table 8: HBV DNA detection in HBsAg positive patients in various group screened by PCR.

Group tested	Number of HBsAg positive	Number of HBV-DNA positive	Number of HBV-DNA% positive
Blood donors	**16**	**11**	**68.7%**
Multi-transfused	**2**	**2**	**100%**
Haemodialysis	**23**	**23**	**100%**
Jaundice patients	**9**	**9**	**100%**
Total	**50**	**45**	**90%**

Table 9: HBV DNA detection in samples negative for HBsAg screened by ELISA and ICT test.

Group Tested	Number of HBsAg Negative	HBV DNA Positive	Number of HBV DNA%	Total of HBV DNA%
Blood Donors	10	2	20 %	20 %

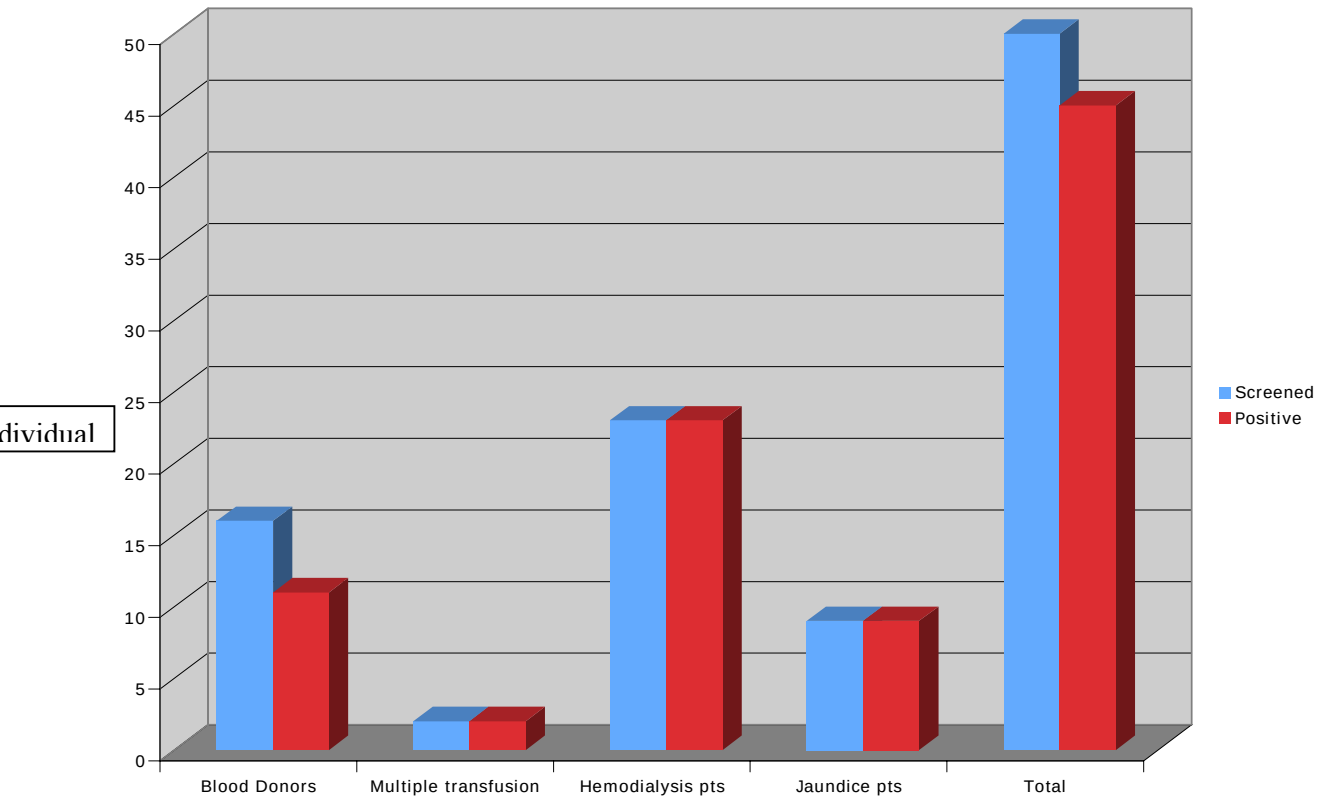

Fig. 6: HBV DNA detection in HBsAg positive patients in various groups.

* Pts = Patient.

Table 10: Sensitivity and Specificity of ELISA and ICT Test.

Test	Sensitivity %	Specificity %
ELISA	**100 %**	**99.07 %**
ICT	**100 %**	**99.07 %**

Table 11: Agreement between ELISA and ICT Test (Kappa Statistic).

Tests	Agreement %	Kappa
ELISA & ICT	**99.79 %**	**0.9***

*** <u>Very Good Agreement</u>**

Fig. 7: Detection of HBsAg by Immune-chromatography Test (ICT).

