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Investigation of Hepatitis B Virus DNA in Rheumatoid Arthritis Patients Khartoum – Sudan

كشف انتشار مرض الكبد الوبائي (ب) في مرضى إلتهاب المفاصل الروماتيدي في الخرطوم - السودان

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بسم الله الرحمن الرحيم

الآيـــــة

قَالَ تَعَالَىٰ:

قَالَ تَعَالَىٰ: أَعُوذُ بِٱللَّهِ مِنَ ٱلشَّيۡطَٰنِ ٱلرَّجِيمِ ﴿ ءَامَنَ ٱلرَّسُولُ بِمَآ أُنزِلَ إِلَيۡهِ مِن رَّبِّهِۦ وَٱلۡمُؤۡمِنُونَۚ كُلٌّ ءَامَنَ بِٱللَّهِ وَمَلَٰٓئِكَتِهِۦ وَكُتُبِهِۦ وَرُسُلِهِۦ لَا نُفَرِّقُ بَيۡنَ أَحَدٖ مِّن رُّسُلِهِۦۚ وَقَالُواْ سَمِعۡنَا وَأَطَعۡنَاۖ غُفۡرَانَكَ رَبَّنَا وَإِلَيۡكَ ٱلۡمَصِيرُ ٢٨٥ ﴾

البقرة: ٢٨٥

صدق الله العظيم

Dedication

This work is dedicated to my parents who mean the world to me.

To all my family members and my friends.

Acknowledgments

All thanks to Allah from the start to the end, and special thanks to my supervisor **DR. Abbas Mohamed Ahmed** who directed and guided me.

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ABSTRACT

Hepatitis B infection is an alarming public health problem .Almost two billion people from the population alive today, were infected at some time in their lives by hepatitis B virus.It has been reported to be associated with liver diseases as well as various types of extrahepatic manifestations. Furthermore the association with other inflammatory syndromes as rheumatoid arthritis, polymyalgia rheumatica and polymyositis.This is a descriptive cross sectional study conducted to determine the prevalence of hepatitis B virus infection related to rheumatoid arthritis at Alrayan , Military hospital and Alya military hospital, Khartoum – Sudan using PCR .The study was carried out from march 2018 to march 2019. Participants comprised 120 randomly selected (40 controls, 40 known rheumatoid cases and 40 suspected with rheumatoid arthritis). Samples were collected in plain containers then centrifuged then the clear serum was separated and stored at 4 C^0 until used, after that DNA was extracted by chloroform method and processed by conventional PCR using the following primers forward (5' – CCC TTG CGG ATG CCAAT -3') and reverse primer (5' – GGC TGA TAT GGA TGC CC - 3'). Out of 120 patients 34 (28 %) was positive for HBV DNA , 22(18.3%)were males, 98(81.7%)were females .Throughout the males population (22) only 6 samples was positive and 28 out of the females population (98) were positive .HBV DNA observed in 3 (2.5%) samples out of 40 cases and 31 (25.8%)samples from 40 suspected which was statistically significant, relying on the results hepatitis B virus its related to rheumatoid arthritis and arthritis symptoms pressed the onset of jaundice .

ملخص الاطروحة

عدوى التهاب الكبد B هي مشكلة صحية عامة تنذر بالخطر ، فقد أصيب ما يقرب من ملياري شخص على قيد الحياة اليوم بفيروس التهاب الكبد B ، وقد تم الإبلاغ عن ارتباطه بأمراض الكبد بالإضافة إلى أنواع مختلفة من المظاهر خارج الكبد. علاوة على ذلك ، فإن الارتباط مع المتلازمات الالتهابية الأخرى مثل التهاب المفاصل الروماتويدي والتهاب العضلات الروماتيزمي والتهاب العضلات. أجريت هذه الدراسة الوصفية المقطعية لتحديد مدى انتشار عدوى فيروس التهاب الكبد B المرتبطة بالتهاب المفاصل الروماتويدي في مستشفى الريان العسكري ومستشفى علياء العسكري بالخرطوم - السودان باستخدام PCR. تم إجراء الدراسة من مارس 2018 إلى مارس 2019. ضمت المشاركين 120 تم اختيارهم عشوائيًا (40 مجموعة تحكم ، 40 حالة روماتيزمية معروفة و 40 حالة مشتبه بإصابتها بالتهاب المفاصل الروماتويدي). تم جمع العينات في حاويات الخطة ثم الطرد المركزي ثم تم فصل المصل الصافي وتخزينه عند 4 درجة مئوية حتى يتم استخدامه ، بعد ذلك تم استخراج الحمض النووي بطريقة الكلوروفورم ومعالجته بواسطة PCR التقليدي باستخدام البادئات التالية للأمام (5 '- CCC TTG CGG ATG CCAAT - 3 ') والتمهيدي العكسي (5' - GGC TGA TAT GGA TGC CC - 3 - '). من بين 80 مريضًا ، كان 34 (28٪) موجبًا للحمض النووي لفيروس التهاب الكبد B ، و 22 (18.3٪) من الذكور ، و 98 (81.7٪) من الإناث ، وفي جميع أنحاء السكان الذكور (22) كانت 6 عينات فقط إيجابية و 28 من الإناث. (98) كانت إيجابية. لوحظ الحمض النووي لفيروس التهاب الكبد B في 3 (2.5٪) عينات من 40 حالة و 31 (25.8٪) عينة من 40 مشتبهاً به والتي كانت ذات دلالة إحصائية ، معتمدين على نتائج فيروس التهاب الكبد B المرتبطة بالتهاب المفاصل الروماتويدي وأعراض التهاب المفاصل التي ضغطت على ظهور اليرقان.

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CHAPTER ONE

1. Introduction

1.1 Introduction

Hepatitis B virus, a member of the *Hepadnaviridae* family, is a circular, partially double-stranded Deoxyribonucleic acid (DNA) virus with a genome of approximatively 3.2 kilobases in length. It contains four overlapping open reading frames (ORFs) PreS/S, PreC/C, P and X (Pourkarim *et al*., 2014).

Transmission of HBV is through the parenteral route, blood transfusion products and sexual intercourse and vertically from infected mothers to neonates. The virus is found in body fluids such as urine, saliva, nasopharyngeal fluids, semen and menstrual fluids, and can be transmitted through contact with these fluids (Mahony, 1999).

Hepatitis B virus (HBV) infects the liver causing acute and chronic liver disease (Akbar *et al*., 2004; McMahon, 2005). Despite the presence of an effective prophylactic vaccine it is estimated that 2 billion people have been infected with HBV during their lifetime and over 350 million are chronic carriers of the virus and have an increased risk of developing liver cirrhosis and hepatocellular carcinoma (HCC), (WHO, 2012), with 65 million chronic carriers residing in Africa. The sub-Saharan region is highly endemic with Hepatitis B surface antigen (HBsAg) carrier rates of between 9-20 % (Kramvis and Kew, 2007).

Hepatitis B virus infection is common in Sudan in all age groups. In a seroprevalence study carried out in Juba, Southern Sudan, in 1993 on 666 patients attending Juba Hospital, 26% were HBsAg positive (McCarthey *et al*,1994) In another study among soldiers in 5 urban localities, 78% had evidence of past infection (McCarthey *et al*,1989)In a study conducted in eastern Sudan on people in high-risk groups (prostitutes, long distance truck drivers and soldiers), positivity for HBsAg was 14% (McCarthey *et al*,1989). The epidemiology of hepatitis B was also studied in central Sudan, Gezira area, where HBsAg positivity was 14% (Hyams *et al*.,1989). In a study conducted in Omdurman among adults with acute hepatitis, HBV infection was 12.6% (El Arabi *et al*.,1987)Similarly, 12.4% of patients attending a surgical unit were positive for HBsAg (El Sanousi,1997)Sudan is considered highly endemic for HBsAg, with prevalence about 16%–20% in the general population(Qirbi and Hal,2001).