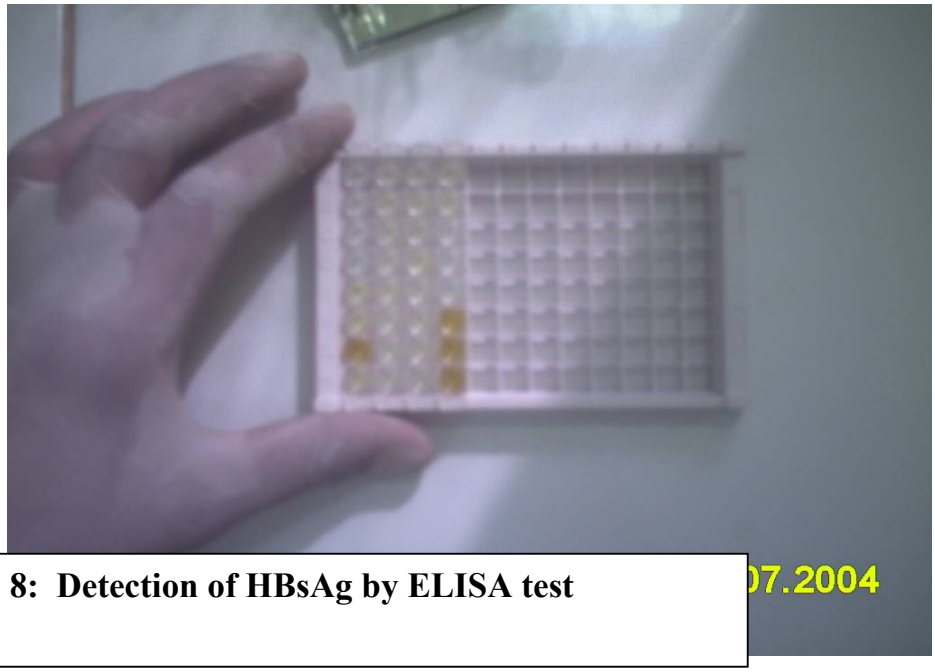

Fig. 8: Detection of HBsAg by ELISA test

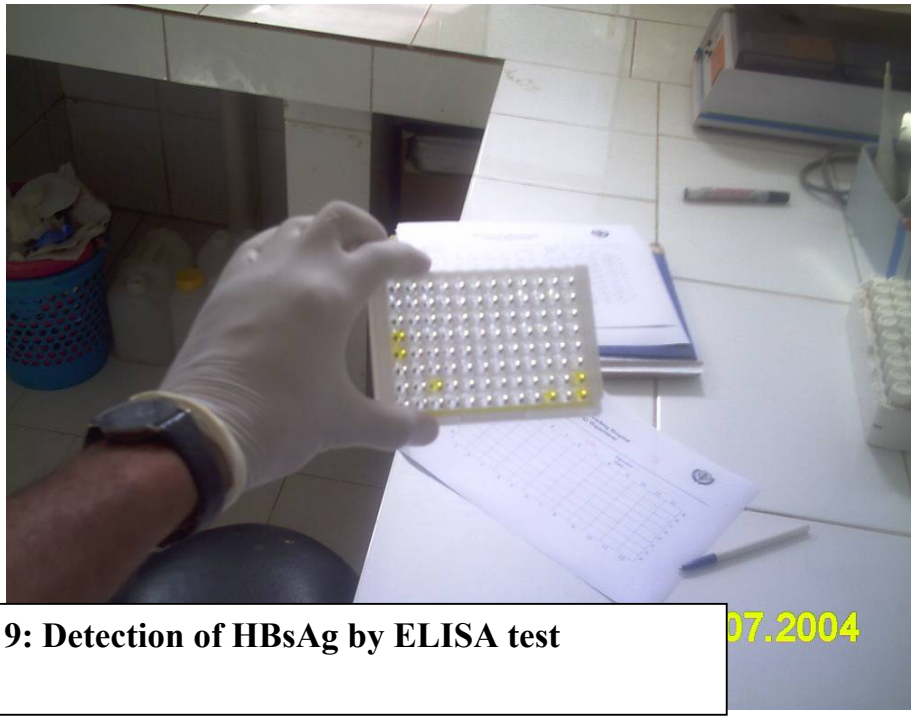

Fig. 9: Detection of HBsAg by ELISA test

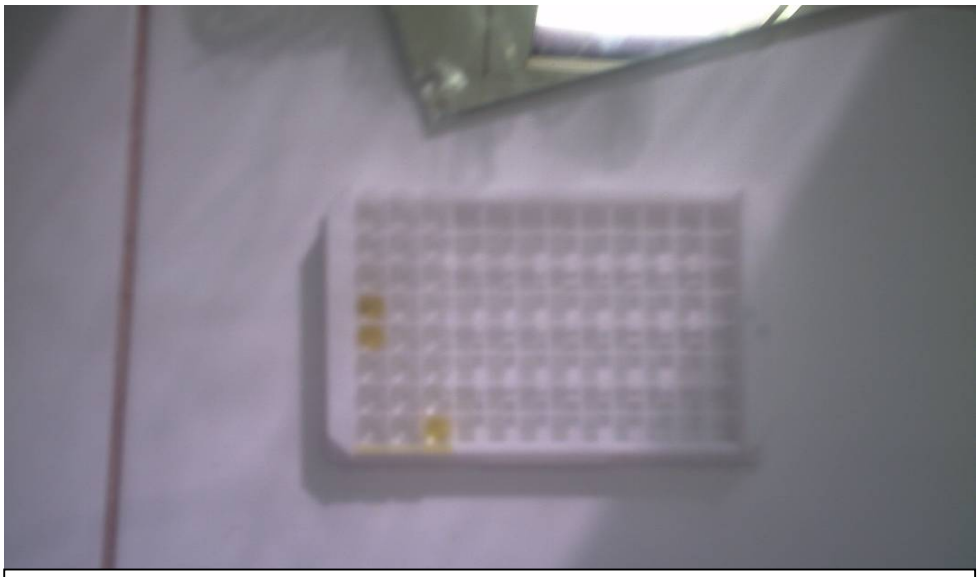

Fig. 10 : Detection of HCV-anti-bodies by ELISA test

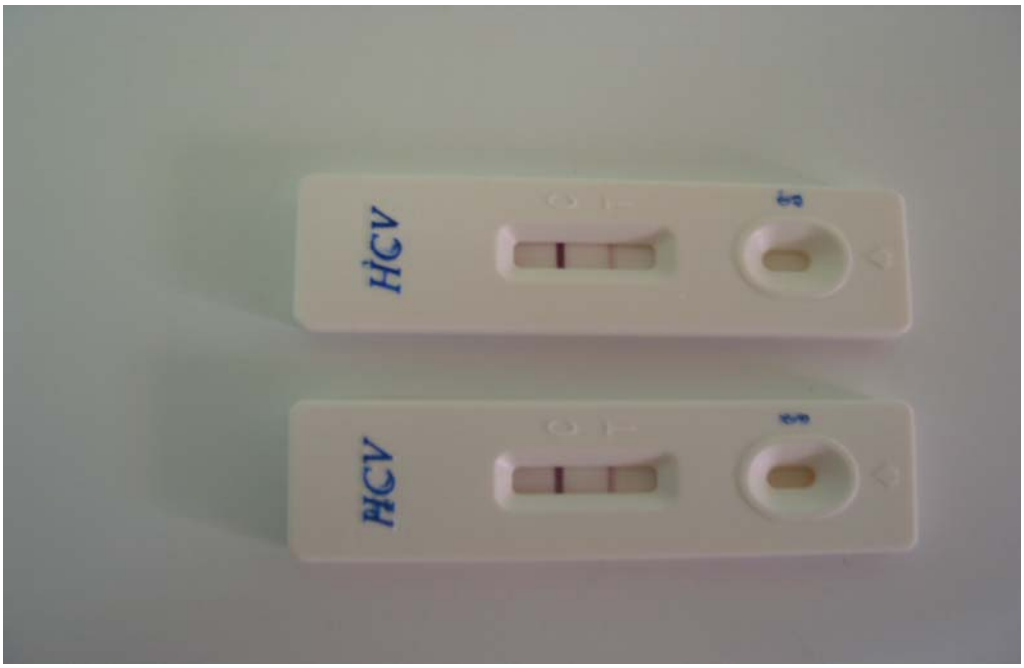

Fig. 11: Detection of HCV–antibodies by Immune-chromatography Test (ICT).

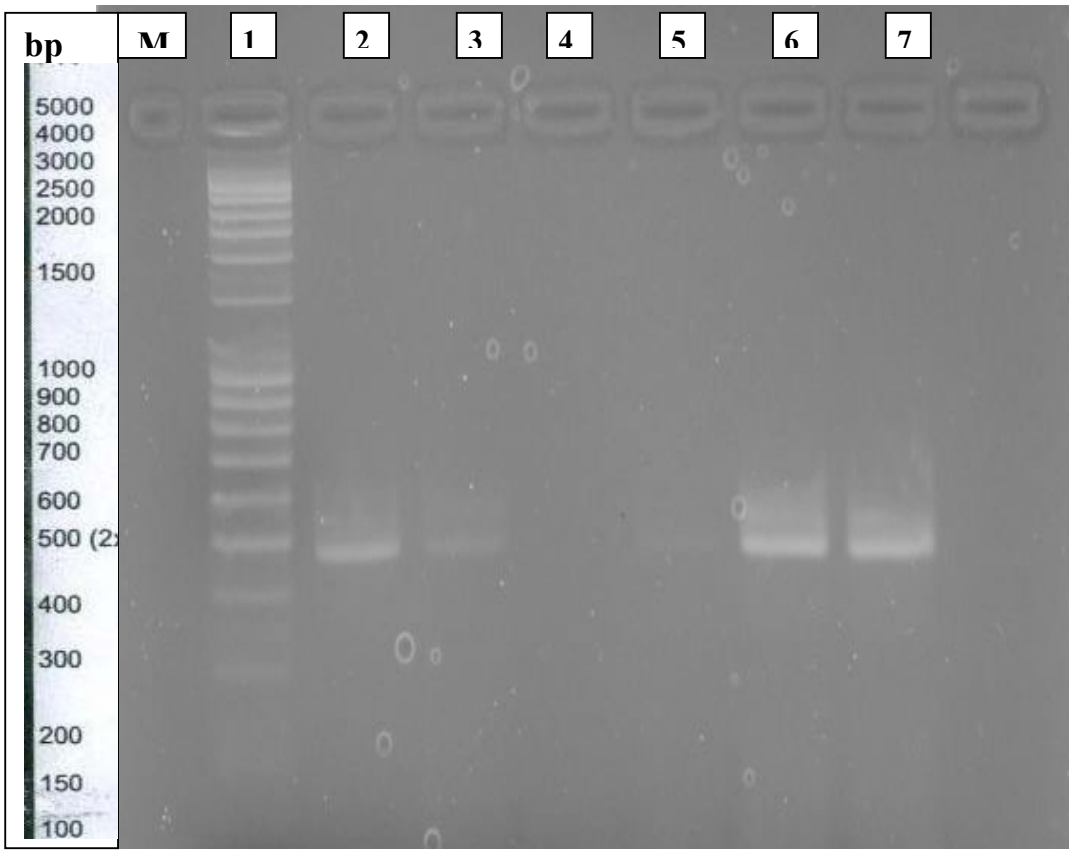

Fig. 12: Ethidium bromide stained agarose gel (1.5%) electrophoresis of H**BV** PCR products with a band of expected size 477 bp, carried out on DNA samples extracted from serum, Lane M: 100 bp ladder, with fragment size indicated along the left edge of the figure, Lane, 1 positive control, Lane, 7 negative control, Lane, 2-3-4-5-6 serum samples, sample 4 showed weak Positive.

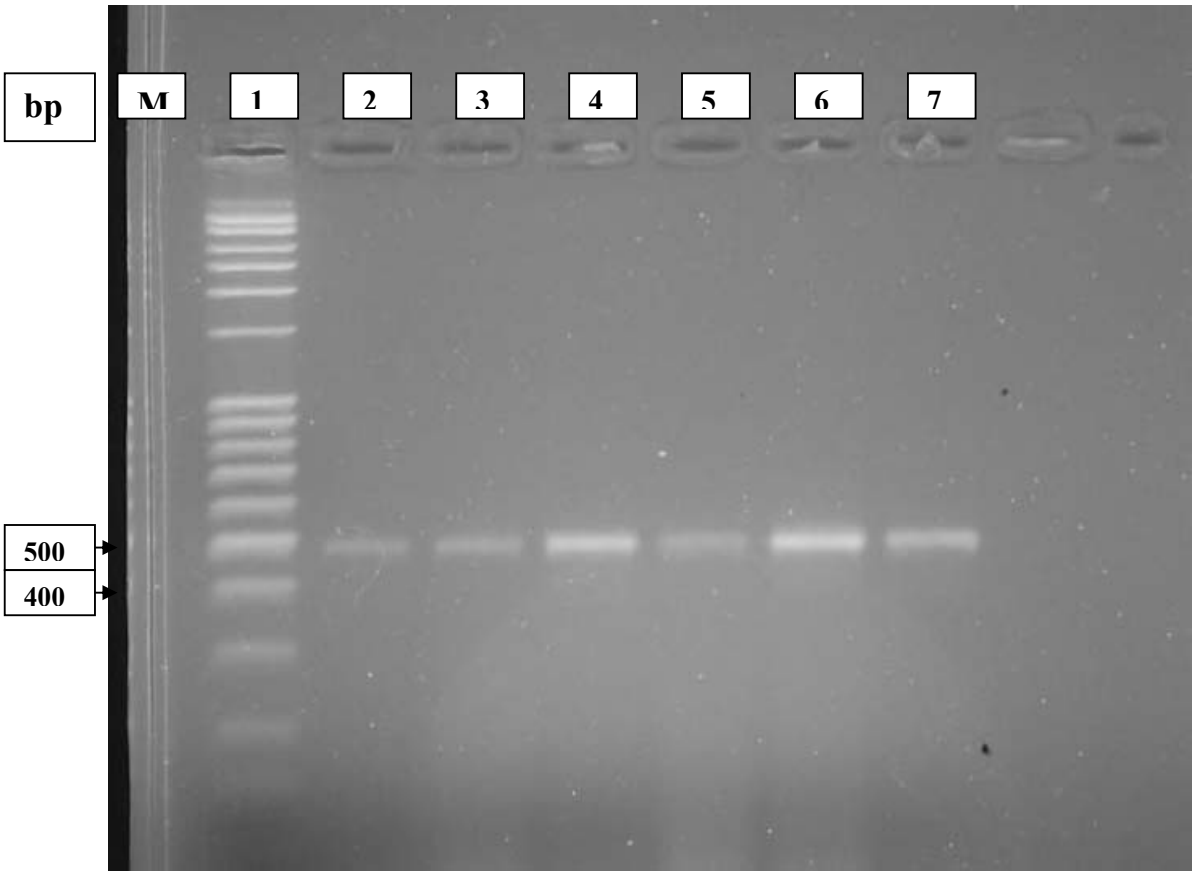

Fig. 13 : Ethidium bromide stained agarose gel (1.5 %) electrophoresis of **HBV** PCR products with a band of expected size 477 bp, carried out on DNA samples extracted from serum, Lane M: 100 bp ladder, **(**Arrow shows 500 and 400 bp band), Lane, 7 negative control, Lane, 1- 2-3-4-5-6 serum samples.