In Africa very high endemicity of HBV is seen in developing regions with large populations and most infections in these highly endemic areas occur during infancy or early childhood .More than 8 % of the population in these areas is chronically infected with HBV and 70-95 % of the population bears serological evidence of past or present HBV infection. Sub-Saharan Africa, most of the West, East, Central and sub-Saharan Africa with chronic infection rates of (7-26%), nonetheless areas like Kenya, Cote d'Ivoire, Liberia, Sierra Leone, Senegal and Zambia are moderately affected with intermediate endimicity rates between 2 and 8 %) (Kramvis and Kew, 2007), this shows mixed patterns of transmission, including infant,early childhood and adult transmission (Hou *et al*., 2005, Ander , 2000). Low endemicity was found in the Northern African countries of Egypt, Tunisia, Algeria and Morocco with prevalence rates of less than 2 % (Figure 1.1) (Kramvis and Kew,2007).

The prevalence of chronic hepatitis B (CHB) varies from regions: a low rate (0.1 – 2%) in the USA and Western Europe, an intermediate rate (2 – 8%) in Mediterranean countries and Japan and a high rate (8 – 20%) in SouthEast Asia and SSA regions where infections are the most common (Liaw and Chu, 2009). HBV-infected children are most at risk to develop CHB, putting them at high risk of developing the complications of chronic infection.

Rheumatoid arthritis (RA) is a chronic inflammatory disorder which affects the joints and is associated with swelling, stiffness and pain. Advanced disease stages can lead to substantial loss of functioning and mobility. RA is an autoimmune disease, whereby the body's immune system attacks its own tissues. The triggers for the onset of RA are only speculated, but it is

expected that a genetic liability to the disorder, several viruses and bacteria (e.g. EpsteinBarr-Virus and Mycobacterium tuberculosis; (Miehle *et al.*, 2000), and [hepatitis B virus (HBV) and hepatitis C virus (HCV)], human immunodeficiency virus, parvovirus B19, human T-cell lymphotropic virus-I, and alpha viruses(Vassilopoulos and Calabrese, 2008) disruption of the immunological tolerance as well as the psychological condition by further weakening the immune system of people concerned could play a major role (Breitenberger, 2008). As the causes for RA are still unknown, cures have not been discovered yet as well. All treatments and therapies which are applied so far are intended largely to reduce symptoms and delay the progress of the disease (Newman and Fitzpatrick, 1995). The onset of RA arises usually between the age of 30 and 50, but may also occur at any other age. Women are three times more affected by it than men and people who are less educated and with fewer socioeconomic resources experience more problems emerging of RA. About 1% of the whole world

population is attacked by rheumatoid arthritis (Majithia and Geraci, 2007) whereas about 790,000 people in the Netherlands are affected (Reumafonds, 2009).

Rheumatic diseases in Sudan are old, in support of this view; is a case of erosive arthritis reported in a skeleton from Kulubnarti, Republic of the Sudan (Kilgore, 1989). but data concerning the pattern of these disorders are scarce in Sudan.

Rheumatic disorders are common diseases worldwide that result in significant mortality and morbidity. In the United States, osteoarthritis affects 27 million, eight are affected by gout arthritis, 1.3 million had rheumatoid arthritis, and up to 322 thousands had systemic lupus erythematosus. The prevalence of doctor diagnosed arthritis is 21%(Helmick *etal*.,2008). The highest prevalence of rheumatic disorders is in the South America and the Caribbean countries where 23.8-56% of the populations are affected, the prevalence is 15-24% in Australia and New Zealand, while up to 13% of the United Kingdom are affected (Charles *etal* .,2008).

1.2 Rationale

According to the World Health Organization (WHO), the virus is responsible for around 2 billion infections worldwide with 250 million chronic carriers, despite the availability of a safe and effective vaccine for more than 20 years (Bertoletti and Gehring, 2013).

Because hepatitis infections are continue to be a global health issue and there was no published data on the prevalence of hepatitis among rheumatoid arthritis patients in Sudan and little is known about the actual mechanism by which HBV initiate the various immunological disorder , the present study was conducted to determine the prevalence of HBV among rheumatoid arthritis patients in Sudan.

It would be a preliminary study of the virus and its relation with immunological disorder that could later form a basis for further comprehensive researches in the development prophylactic protocols for HBVinfections .

1.3 Objectives

1.3.1 General Objective

To determine the prevalence of HBV among Sudanese rheumatoid arthritis patients in Khartoum, Sudan .

1.3.2 Specific objectives

I .To determine the prevalence of HBV among Sudanese rheumatoid arthritis patients by using polymers chain reaction (PCR).

II . To find out the relationship between HBV and rheumatoid arthritis .

CHAPTER TWO

Literature review

2.1.Hepatitis B Virus

2.1.1.Classification

HBV, with a genome of 3.1-3.2 kb is the smallest DNA virus infecting man and is the prototype member of the family Hepadnaviridae. This family consists of 2 genera: the Orthrohepadnaviridae, which infect mammals and the Avihepadnaviridae, which infect birds .

The members of the genus Orthohepadnavirus include: HBV, the woodchuck hepatitis virus) WHV) (Hodgson and Michalak, 2001), the ground squirrel hepatitis virus (GSHV) (Marion *et al*., 1980), the arctic squirrel hepatitis virus (ASHV) (Testut *et al*., 1996), the chimpanzee HBV (ChHBV) (Takahashi *et al*., 2000), the gorilla HBV (GoHBV) (Makuwa *et al*., 2003 ,)the gibbon HBV (GiHBV) (Norder *et al*., 1996), the orang-utan hepatitis (OuH) (Takahashi et al., 2000), the woolly monkey hepatitis B virus (WMHV) (Lanford *et al.*, 1998), the Barbay macaques hepatitis virus (Gheit *et al*., 2002), the tree shrew hepatitis virus (Cao *et al*., 2003)and recently, Dickens *et al*., 2013 provided evidence for occult HBV infection (OBI) in chacma baboons (Dickens *et al*., 2013). The genus Avihepadnavirus consists of the duck hepatitis B virus (DHBV) (Mason *et al*., 1980), the heron hepatitis B virus (HHBV) (Sprengel *et al*., 1988), the snow goose HBV (SGHBV) (Chang *et al*., 1999), the Ross‘s goose HBV (RGHBV) (Pult *et al.*, 2001), the white stork HBV (STHBV) (Pult *et al*., 2001)and grey crowned crane HBV (CHBV) (Prassolov *et al*., 2003).

2.1.2. HBV structure

The viral agent responsible for hepatitis B was first isolated by Blumberg *et al*. in .1965 Five years later, in 1970, Dane *et al*. described the 42 nm complete HBV virion particle, which they called the Dane particle (Kann, 2002). Electron microscopy of the serum of infected individuals has shown two types of particles – the 42 nm to 47 nm Dane particles at a titre of up to 109 particles per ml, as well as an excess of 20 nm spherical subviral particles at a concentration of up to 1014 spheres per ml (Kann, 2002).