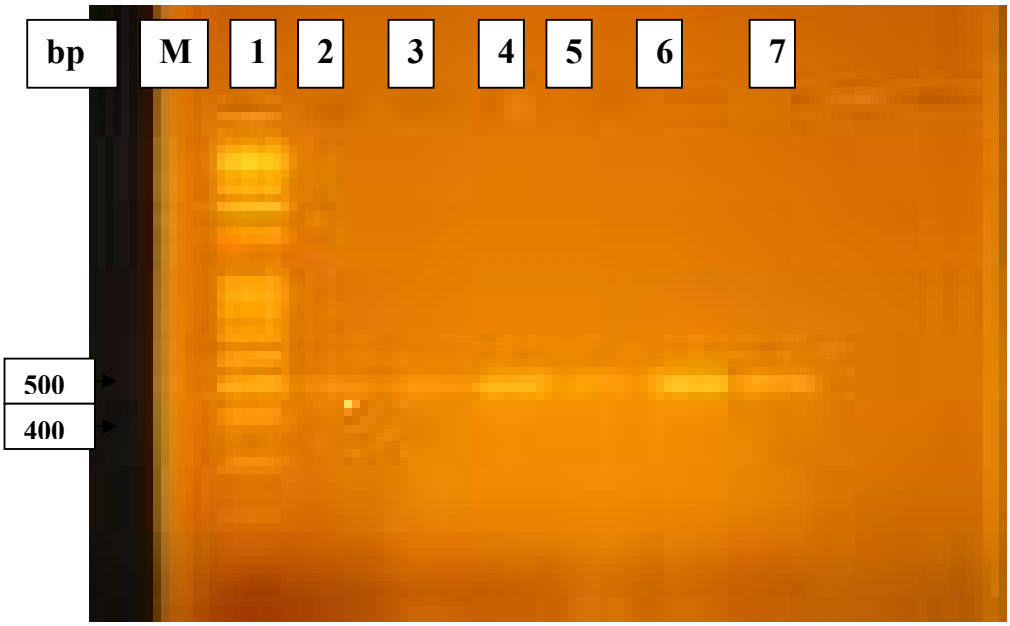

Fig. 14 : Ethidium bromide stained agarose gel (1.5 %) electrophoresis of **HBV** PCR products with a band of expected size 477 bp, carried out on DNA samples extracted from serum, Lane M: 100 bp ladder, **(**Arrow shows 500 and 400 bp band), Lane,7 negative control, Lane, 1- 2-3-4-5-6 serum samples .

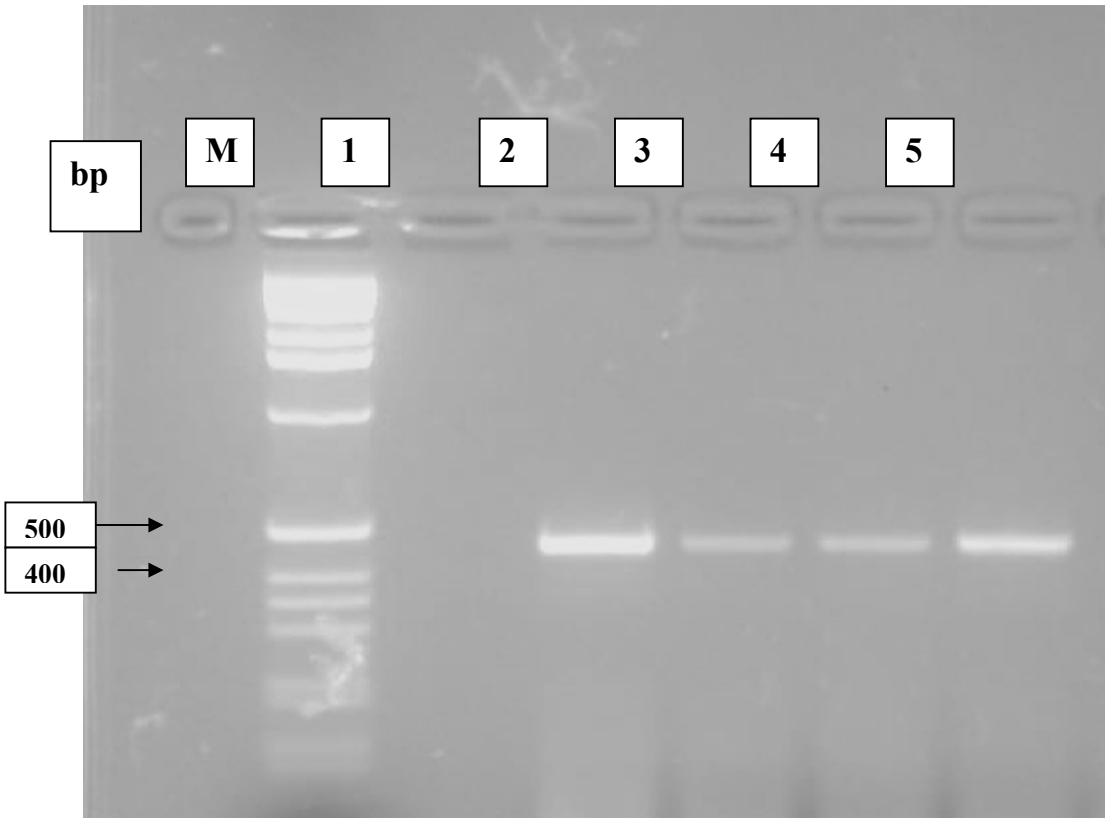

Fig. 15 : Ethidium bromide stained agarose gel (1.5 %) electrophoresis of **HBV** PCR products with a band of expected size 477 bp, carried out on DNA samples extracted from serum, Lane M: 100 bp ladder, **(**Arrow shows 500 and 400 bp band), Lane, 1 negative control, Lane, 2-3-4-5 serum samples.

CHAPTER FIVE

DISCUSSION

Hepatitis B (HB) is a major cause of death worldwide, with more than 2 million deaths per year mainly because of the sequelae of chronic liver disease, liver cirrhosis and hepatocellular carcinoma (HCC). The disease 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 (Shobokshi, 1990; Gill and Beeching, 2004).

Several studies were published on sero-prevalence of HB in the Sudan (Hymas *et al*., 1989; McCarthy *et al*.,1989; Elshafie.,1992; Khalil *et al*., 1996; Fahal *et al*., 1997; and Suliaman *et al*.,1997).)

This study is focused on the molecular and serological diagnosis of HBV and determination of co-infection with HCV in risk groups in Khartoum state, Sudan. A number of 480 serum samples were collected from blood donors, haemodialysis, multi-transfused and jaundice patients.

Out of 480 samples 49 (10.2%) were found positive for HBsAg, when tested by ICT test, while 50 samples (10.4%) were found positive for HBsAg, when tested by ELISA test .

According to Pawlotskey *et al.* (1998) HBsAg one step test will only indicate the presence of HBsAg in the sample and should not be used as the sole criteria for diagnosis of hepatitis B infection. The intensity of the red colour in the test region (T) in ICT may vary depending on the concentration of HBsAg present in the sample. Therefore, any shade of red color that develops in the test region should be considered positive.

When we compared the ELISA and ICT test the ELISA test detected one more positive sample that were negative by ICT test. Our study showed a very high agreement between ICT assay and ELISA test 99.79 % and no statistical significant difference was obtained by the application of Stata NT/98 and both assay were sensitive, specific and reliable tests.

The ELISA test is very sensitive in detecting HBs antigen, the presence of these antigens is considered as marker for HBV infection (Pawlotskey *et al,* 1998).

In this study HBV infection is found high among haemodialysis patients (23%). This figure is slightly lower than that obtained by Suliman *et al.* (1997) who found a prevalence of 27.7% of HBV among haemodialysis patients in Khartoum Kidney Dialysis Center. However, the dialysis process itself and the level of hygienic standard

may influence the risk of HBV infection, this may explain differences found between dialysis centers in one country.

Dialysis patients have an increased risk of exposure to parenterally transmitted hepatitis virus and that haemodialysis machine and parenteral medicine may be considered risk factors in HBV transmission among HBsAg positive patients (Pereira, 1992). The present study is in support of this assumption and 46% of the positive HBsAg have a history of exposure to haemodialysis machine.

Multiple blood transfusions seems to be an important risk factor in the acquisition of HB infection. Nonetheless 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 (Chopra, 1985).

In the present study we detected HBV-DNA by PCR in 45 samples out of 50 samples (90%) positive for HBsAg by ELISA test. Search of the literature revealed lower detection rate. Chen *et al.* (1995) detected Hepatitis B virus (HBV) DNA in 39 of the 116 samples with HBsAg positive (34%). Mas *et al.* (1990) reported that Hepatitis B virus DNA was found in only two patients out of 33 patients, one was a chronic HBsAg carrier. However high detection rate (98.5%) of HBV DNA during HBsAg and HBeAg antigenemia was reported By Quint *et al.* (1990).

In out of 10 samples the PCR detected two (20%) positive HBV DNA samples collected from blood donors that were negative for HBsAg by ICT and ELISA tests. This result agrees with Rodrigues. (2001) who reported that the presence of HBV DNA in 2/32 patients with past HBV infection in a group that indicates the low replicative state of HBV even after the disappearance of HBsAg. Chen *et al.* (1995) reported that the HBV DNA can be detected in the absence of HBsAg.

Similar previous study was done by Brechot *et al.* (1985) who reported that HBV DNA was detected in only 5 of 88 samples, 3 of which were from patients with no serologic marker for HBV.

In this study we found that there is a good evidence that some individuals with HBV infection as detected by PCR do not have detectable HBsAg in their serum. We suggest that such cases may be either from patients with recent infection, when the sample was obtained during the sero-negative window phase or from patients injected with mutated hepatitis BV surface antigen or the concentration of HBsAg in the samples was below the detectable level.

Our results show a small but significant fraction of blood donors whose sera are HBsAg negative by the most sensitive tests (ELISA) are positive by PCR. Accordingly, these blood donors are potential transmitters of HBV infection to recipients of their blood. Accordingly, PCR should be introduced to screen blood donors negative by ELISA test to be sure that their blood is not infectious.

HBV and HCV co-infection, causes frequently chronic active hepatitis. Both types of infections may cause fulminant hepatitis (Robinson, 1995). In this study out of 50 samples positive for HBsAg only two (4%) were found positive for HCV-antibodies when tested by ICT. However, these 2 samples in addition to another one (6%) were positive by ELISA using recombinant HCV-antigen. Vary. (2006) reported that one serum sample was positive to anti-HCV out of 175 HBsAg-positive blood donors.

Chiaramonte *et al.* (1999) reported that HBV and HCV co-infection convey increased risk for develop cirrhosis and hepatocellular carcinoma. Accordingly, the 3 patients with HBV and HCV co-infection detected in the present study are likely to develop cirrhosis and hepatocellular carcinoma.

The presence of HCV infection in Sudan was previously reported. McCarthy *et al*. (1994) reported that the 3% of studied population were found positive for HCV-antibodies in Juba, southern Sudan. Mohamed. (2006) reported the prevalence of HCV 3.4% among blood donors, mluti-transfused and haemodialysis patients and Suliman. (1995) reported that the prevalence of HCV infection in Sudanese blood donors, multi-transfused and dialysis patients is relatively low compared with those shown by data from

other countries. However, co-infection of HBV and HCV in the Sudan was reported for the first time in the present study.

Among the individuals positive for HBsAg by ELISA 30% of them have been exposed to different surgical operation. These results showed that surgical instruments might be also implicated in transmission of HB infection in Sudan. Tattooing and intravenous drug users were not found to be risk factors in this study. Accordingly these two risk factors seem not to play any role in transmission of HBV in Sudan. Our results disagree with Chopra (1985) who considered tattooing and intravenous drug users as risk groups.

In this study haemodialysis machine (46%), injury by contaminated needles (14%), sexual practice (12%), blood transfused (66%) and surgical instruments (30%) represent risk factors for transmission of HBV infection.

In this study only 4 patients (8%) have background about how HBV is transmitted and how it is prevented. According to the data obtained from the questionnaire this is probably due to the lack of professional and public education. In this study it has been found that infection with hepatitis viruses may be predisposed by more than one risk factors.

CHAPTER Six

CONCLUSION

- There was a very high agreement between ICT and ELISA and no statistical significant difference was obtained by the application of Stata NT/98 and both assays were sensitive, specific and reliable.
- High incidence of HBsAg (23%) was found in haemodialysis patients and probably haemodialysis machine have a role in the transmission of the disease.
- In this study we detected the HBV DNA in HBsAg-negative samples and suggests that multiplication of HBV may occur in the absence of any conventional serologic marker for HBV.
- In this study only 4 patients (8%) have background about how HBV is transmitted and how it is prevented. According to the data obtained from the questionnaire this is probably due to the lack of professional and public education. In this study it has been found that infection with hepatitis viruses may be predisposed by more than one risk factors.
- ICT is good as ELISA for screening for HBsAg and HCV-anti-bodies.
- There is a serious lack of information and meaningful data due to stigmatization view.
- Public awareness regarding HBV infection is low.

RECOMMENDATIONS

* PCR should be introduced to screen blood donors negative by ELISA test to be sure that their blood is not infectious.

* In order to avoid unnecessary risk of HBV infection, patients who depend on recurrent transfusion should be vaccinated .
* Blood donors must be tested for HBsAg before transfusion to recipient.
* Haemodialysis patients positive to HBsAg, must have separate machine.
* Surgical and tattooing instruments must be well sterilized before each operation.
* Isolation of patients with HBV DNA could be beneficial for preventing the dissemination of HBV among haemodialysis patients.
* HBsAg carriers should be prohibited from donating blood for transfused.
* persons with antigen-positive hepatitis B should be considered infectious and control measures by taken with respect to potentially infectious material, such as blood and blood-contaminated secretions and body secretions.
* Persons found to have a positive antigen test in the course of diagnostic studies, blood donors testing or testing after know exposure to infection with hepatitis B should be informed and the test should be repeated.
* Persons with confirmed positive test should be evaluated for the presence of liver disease and monitored to determine whether the antigen persists.
* Renal dialysis patients must be tested for hepatitis B antigen and HCV-antibodies before each dialysis or at least monthly.
* Education programmes : a comprehensive information, education and communication approach is needed to raise awareness levels in the general population and in high risk groups using all communication channels to provide appropriate messages on HBV and HCV transmission, prevention and control.
* For person whose sexual behaviors place them at risk for HBV and HCV infection, correct and consistent use of the male latex condom can reduce the risk of HBV

and HCV transmission. However, no protective method is 100 percent effective, and condom use cannot guarantee absolute protection against any sexually transmitted disease (STDs)

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Appendix (1)

1. Preparation of cell lysis buffer (ph.7.4):

1. 10 mM Tris Hcl.	0.121 gm
2. 10 mM EDTA.	0.29gm
3. SDS. (0.5%)	0.5 gm
4. Mercaptoethanol 2%	2ml
5. 100mM NaCl.	0.58gm

Complete to 100ml of distil water.