These subviral particles consist of viral surface proteins and host-derived lipid components and are not infectious as they lack nucleic acids (Ganem, 1996). The Dane particle (figure 1) is double shelled with the outer shell or envelope consisting of viral surface particles embedded in host derived lipid and the inner shell consisting of an icosahedral nucleocapsid composed of viral core protein (Kann, 2002; Huang *etal*., 2006) A 3,2 kb relaxed circular partially double stranded DNA molecule, together with a covalently bound viral polymerase protein on the 5' end of the negative strand, is contained within the nucleocapsid.

The negative or minus strand of the DNA molecule has a defined nick and a terminal redundancy of seven to nine nucleotides (Kann, 2002; Huang *et al*., 2006). The positive or plus strand is of variable length and shorter than the negative strand (Kann, 2002; Yokosuka and Arai, 2006). The binding of the viral polymerase protein to the 5'-end of the negative strand, means that the negative strand is not covalently closed. The positive strand bridges this gap and keeps the genome circular by base pairing on either side of the gap to the complementary negative strand.

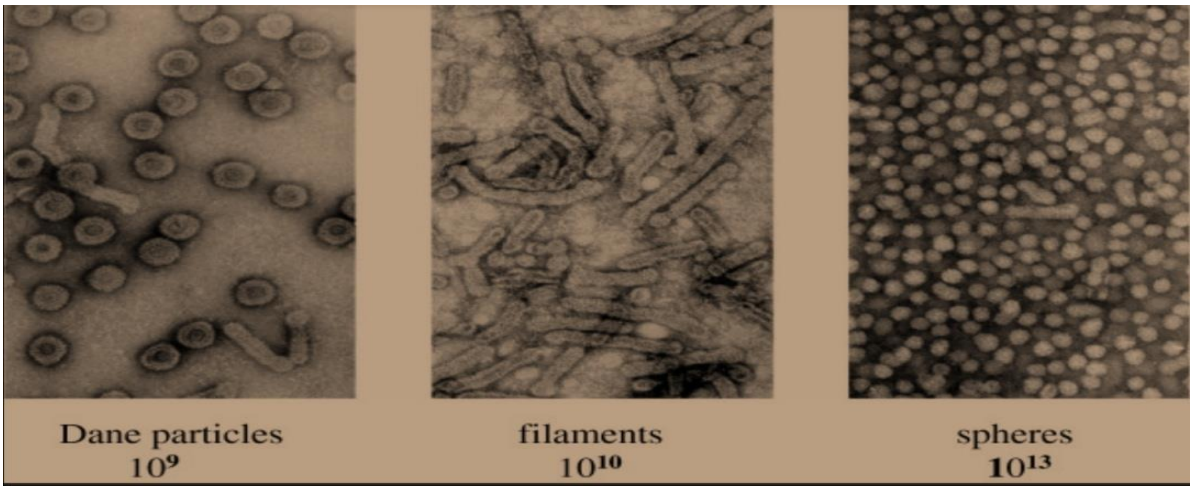


Figure 1: Research Gate: electron microscopy images (negative staining) and approximate numbers of HBV associated particles in 1 ml of the serum from a highly viremic chronically infected HBV carrier

2.1.3. HBV Genome Structure

As shown in Figure 2 the HBV genome is composed of circular, partially double-stranded DNA, ranging in size between 3182 to 3248 nucleotides, depending on the genotype (Feitelson, 1994). The partially double-stranded DNA is composed of a full-length negative strand (3020 to nucleotides) (Beck and Nassal, 2007, Seeger and Mason, 2000). This compact genome consists of four partially overlapping open reading frames (ORFs), which include :

i) Precore/Core gene (PreC/C), which encodes the hepatitis B e antigen (HBeAg) and the core protein (HBcAg).

ii) Polymerase gene (P), which encodes the viral polymerase (reverse transcriptase).

iii) PreS/Surface gene (PreS/S), which encodes: LHBs, MHBs, SHBs envelopeproteins

iv) X gene, which encodes the small regulatory hepatitis B X protein (HBx) (Tiollais *et al*., 1985, Ganem and Varmus,1987) .

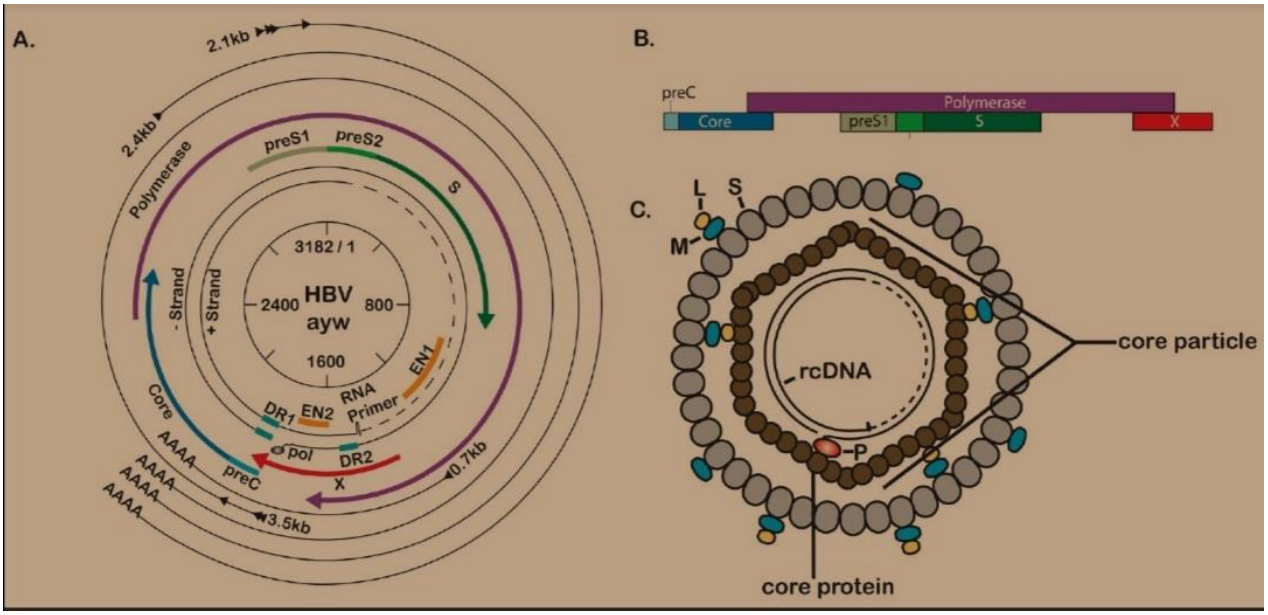


Figure 2: Hepatoma Res 2016 ;2:163-186

2.1.4. HBV Replication

The exact mechanism by which HBV replicates is not fully understood. The replication cycle ,as illustrated in figure 3, begins when the infectious virion attaches to a hepatocyte-specific preS1 receptor (Yan *et al*., 2012).