2. Preparation of protinase K (10mg/ml):

0.01gm of protinase K was added to 1 ml of distill water.

3. Preparation of materials for gel electrophoresis:

3.1 Agarose 1.5%:

1.5 gm of agarose (SIGMA) was dissolved in 100 ml TAE buffer heated in a microwave for 45seconds, left to cool and poured onto electrophoresis.

3.2 TAE Buffer:

40mM Tris-Hcl (ph 8.0)	40ml
20mM Na-acetate	20ml
EDTA Powder (292.25MW)	5843gm

Complete to one liter.

3.3 Loading dye:

Bromophenol Blue	11%
Glycerol	40µl
DDW	50µl

3.4 100 bp Ladder (INVITROGEN):

Ladder (1.0 ug/ul)	20ml
Blue dye	80ml

3.5.Ethidium bromide:

Stock solution (PROMEGA, Madison) 10mg/ml

And protected from light.

Table 12: Data obtained from the questionnaire

NO Of Sample	sex	age	PBT	BD	op	ta	Ivdu	Ad	RPsc	HM	HB vac	AH BT	injcn	HBTw	sc
14	M	26	Yes	No	NO	NO	NO	NO	Yes	Yes	NO	NO	NO	NO	NO
30	M	31	yes	Yes	Yes	NO	NO	NO	Yes	Yes	NO	NO	NO	NO	NO
36	M	17	Yes	NO	NO	NO	NO	NO	NO	Yes	NO	NO	NO	NO	NO
37	F	25	Yes	NO	NO	NO	NO	NO	NO	Yes	NO	NO	NO	Yes	NO
40	M	17	Yes	NO	NO	NO	NO	NO	NO	Yes	NO	NO	NO	NO	NO
41	M	45	Yes	NO	Yes	NO	NO	NO	Yes	Yes	NO	NO	NO	NO	NO
42	F	27	Yes	NO	NO	NO	NO	NO	Yes	Yes	NO	NO	NO	NO	NO
43	M	31	Yes	NO	NO	NO	NO	Yes	Yes	Yes	NO	NO	NO	NO	NO
44	M	22	Yes	Yes	NO	NO	NO	NO	NO	Yes	NO	NO	NO	NO	NO
50	F	35	Yes	NO	Yes	NO	NO	NO	Yes	Yes	NO	NO	NO	Yes	NO
57	M	40	Yes	NO	NO	NO	NO	NO	Yes	Yes	NO	NO	NO	NO	NO
58	M	35	Yes	Yes	NO	NO	NO	NO	Yes	Yes	NO	NO	NO	NO	NO
59	M	41	Yes	NO	NO	NO	NO	Yes	Yes	Yes	NO	NO	NO	NO	NO
60	M	38	Yes	NO	NO	NO	NO	NO	Yes	Yes	NO	NO	NO	NO	NO
70	F	52	Yes	NO	Yes	NO	NO	NO	Yes	Yes	NO	NO	NO	NO	NO
78	M	49	Yes	NO	NO	NO	NO	Yes	Yes	Yes	NO	NO	NO	NO	NO
83	M	39	Yes	NO	NO	NO	NO	NO	Yes	Yes	NO	NO	NO	NO	NO
84	M	47	Yes	NO	NO	NO	NO	NO	Yes	Yes	NO	NO	NO	NO	NO
85	M	20	Yes	Yes	NO	NO	NO	NO	NO	Yes	NO	NO	NO	NO	NO
89	M	34	Yes	NO	NO	NO	NO	NO	NO	Yes	NO	NO	NO	NO	NO
92	M	28	Yes	Yes	NO	NO	NO	NO	Yes	Yes	NO	NO	NO	NO	NO
95	M	37	Yes	NO	NO	NO	NO	NO	Yes	Yes	NO	NO	NO	NO	NO
98	M	29	Yes	NO	NO	NO	NO	NO	NO	Yes	NO	NO	NO	NO	NO
109	F	43	Yes	NO	Yes	NO	NO	NO	Yes	NO	NO	NO	NO	NO	NO
118	M	63	Yes	NO	NO	NO	NO	NO	Yes	NO	NO	NO	NO	NO	NO
119	F	26	No	NO	NO	NO	NO	NO	NO	NO	NO	NO	Yes	NO	NO
143	M	50	NO	NO	Yes	NO	NO	NO	Yes	NO	NO	NO	NO	NO	NO
149	F	27	NO	NO	NO	NO	NO	NO	Yes	NO	NO	NO	Yes	Yes	NO
159	F	35	Yes	NO	NO	NO	NO	NO	Yes	NO	NO	NO	NO	NO	NO
167	M	60	Yes	NO	Yes	NO	NO	NO	Yes	NO	NO	NO	NO	NO	Yes
171	M	31	NO	Yes	NO	NO	NO	Yes	NO	NO	NO	NO	Yes	NO	NO
174	M	40	Yes	NO	NO	NO	NO	NO	Yes	NO	NO	NO	NO	NO	NO
181	M	38	NO	NO	NO	NO	NO	NO	Yes	NO	NO	NO	Yes	NO	NO
190	M	35	Yes	NO	Yes	NO	NO	NO	Yes	NO	NO	NO	NO	NO	Yes
211	M	30	NO	Yes	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO
234	M	27	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	Yes	NO	Yes
250	M	18	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO
282	M	25	NO	Yes	NO	NO	NO	NO	NO	NO	NO	NO	Yes	NO	NO
307	M	36	Yes	NO	Yes	NO	NO	NO	Yes	NO	NO	NO	NO	Yes	NO
320	M	39	NO	Yes	Yes	NO	NO	NO	Yes	NO	NO	NO	NO	NO	Yes
380	M	28	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	Yes
392	M	21	NO	NO	NO	NO	NO	Yes	NO	NO	NO	NO	NO	NO	NO
393	M	37	NO	Yes	Yes	NO	NO	NO	Yes	NO	NO	NO	NO	NO	NO
394	M	32	NO	NO	Yes	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO
410	M	26	NO	Yes	NO	NO	NO	NO	NO	NO	NO	NO	Yes	NO	NO
426	M	24	NO	NO	Yes	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO
430	F	44	Yes	Yes	Yes	NO	NO	NO	Yes	NO	NO	NO	NO	NO	Yes
437	M	33	NO	NO	NO	NO	NO	NO	Yes	NO	NO	NO	NO	NO	NO
445	M	30	Yes	Yes	NO	NO	NO	NO	Yes	NO	NO	NO	NO	NO	NO
448	M	39	Yes	Yes	Yes	NO	NO	NO	Yes	NO	NO	NO	NO	NO	NO

PBT = Previous Blood Transfused __ BD = Blood donate __OP = operations __ ta = tattooing __ ivdu = intravenous drug users __ ad = alcoholic drink ___ Rpsc = regular partener sexual contact __ HM = heamodialysis Machine ___ HBvac = Hepatitis B vaccine AHBT = Anti-hepatitis B treatment __ injcn = injured by contaminated needles ___HBTw = Hepatitis B Transmission ways ___ Sc = sexual contact __M = Male ____ F = female

Table 13: Risk factor associated with HBV positive samples in each group.

NO Of Sample	PBT	BD	OP	TA	IVDU	Ad	RPsc	HM	INJ CN	Sc
14	+	-	-	-	-	-	+	+	-	-

30	+	+	+	-	-	-	+	+	-	-
36	+	-	-	-	-	-	-	+	-	-
37	+	-	-	-	-	-	-	+	-	-
40	+	-	-	-	-	-	-	+	-	-
41	+	-	+	-	-	-	+	+	-	-
42	+	-	-	-	-	-	+	+	-	-
43	+	-	-	-	-	+	+	+	-	-
44	+	+	-	-	-	-	-	+	-	-
50	+	-	+	-	-	-	+	+	-	-
57	+	-	-	-	-	-	+	+	-	-
58	+	+	-	-	-	-	+	+	-	-
59	+	-	-	-	-	+	+	+	-	-
60	+	-	-	-	-	-	+	+	-	-
70	+	-	+	-	-	-	+	+	-	-
78	+	-	-	-	-	+	+	+	-	-
83	+	-	-	-	-	-	+	+	-	-
84	+	-	-	-	-	-	+	+	-	-
85	+	+	-	-	-	-	-	+	-	-
89	+	-	-	-	-	-	-	+	-	-
92	+	+	-	-	-	-	+	+	-	-
95	+	-	-	-	-	-	+	+	-	-
98	+	-	-	-	-	-	-	+	-	-
109	+	-	+	-	-	-	+	-	-	-
118	+	-	-	-	-	-	+	-	-	-
119	-	-	-	-	-	-	-	-	+	-
143	-	-	+	-	-	-	+	-	-	-
149	-	-	-	-	-	-	+	-	+	-
159	+	-	-	-	-	-	+	-	-	-
167	+	-	+	-	-	-	+	-	-	+
171	-	+	-	-	-	+	-	-	+	-
174	+	-	-	-	-	-	+	-	-	-
181	-	-	-	-	-	-	+	-	+	-
190	+	-	+	-	-	-	+	-	-	+
211	-	+	-	-	-	-	-	-	-	-
234	-	-	-	-	-	-	-	-	+	+
250	-	-	-	-	-	-	-	-	-	-
282	-	+	-	-	-	-	-	-	+	-
307	+	-	+	-	-	-	+	-	-	-
320	-	+	+	-	-	-	+	-	-	+
380	-	-	-	-	-	-	-	-	-	+
392	-	-	-	-	-	+	-	-	-	-
393	-	+	+	-	-	-	+	-	-	-
394	-	-	+	-	-	-	-	-	-	-
410	-	+	-	-	-	-	-	-	+	-
426	-	-	+	-	-	-	-	-	-	-
430	+	-	+	-	-	-	+	-	-	-
437	-	-	-	-	-	-	+	-	-	+
445	+	-	-	-	-	-	+	-	-	-
448	+	+	+	-	-	-	+	-	-	-
Total	**66%**	**24%**	**30%**	**0**	**0**	**10%**	**64%**	**46%**	**14%**	**12%**

50										

PBT = Previous Blood Transfused __ BD = Blood donate __OP = operations

TA = tattooing __ IVDU = intravenous drug users __ AD = alcoholic drink

RPsc = regular partener sexual contact __ HM = haemodialysis Machine

INJCN = injured by contaminated needles ___ Sc = sexual contact.

Table.14: HBV-DNA and HBsAg in each group.

No Of Sample	HBsAg	HBV -DNA	Tested Groups
14	+	+	Haemodialysis Patients
30	+	+	Haemodialysis Patients
36	+	+	Haemodialysis Patients
37	+	+	Haemodialysis Patients
40	+	+	Haemodialysis Patients
41	+	+	Haemodialysis Patients
42	+	+	Haemodialysis Patients
43	+	+	Haemodialysis Patients
44	+	+	Haemodialysis Patients
50	+	+	Haemodialysis Patients
57	+	+	Haemodialysis Patients

58	+	+	Haemodialysis Patients
59	+	+	Haemodialysis Patients
60	+	+	Haemodialysis Patients
70	+	+	Haemodialysis Patients
78	+	+	Haemodialysis Patients
83	+	+	Haemodialysis Patients
84	+	+	Haemodialysis Patients
85	+	+	Haemodialysis Patients
89	+	+	Haemodialysis Patients
92	+	+	Haemodialysis Patients
95	+	+	Haemodialysis Patients
98	+	+	Haemodialysis Patients
109	+	+	Multi-transfused Patients
118	+	+	Multi-transfused Patients
119	+	+	Jaundiced Patients
143	+	+	Jaundiced Patients
149	+	+	Blood Donors
159	+	+	Blood Donors
167	+	+	Blood Donors
171	+	+	Blood Donors
174	+	+	Blood Donors
181	+	+	Blood Donors
190	+	+	Blood Donors
211	+	+	Blood Donors
234	+	+	Blood Donors
250	+	+	Blood Donors
282	+	+	Blood Donors
307	+	+	Blood Donors
320	+	+	Blood Donors
380	+	+	Blood Donors
392	+	+	Blood Donors
393	+	+	Blood Donors
394	+	+	Blood Donors
410	+	+	Blood Donors
426	+	-	Blood Donors
430	+	-	Blood Donors
437	+	-	Blood Donors
445	+	-	Blood Donors
448	+	-	Blood Donors
Total 50	**50**	**45**	

Table .15: ELISA, ICT test and PCR results

No Of Sample	ELISA Test	ICT Test	PCR	Tested Groups
14	+	+	+	Haemodialysis Patients
30	+	+	+	Haemodialysis Patients
36	+	+	+	Haemodialysis Patients
37	+	+	+	Haemodialysis Patients
40	+	+	+	Haemodialysis Patients
41	+	+	+	Haemodialysis Patients
42	+	+	+	Haemodialysis Patients
43	+	+	+	Haemodialysis Patients
44	+	+	+	Haemodialysis Patients
50	+	+	+	Haemodialysis Patients
57	+	+	+	Haemodialysis Patients
58	+	+	+	Haemodialysis Patients
59	+	+	+	Haemodialysis Patients
60	+	+	+	Haemodialysis Patients
70	+	+	+	Haemodialysis Patients
78	+	+	+	Haemodialysis Patients
83	+	+	+	Haemodialysis Patients
84	+	+	+	Haemodialysis Patients