Evidence, from a recent study, revealed that the receptor-binding region ofpre-S1 interacts specifically with sodium taurocholate cotransportin

polypeptide (NTCP), which is a multiple trans-membrane transporter, expressed mainly in the liver (Yan *et al*., 2012). The virus enters the cell in one of two ways: either by fusion and penetration at the plasma membrane or by endocytosis of the viral nuclocapsid followed by fusion to the cell (Pawlotsky, 2005, Xie *et al*., 2010, Urban *et al*., 2010). Entry of the virus isfollowed by the release of the viral nucleocapsid into the cytoplasm (Urban *et al*., 2010). The nucleocapsid is transported to the nucleus, where the relaxed circular DNA (rcDNA) is released into the nucloplasm (Kann *et al*., 2007). Here the rcDNA is repaired by the viral polymerase, which completes the synthesis of the positive strand (Nassal, 2008). Thereafterthe ligation of both positive and negative DNA strands occurs and the covalently closed circular DNA (cccDNA) is formed (Nassal, 2008), which serves as the transcriptional template for RNA polymerase II to produce six genomic and subgenomic RNAs :

i) 3.5 kb pregenomic RNA (pgRNA) translated into the HBcAg and polymerase.

ii) 3.5 kb preC/C mRNA translated into the HBeAg.

iii) 2.4 kb preS1 mRNA translated into LHBs (preS1/preS2/S).

iv) 2.1 kb preS2 mRNA translated into MHBs(preS2/S).

v) 2.1 kb S mRNA translated into the SHBs(S).

vi) 0.7 kb X mRNA translated into the X protein (Summers and Mason, 1982, Moolla *et al*., 2002, Feitelson, 1992, Ganem and Varmus, 1987, Beck and Nassal, 2007 .)This transcription is regulated by both viral and host factors such as viral proteins (core, the regulatory X protein)and CCAAT/enhancer-binding protein (C/EBP)/hepatocyte nuclear factors (HNF), respectively (Levrero *et al*.,2009).

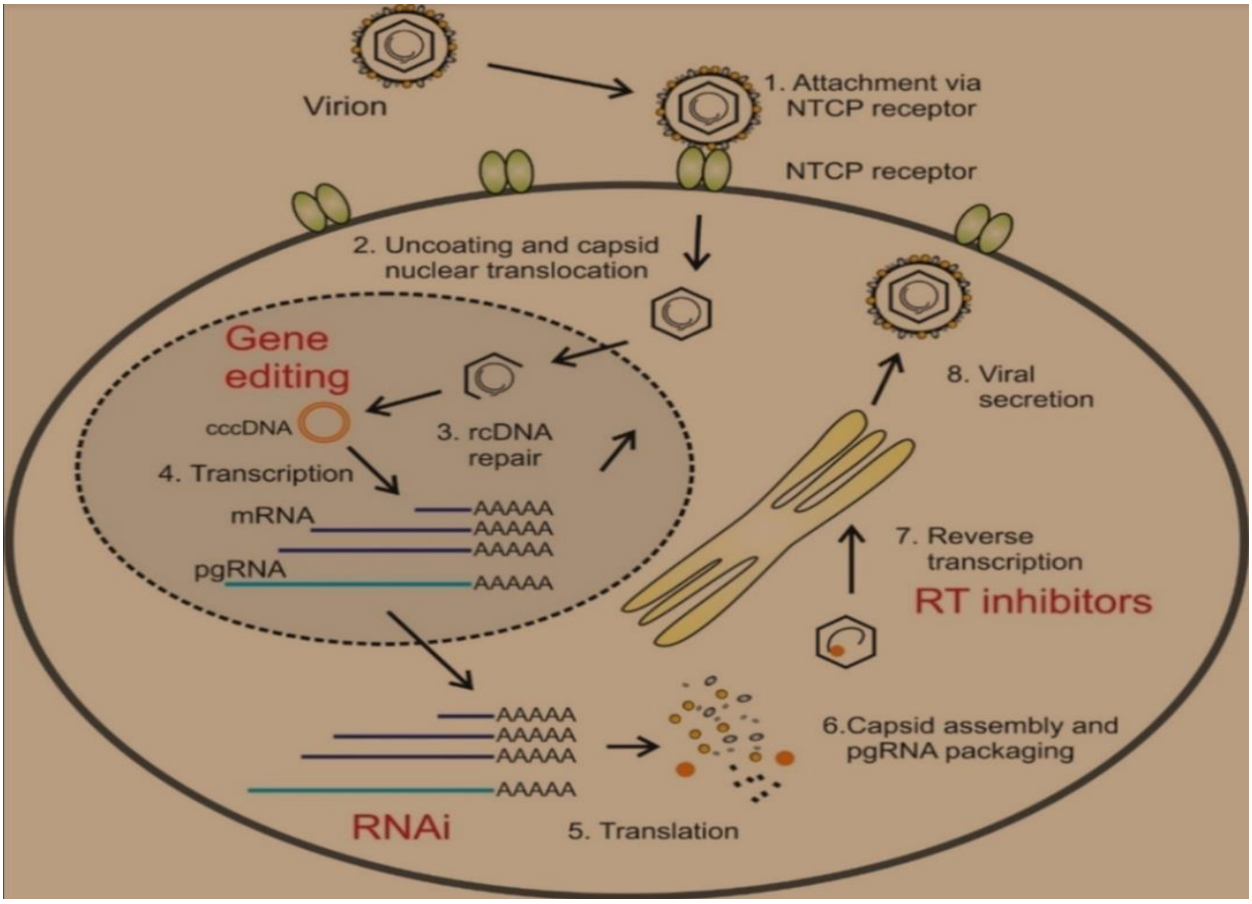


Figure 3: Research Gate : diagram of hepatitis B virus replication cycle.

2.1.5.Transmission

Blood is the main carrier for HBV infection, although other body fluids, like semen and saliva, may also play a role in transmission. There is no evidence that HBV can be transmitted by airborne means or through the oral faecal route (Bancroft *et al*., 1977, Hou *et al*., 2005). HBV transmission routes can be divided into two types :

i) Perinatal transmission: when the infection passes from the mother to the child ,through percutaneous and per mucosal exposure to the mother‘s blood from 28 weeks of gestation up to 7 days after birth (Beasley and Hwang, 1983, Jonas, 2009 ,Burnett *et al*., 2012). This type of transmission is also called vertical infection .Usually it occurs in HBeAg positive mothers and leads to chronic infection in the infant. This type of transmission has been observed mainly in China and southeast Asia (Kramvis and Kew, 2007, Hou *et al*., 2005).

ii) Horizontal transmission occurs when HBV is passed from person to person or from person to surface to person. This route can be divided into four routes:

a) Sexual, and especially homosexual activity, can transmit HBV, and this mode

of transmission occurs in all areas of the world but is very common in regions of low endemicity (Alter,. 2003, Wasmuth, 2009) .

Transmission between toddlers has been found to be the major route of infection in Africa, where there is a high prevalence of HBV. Infection during childhood can lead to chronic HBV infection and once these individuals become sexually active, sexual transmission becomes the dominant route of infection (Burnett *et al*., 2012).

b) Percutaneous HBV transmission includes injection drug use,transfusions and dialysis acupuncture, working in a health-care setting, tattooing, circumcision and scarification, and household contact. (Bancroft *et al*.,1977).

c) Transmission via HBV contaminated blood products in patients.

2.1.6.Immuneresponse

2.1.6.1. Characteristics of the Immune Response to HBV

2.1.6.1.1 Absence of an Innate Response by Infected Cells

Virus replication often results in the induction of an innate immune response which is heralded by rapid induction of IFNα/β by the infected cell (Samuel.,1991). Production of IFNα/β induces the transcriptional expression of a large number of interferon inducible genes (ISGs) which in turn exert a variety of intracellular antiviral mechanisms that have the potential to minimize pathogenetic processes by limiting viral production and spread (Samuel.,1991,Wieland *et al* .,2005). Surprisingly, as shown in, intrahepatic gene expression profiling in acutely HBV infected chimpanzees revealed that HBV acts like a stealth virus early after infection since it does not induce any cellular gene expression including ISGs as it spreads through the liver(Wieland *et al*., 2004). This is in stark contrast to the induction of many ISGs during the spread of hepatitis C virus (HCV) infection in chimpanzees, as shown in suggesting

that, unlike HBV, HCV is highly visible to the innate immune system (Wieland *et al* .,2005, Su *et al* .,2002). The relative invisibility of HBV to the innate sensing machinery of the cells likely reflects its replication strategy, which retains the transcriptional template in the nucleus, involves the production of capped and polyadenylated viral mRNAs that resemble the structure of normal cellular transcripts, and sequesters its replicating genome within viral capsid particles in the cytoplasm (Ganem *and* 2004, Summers.,1988 and Seeger *et al*., 2000). Thus, the typical widespread expansion of HBV in the liver may reflect the absence of IFNα/β production to which the virus is exquisitely sensitive as has been shown in HBV transgenic mice (Guidotti *et al*.,1996, Wieland *et al*.,2000).

2.1.6.1.2The Adoptive Immune Response

The Antibody Response—The antibody response to the HBV envelope antigens is a T cell-dependent process (Tsui *et al.*,1995). Because these anti-envelope antibodies are readily

detectable inpatients who clear the virus and recover from acute hepatitis, and they are usually undetectable in patients with chronic HBV infection, they are thought to play a critical role in viral clearance by complexing with free viral particles and removing them from circulation or by preventing their attachment and uptake by hepatocytes. This notion is supported by the observation that chimpanzees that resolved a previous infection are completely protected from rechallenge (Moss *et al*.,1984). The appearance of neutralizing antibodies, however, occurs relatively late after HBV exposure and, thus, it is unlikely to contribute to the early phase of viral clearance during acute infection.Instead they probably prevent viral spread from rare cells that remain infected after resolution of HBV infection.

The CD4 T Cell Response—The peripheral blood CD4 T cell response to HBV is vigorous, and multispecific in patients with acute hepatitis who ultimately clear the virus, while it is relatively weak in persistently infected patients with chronic hepatitis (Ferrari *et al*.,1990). Although the association between a strong CD4 T cell response, acute hepatitis, and viral clearance suggests that a relationship exists between these events (Tsui *et al*.,1995, Ferrari *et al*.,1990 and Jung *et al*.,1991) , CD4 T cell depletion at the peak of HBV infection had no effect on viral clearance and liver disease in infected chimpanzees (Thimme *et al*., 2003), suggesting that CD4 T cells do not directly participate in viral clearance and tissue damage. As we will discuss in more detail later in this review CD4 T cells probably contribute indirectly to the control of HBV infection by facilitating the induction and maintenance of the virus-specific (B cell) and CD8 T cell response.

The CD8 T Cell Response—The HBV specific CD8 T cell response plays a fundamental role in viral clearance and the pathogenesis of liver disease. A vigorous polyclonal CD8 T cell response is readily detectable in the peripheral blood of patients with acute hepatitis who ultimately clear HBV. In contrast, the peripheral blood T cell response in chronically infected patients is weak and narrowly focused (Tsui *et al*.,1995, Penna *et al*.,1991, Rehermann *et al*.,1995and Bertoletti,*et al*.,1991). The livers of these patients contain virus-specific T cells that likely contribute to disease pathogenesis, but for functional and/or quantitative reasons are unable to clear the infection. Interestingly, a recent study that examined a relationship between the number of intrahepatic HBV specific CD8 T cells, extent of liver disease, and levels of HBV replication in chronically infected patients indicated that inhibition of virus replication could be independent of liver damage, and that the functionality of HBV-specific CD8 T cells was more important than the number of T cells to control HBV replication (Maini *et al*,.2000). Experiments in chimpanzees have

shown that the viral clearance and the onset of liver disease coincide with the accumulation of virus-specific CD8 T cells and the induction of IFNγ and IFNγ-inducible genes in the liver (Guidotti,1999, Thimme *et al*., 2003). Importantly, depletion of CD8 T cells at the peak of viremia delays viral clearance and onset of viral hepatitis until the T cells return, proving that the viral clearance and liver disease are mediated by virus specific CD8 T cells (Thimme *et al*., 2003).

2.1.6.1.3.Mechanisms of HBV Clearance

It is widely believed that the CTL response clears viral infections by killing infected cells. CTL killing is an inefficient process, however, requiring direct physical contact between the CTLs and the infected cells. Thus, it may not be possible for CTLs to kill all HBV infected cells if the CTLs are greatly outnumbered as occurs during HBV infections in which as many as 1011 hepatocytes can be infected (Thimme *et al*., 2003, Guidotti *et al*.,1999 and Asabe *et al*.,2009). Thus, although the liver disease in HBV infection is clearly due to the cytopathic activity of the CTL response, viral clearance may require more efficient CTL functions than killing. Important insights into the pathogenetic and noncytopathic antiviral functions of the CTL response have come from studies in HBV transgenic mice that develop an acute necroinflammatory liver diseaseafter adoptive transfer of HBsAg specific CTL clones (Ando *et al*.,1994, Guidotti *et al*.,1996). the CTLs rapidly enter the liver and recognize viral antigen which triggers two events: (a) apoptosis of the hepatocytes that are physically engaged by the CTLs,and

(b) secretion of interferon gamma(IFNγ) which noncytopathically inhibits HBV gene expression and replication in the rest of the hepatocytes (Guidotti *et al*.,1996, Guidotti *et al*.,1994) by preventing the assembly of HBV RNA-containing capsids in the cytoplasm (Wieland *et al*.,2000, Wieland *et al*., 2005) in a proteasome (Robek *et al*.,2002) and kinase-dependent (Robek *et al*.,2004) process. During this remarkable process, the viral nucleocapsids disappear from the cytoplasm of the hepatocytes (Guidotti *et al*.,1996 Wieland *et al*., 2002,) and the viral RNAs are destabilized by a SSB/La-dependent mechanism in the nucleus (Tsui *et al*.,1995, Heise *et al*.,2001 and Heise *et al*.,1999), yet the hepatocytes remain perfectly healthy (Tsui *et al*.,1995, Guidotti *et al*.,1994). Antibody blocking and knockout experiments in the HBV transgenic mouse model further demonstrated that the cytopathic and antiviral functions of CTLs are completely independent of each other (Guidotti *et al*.,1996). These results suggest that a strong intrahepatic CTL response to HBV can suppress viral gene expression and replication noncytopathically.

2.1.7.Diagnosis

In March 2015, the World Health Organization published the first guidelines for the prevention, care, and treatment of individuals with chronic HBV infection.(WHO.,2015 virus) These guidelines focused on assessment for treatment eligibility, initiation of first-line therapies, switching, and monitoring. These initial guidelines did not include recommendations on testing strategies that included what test to use and how to test. Given the large burden of HBV in low- and middle-income settings where there are limited or no existing HBV testing guidelines, there is a substantial need for HBV testing guidelines.

2.1.7.1.Description of HBV Ag detection

Chronic HBV infection is defined as persistence of hepatitis B surface antigen (HBSAg) for at least six months. However, interpretation of HBV serologies is complex (Table 1). The serological markers most frequently used for HBV testing include HBsAg, total anti-HBc, and anti-HBs (Table 1). Table 1. Hepatitis B serological marker interpretation Serological marker Interpretation HBsAg (hepatitis B surface antigen),Total anti-HBc (antibody to hepatitis B core antigen), IgM anti-HBc (immunoglobulin M to anti-HBc),Anti-HBs (antibody to HBsAg).