85	+	+	+	Haemodialysis Patients
89	+	+	+	Haemodialysis Patients
92	+	+	+	Haemodialysis Patients
95	+	+	+	Haemodialysis Patients
98	+	+	+	Haemodialysis Patients
109	+	+	+	Multi-transfused Patients
118	+	+	+	Multi-transfused Patients
119	+	+	+	Jaundiced Patients
143	+	+	+	Jaundiced Patients
149	+	+	+	Blood Donors
159	+	+	+	Blood Donors
167	+	+	+	Blood Donors
171	+	+	+	Blood Donors
174	+	+	+	Blood Donors
181	+	+	+	Blood Donors
190	+	+	+	Blood Donors
211	+	+	+	Blood Donors
234	+	+	+	Blood Donors
250	+	+	+	Blood Donors
282	+	+	+	Blood Donors
307	+	+	+	Blood Donors
320	+	+	+	Blood Donors
380	+	+	+	Blood Donors
392	+	+	+	Blood Donors
393	+	+	+	Blood Donors
394	+	+	+	Blood Donors
410	+	-	+	Blood Donors
426	+	+	-	Blood Donors
430	+	+	-	Blood Donors
437	+	+	-	Blood Donors
445	+	+	-	Blood Donors
448	+	+	-	Blood Donors
Total 5o samples	**50 positive**	**49 positive**	**45 positive**	50 individuals

Appendix (2)
Questionnaire

Molecular and Serological Detection of Hepatitis B Virus in Risk Groups and Blood donors in Khartoum state and Determination of Co-infection with Hepatitis C Virus.

KHARTOUM STATE

Questionnaire

IDENTIFICATION
Lace NAME:…….
State:……..
Sample number:……..
Name of respondent (in pencil):…….
Address:…….

Supervisor:_____
Date: / /

Consent form

Good morning/afternoon /evening my name is ***MOAWIA_ELEAD HAMED***

Thank you for taking the time to talk with me. We are from Khartoum University - Graduate college. We asking questions to people such as your self at different parts of Khartoum state in our research is detect HBV DNA . This research in expected to provide data that may help in medical care of people, who contacted this disease. if you agree to be interviewed, I will be asking you questions about your self, your ideas, attitudes and behavior on various issues. Most of them are related to your disease. in addition to this we will be taking some samples (blood), to be for laboratory investigations.

Your opinion or experiences are important to us. We want you to be honest and truthful in answering our questions. Your answers will be confidential and secret. if you agree to be interviewed, (you can suggest a place to go where no one can hear us talking.)

Your participation in the study is voluntary. Some of the questions I will ask you some personal and about reproductive health, sexuality, blood transfusion and haemodialysis. If you are uncomfortable with a question, you don't have to answer if you wish. You may also stop the interview at any time.

I will first ask you my questions. It will take some time to complete. After that you may ask me questions if you want. Is there a place we can go where we can talk? (Suggest a place if the participant does not.) Do you have any questions?

Do you agree to participate in this interview?
Yes/ No
Signature of the interviewer:_____
Name of the interviewer:_____ Date:_____

Section 1. Respondent's Background

No.	Questions	Answers
1	Record the time use 24-hour time	Hour: Minute:
2	Sex of respondent	Male: Female:
3	In what month and year were you born?	Month: Don't know month: Year : Don't know year:
4	Have you ever attended school?	Yes: No:
5	Are you currently in school?	Yes: No:
6	What is the highest normal school you completed?	Less than one year: primary: intermediate: secondary: University: Other:
7	Can you read or understand a letter or news easily, with difficulty, or not at all?	Easily: With difficulty: No at all:
8	What is your religion?	Moslem: Christian: None: Other: (specify)
9	Are you employed?	Yes: No:
10	Tribe	
11	Original place	
12	Have you ever been married or lived with a person?	Yes married: Yes lived with a person: No:
13	Are you currently married or living with someone?	Yes currently married: Yes, living with someone: No, Not in union:
14	Do you currently have a regular sexual partner, an occasional sexual partner, or no sexual partner at all?	Regular sexual partner: Occasional sexual partner: No sexual partner:
15	Is your partner living with you now or staying elsewhere?	Living with : Staying elsewhere:
16	Have you ever had sexual intercourse?	Yes: No:
17	When was the last time you had sexual intercourse? Record "years ago" only if last intercourse was one or more years ago (12 or more months ago) Record "00" days ago if last intercourse was on	Days ago : Weeks ago: Months ago: Years ago:

	same day as interview	
18	The last time you had sexual intercourse was a condom used?	Yes: No: Never heard of condoms: Don't know:

Section(2) HBV

19	HB vaccines	Yes: No:
20	When did this happen	Specify:
21	Haemodialysis	Yes: No:
22	History of Jaundice	Yes: No:
23	History of blood transfusion	Yes: No:
24	Do you know how HBV transmission? (DONOT READ LIST)	Sexual intercourse: Sharing needles and blades: Blood transfusion: Mother to fetus: Haemodialysis: Other: Don't know:
25	When frequent do you visit your doctor?	Once month: More than once month: When I feel sick:
26	Do you follow a specific lifestyle advised by health professional?	Yes: No:
27	**Using anti-HBV treatments** Have you ever taken any Anti-HBV treatments	Yes: No:
28	Have you ever experienced any side effects from anti – HBV treatments?	Yes: No:
29	Are you taking any Anti-HBV treatments, at the moment?	Yes: No:
30	At the moment, how many times a day do you have to take any kind of anti-HBV treatment?	Once a day: Twice a day: More: (specify)

Section (3)Media

31	Do you usually read a newspaper or magazine almost every day, at least once a week, less than once a week or not at all?	Almost every day: At least once a week: Less than once a week: Not at all:
32	Do you listen to the radio almost every day, at least once a week, less than once a week or not at all?	Almost every day: At least once a week: Less than once a week:

		Not at all:
33	Do you watch television almost every day, at least once a week, less than once a week or not at all?	Almost every day: At least once a week: Less than once a week: Not at all:
34	Do you use internet almost every day, at least once a week, less than once a week or not at all?	Almost every day: At least once a week: Less than once a week: Not at all:
35	In the past year, have you heard or seen any message about HB?	Yes: No: Don't know:
36	What slogans or messages about HB did you hear or see?	
37	Where did you hear or see these messages? (Prompt any thing else Record all mentioned Selections not mentioned spontaneously)?	Television Radio Newspapers Magazines Billboards Football match Concert Community Rally Road show Tee shirts/ caps Moible video Tire protector Calendar Poster Sticker
38	In the past year have you heard or seen any messages about transfusion and haemodealysis?	Yes: No: Don't know
39	Where did you here or see these messages about haemodealysis (Prompt any thing else? Record all mentioned Selections not mentioned spontaneously)	Television Radio Newspapers Magazines Billboards Football match Concert Community Rally Road show Tee shirts/ caps Moible video Tire protector Calendar Poster Sticker