Table 1: HBV serological marker interpretation.

	Serological marker				
	HBsAg	Total anti-HBc	IgM anti-HBc	Anti-HBs	Interpretation
Test results	–	–	–	–	Never infected and susceptible to infection
	+	+	–	–	Chronic infection
	–	+	–	+	Recovered from past infection and immune
	+	+	+	–	Acute infection
	–	+	–	+	Immune by natural infection
	–	–	–	+	Immune by HB vaccine

Source: US Centers for Disease Control and Prevention. (Available at:http://www.cdc.gov/immigrantrefugeehealth/ guidelines/ domestic/hepatitis-screening- guidelines.html)

2.1.7.2.One test vs two test serological testing strategy

The most important marker for the diagnosis of chronic hepatitis B infection requiring further assessment or treatment remains HBsAg. The case definition of chronic hepatitis B is the detection of HBsAg twice six months apart. After an initial positive result for HBsAg, supplementary testing can be undertaken in order to facilitate entry into a care pathway. The detection of HBsAg in blood can include rapid diagnostic tests, or enzyme immunoassays. Confirmation of the specificity of a reactive HBsAg first-line test result is usually carried out by either:

i) repeating the HBsAg testing in a different assay of similar sensitivity,or

ii) performing a neutralization test using a specific anti-HBs-containing reagent in the same first-line assay after appropriate dilution of the specimen under test. Specificity is confirmed when this reagent abolishes reactivity in the assay.

Molecular assays like conventional PCR and Real Time PCR are rapid, sensitive and specific but Real Time PCR is costly than the conventional PCR. Loop mediated Isothermal amplification (LAMP) is also used to detect viruses DNA/RNA (Notomi *et al*.,2000).

LAMP is a novel method for detection of viral pathogens and also it has similar sensitivity and specificity as real time PCR (Notomi *et al*.,2000, Nyan *et al*.,2014). Serological assays based on HBsAg are very rapid and cost effective in the diagnosis of HBV infection but sensitivity and specificity of serological assays are poor when compared with molecular methods

HBVDNAsometimesmay bethonlymarkerpresentin early in. fections.

2.1.8. Treatment Indications

Normalization of alanine transaminase (ALT), loss of HBeAg (seroconversion), decrease in serum HBVDNAlevel,and improvement in liverhistology indicate treatment effectiveness. (Dienstag.,2008 , Lok and Mcmahon.,2007 , Delaney et al.,2008 , Keeffe et al.,2008) A recent systematic review found insufficient evidence to assess treatment effectiveness on patient-oriented outcomes, such as decreased mortality and improved quality of life. (Shamliyan et al.,2009) A disease-orientedoutcome, suppression ofHBVDNAlevels, is oftenusedas an end point of treatment.(Lai and Yuen.,2007)

2.1.9. TreatmentOptions

Several medications are approved in the United States for the treatment of HBVinfection .(Dienstag.,2008) Although interferon is approved for treatment, pegylated interferon alfa-2a has higher effectiveness, with a similar adverse effect profile, and is preferred overinterferon.

2.2. Rheumatoid arthritis(RA)

Rheumatoid arthritis (RA) is a chronic, autoimmune inflammatory disease with a female predominance, and is estimated to affect approximately 1% of the world's population (Silman&Hochberg,2001; Gibofsky,2012). The etiology of RA is unknown, but genetic factors are associated with the condition and its severity(Silman&Hochberg,2001;Begovich *et al*.,2004; Barton&Worthington,2009; Gibofsky,2012), and multiple environmental and life style factors have been shown to be associated with its development (Silman&Hochberg, 2001; Begovich *etal*.,2004; Barton&Worthington,2009; Gibofsky,2012). In RA, inflammation of the synovium leads to cartilage and bone destruction, with the joints of the hand and feet being the first affected (Lindhardsen *et al*.,2012; Ong *et al*.,2013). Other joints in the body are subsequently affected.

Classification criteria for RA were first proposed by the American Rheumatism Association (ARA) in 1958 (Ropes *et al.*,1958). The separation by these criteria into possible, probable, definite and classical RA has been used for the majority of published studies of RA. The 1958 criteria included a number of histological features which were not applicable in the populations setting. An adaption - called the Rome criteria

- excluding these features was produced for epidemiological studies (Kellgren.,1962) (Tables 2.3) and broadly reflected the actual use of the ARA criteria in field studies. The time when a sufficient number of criteria are reached should be considered the onset of RA. The Rome criteria also account for past arthritis (Table 3).

Table 2 :Rome Criteria for active rheumatoid arthritis Criterion.

1	Morning stiffness
2	Pain on movement or tenderness in a joint (a)
3	Soft tissue swelling in a joint
4	Soft tissue swelling of another joint
5	Symmetrical tissue swelling simultaneously (a)
6	Subcutaneous nodules (a)
7	X –ray change (b)
8	Positive rheumatoid factor
3-4 criteria positive	Probable rheumatoid arthritis
5-6 criteria positive	Definite rheumatoid arthritis
7-8 criteria positive	Classical rheumatoid arthritis

(a) Must be observed by a physician but does not include terminal inter phalangeal joints. (b) Can include juxta-articular osteoporosis

Table 3 : Rome Criteria for inactive rheumatoid arthritis Criterion.

1	Past history polyarthritis
2	Symmetrical deformity of a hand or feet joints
3	Radiological change
4	Positive rheumatoid factor
2 positive criteria	Positive possible RA
3-4 positive criteria	Positive definite RA

2.2.1 Genetic and Environmental Factors

Rheumatoid arthritis involves a complex interplay among genotype, environmental triggers, and chance.

Findings from studies of gene–environment interactions complement these observations. Smoking and other forms of bronchial stress (e.g., exposure to silica) increase the risk of rheumatoid arthritis among persons with susceptibility HLA– DR4 alleles.(Symmons *et al*.,1997) Moreover, smoking and HLA-DRB1 alleles synergistically increase one's risk of having ACPA.(Klareskog *et al*.,2006) Unifying these observations is the finding that environmental stressors of pulmonary and other barrier tissues may promote posttranslational modifications, through peptidyl arginine deiminase, type IV (PADI4), that result in quantitative or qualitative alteration in citrullination of mucosal proteins. Loss of tolerance to such neoepitopes elicits an ACPA response (which can be detected with a diagnostic anti–cyclic citrullinated peptide [CCP] assay) .(De Rycke *et al*.,2004, Mahdi *et al*.,2009) Several citrullinated self-proteins are recognized in anti-CCP assays, including α-enolase, keratin, fibrinogen, fibronectin, collagen, and vimentin. Characterization of subsets of seropositive patients to elicit true disease autoantigens is ongoing. An estimated 43 to 63% of patients with ACPA-positive rheumatoid arthritis are seropositive for citrullinated α-enolase, which is strongly associated

with HLA-DRB104, PTPN22, and smoking.(Mahdi *et al*.,2009) Similar interactions are reported for citrullinated vimentin and fibrinogen epitopes.(van Woude *etal*.,2010) Infectious agents (e.g., Epstein–Barr virus, cytomegalovirus, Proteus species, and Escherichia coli) and their products (e.g., heat-shock proteins) have long been linked with rheumatoid arthritis, and although unifying mechanisms remain elusive, some form of molecular mimicry is postulated.(Kamphuis *etal*.,2005, Auger and Roudier.,1997) The formation of immune complexes during infection may trigger the induction of rheumatoid factor, a high-affinity autoantibody against the Fc portion of immunoglobulin, which has long served as a diagnostic marker of rheumatoid arthritis and is implicated in its pathogenesis.

2.2.2.Synovial Immunologic Processes and Inflammation

Synovitis occurs when leukocytes infiltrate the synovial compartment. Leukocyte accumulation primarily reflects migration rather than local proliferation. Cell migration is enabled by endothelial activation in synovial microvessels, which increases the expression of adhesion molecules (including integrins, selectins, and members of the immunoglobulin superfamily) and chemokines.

Accordingly, neoangiogenesis, which is induced by local hypoxic conditions and cytokines, and insufficient lymphangiogenesis, which limits cellular egress, are characteristic features of early and established synovitis.(Szekanecz *et al*.,2009, Polzer *et al*.,2008) These microenvironmental changes, combined with profound synovial architectural reorganization and local fibroblast activation, permit the buildup of synovial inflammatory tissue in rheumatoid arthritis.

2.2.3.Rheumatoid arthritis association with hepatitis B virus

RA is a systemic inflammatory disease involving altered immunologic function, and RA patients have increased risks of several types of bacterial and viral infection.(Cobb *et al*.,1953, Listing *et al*.,2013) Although RA patients receiving biologic agents for immunomodulatory treatment have been associated with the reactivation of HBV infection(Shouval and Shibolet.,2013) that may result in liver failure and death,(Rubbert-Roth .,2012) the association of HBV infection and RA remains largely unknown. A few studies have investigated the prevalence of HBV infection in RA patients; most of the these studies have been limited to small sample sizes and specific populations(Zou *et al.*,2013, Permin *et al*,.1982 and. Varache *et al*,.2011) focused on patients who received biologic agents,(Rubbert- Roth.,2012) and could not attain conclusive results.(Singh *et al*.,2012) Whether RA has a pathogenic association with HBV infection remains unanswered.

CHAPTER THREE

3. MATERIALS AND METHODS

3.1. Study design

This was a descriptive cases control study, to determine the prevalence of HBV among rheumatoid arthritis patients at Alrayan , Military hospital and Alya Military Hospital.

3.2. Study area

The study was conducted at Alrayan, Military Hospital and Alya Military Hospital, Khartoum-Sudan.

3.3.Study period

The study was carried out during the period from August (2019) to January (2020)

3.4 Study population

The known rheumatoid arthritis patients were 40, suspected patients with rheumatoid arthritis 40 and known negative control were 40.

3.5. Inclusion criteria

All patients with rheumatoid arthritis and the suspected patients with rheumatoid arthritis at Alrayan, Military hospital and Alya Military Hospital, Khartoum-Sudan were included.

3.6.Exclusion criteria

All healthy individuals or patients with disorder rather than autoimmune diseases were excluded from the study.

3.7.Ethical consideration

This study was approved by the ethical committee of Sudan University of Science and Technology, department of medical microbiology

3.8.Data collection

Persona

l data were collected from the medical records of the patients based on constructed questionnaire that included name, age, gender and type of autoimmune disorder.

3.9 Sampling

3.9.1.Sample size

One hundred and twenty samples were collected from patients with rheumatoid arthritis (40), and suspected patients with rheumatoid arthritis (40) and controls(40)

3.9.2.Sampling collection

Whole blood was collected in plain Vacontainers ,let ot clot at room temperature then centrifuged at 4000R\M for 2 minutesto obtain the serum , the clear serum was separated and stored at 4 C until used.

3.10.DNA extraction method

DNA was extracted from sera samples,250 µl of serum samples were added to sterile tube, then500 µl 5% SDS and 20 µl proteinase K were added to the tube (incubated at 56 C^0 for 1 hour in water bath or 37C over night). The samples were incubated at 95C^0 for 10 minutes (inactivate proteinase K) after that 500 µl chloroform was added and centrifuged at 12000 rpm for 10 minutes. Then the upper layer was transferred into new eppendrof tube. One ml absolute ethanol (cold) was added and incubated at -20C^0 for 2 hour in refrigerator or over night.Thereafter, the samples were centrifuged at 16000 rpm for 15 minutes, then the samples were Discorded leaving dry pelet , 200 µl from 70% ethanol was added to the dry pelet and shaken until it disappeared then centrifuged at 16000 rpm for 15 minutes. The samples were then Discorded and the tubes were inverted opened cap at least for 2 hours to dry. Finally 25 µl water for injection was added and stored at -20 C^0 .(Center of Research – Khartoum extraction method)

3.11.Polymerase Chain Reaction (PCR)

The PCR reactions were performed in a total volume of 25 µl as follows: 6 µl Green master mix (Maxime PCR premix), containing dNTPs, PCR buffer and Taq polymerase, 13 µl

H2O, 1 µl of each primer at a dilution of 100 pmol and 5 µl genomic DNA. A conventional PCR was carried out with the following primers(intron biotechnology - ligo) common forward (5' – CCC TTG CGG ATG CCAAT -3')and reverse primer (5' – GGC TGA TAT GGA TGC CC -3').The thermo cycler (MJ Research PCT-200 thermo cycler)program was as follow: 94 °C for 3 minute, then 35 cycles at 94 °C for 1 minute, 62 °C for 1 minute, and 72 °C for 1 minute, a final extension step 72 °C for 7 minute, and then held at 4°C.

3.12.Gel electrophoresis

To carry out electrophoresis 1.5 g of agarose powder was measured and mixed with 100 ml TBE in a microwavable flask, then dissolved in microwave for (1-3) min. Aftre that the solution was left to cool about 3 min , then 2 µl ethidium bromide was added and was shaken gently. Then the solution was poured slowly in the tray to avoid bubbles, combs was placed and finally the solution was left to sat for 20-30 min until it completely solidified.

3.13.Detection of PCR product

The amplified fragments were separated by electrophoresis on 1.5% agarose gels. A volume of 5 µl from each PCR reaction (containing the loading dye) was loaded on the agarose gel. A volume of 2 µl of 100 bp DNA-ladder was also loaded. The gel was run at 40 V for 1 h and the separated fragments were viewed using a UV- transilluminater and the results were documented.

3.14.Data analysis

The statistical analysis of the results was performed using the statistical package for social science (SPSS) version 21 ,Chi-square test was used for testing difference significance P. value less than 0.005 was considered statistically significant.

CHAPTER FOUR

4.RESULTS

Out of 80 patients 34(28.3%) were positive for HBV DNA, Out of them 22 (18.3%) were male, 98(81.7%) were females with a mean age of 41 years, in range from 12- 75years. They were divided into three groups: Controls, Rheumatoid arthritis patients (cases) and suspected patients with rheumatoid arthritis with sample percentage (33.3%,33.3 %and 33.3%) respectively.

4.1.Detection of hepatitis B virus DNA according to gender:

The number of HBV positive males from the whole population was 6 (5%) from 120 participant (table 4) and the percentage of them among the total number of males was 6(27.3%).

The number of the positive females from the whole population was 28(23.3%) out of 120 (table 4) and 28 (28.6%) out of 98 from the total female number.

Table (4) detection of hepatitis B virus DNA according to gender:

Sex	**HBV**		**Total**
	Positive	Negative	
Male	6	16	22
% of total	5.0%	13.3%	18.3%
Female	28	70	98
% of total	23.3%	58.3%	81.7%
Total	34	86	120
% of total	28.3%	71.7%	100.0%

4.2. Detection of Rheumatoid according to age groups

Out of 120 samples, 8(6.7%) were between 10-20 years old, 56(46.7%) were between 21-40 years old, 39(32,5)were between 41-60 years old and 17(14.2%) were between 61-80 years old.

Table 5 : Percentage of positive , negative and suspected rheumatoid among different age group.

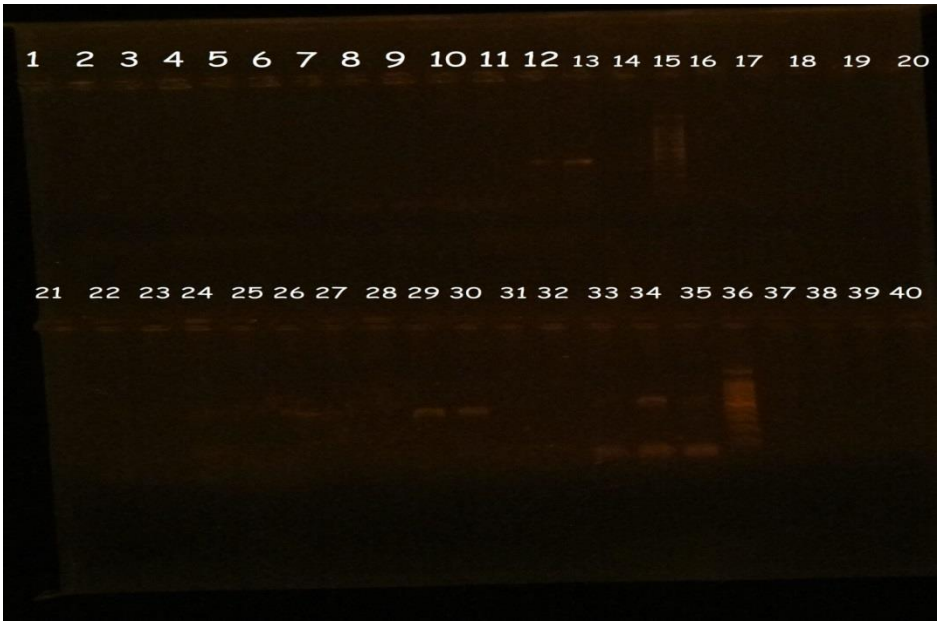
Age group	Rheumatoid			Total
	Positive	Negative	Suspected	
10 – 20	3	3	2	8
% of total	2.5%	2.5%	1.7%	6.7%
21 – 40	18	25	13	56
% of total	15.0%	20.8%	10.8%	46.7%
41 – 60	15	9	15	39
% of total	12.5%	7.5%	12.5%	32.5%
61 – 80	4	3	10	17
% of total	3.3%	2.5%	8.3%	14.2%
Total	40	40	40	120
% of total	33.3%	33.3%	33.3%	100.0%

4.3. Prevalence of HBV related to the population under study

Positive HBV was observed in 3(7.5%) out of 40 of cases when tested by PCR (fig:4). it was observed in 31(77.5%) out of 40 in suspected population (fig:6,7)and no positive results was detected in controls, compared to the total population , the positive in case was 3(3.75%) out of 80 and in suspected case was 31(38.75%) table(7). And this result was significant according to the p value 0.00

Table 6: percentage of HBV positive and negative among case, and suspected objects.

Subjects	HBV		Total
	Positive	Negative	
Case	3	37	40
% of total	3.75%	46.25%	33.3%
Suspected	31	9	40
% of total	38.75%	11.25%	33.3%
Total	34	86	80
% of total	28.3%	71.7%	100.0%

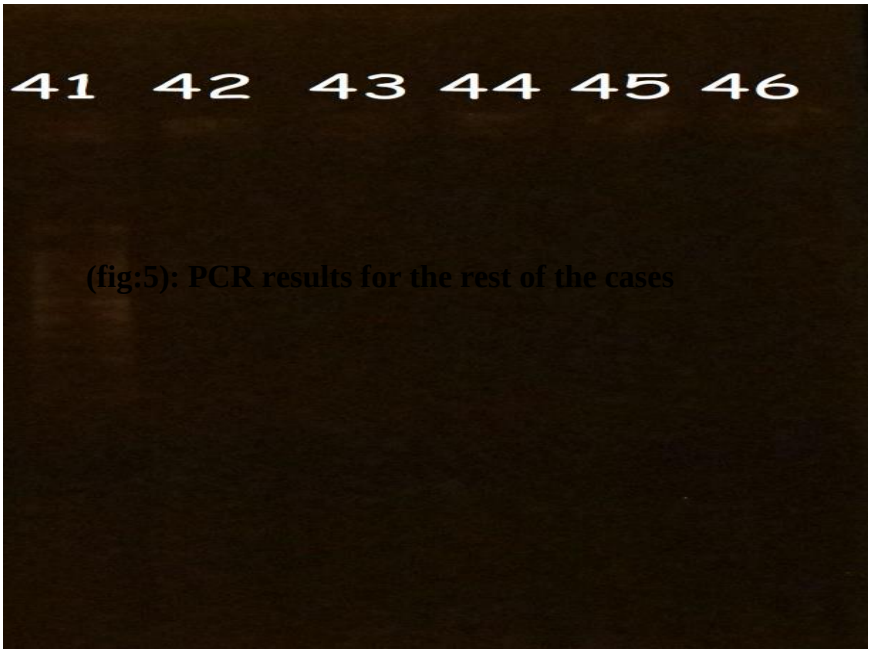


(fig:4): PCR result for cases

Keys:

15,36 = ladder(100pb). 29,30,34= positive cases (350pb) , 14 control(-ve), 12,13control(+ve)

The rest 35 well = the negative cases.

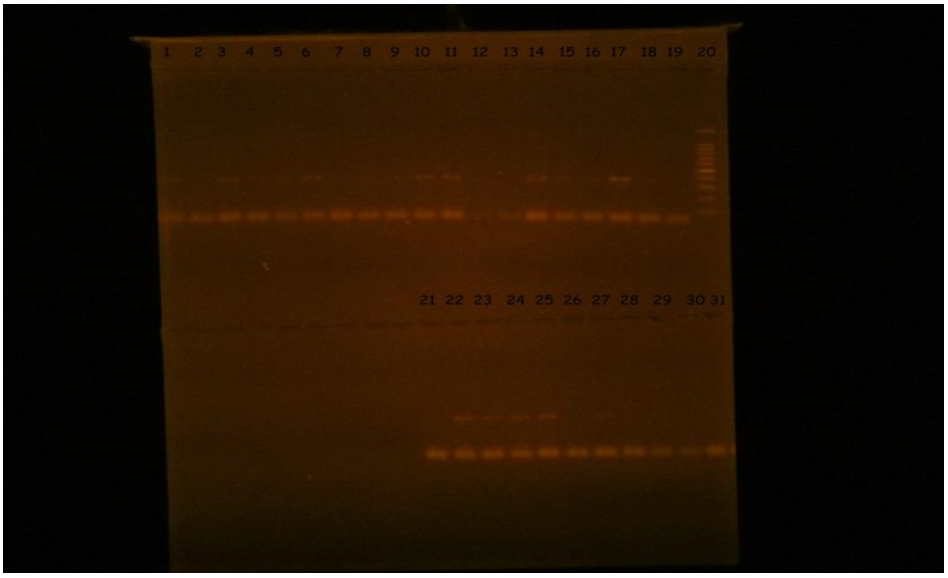


(fig:5): PCR results for the rest of the cases

Keys:

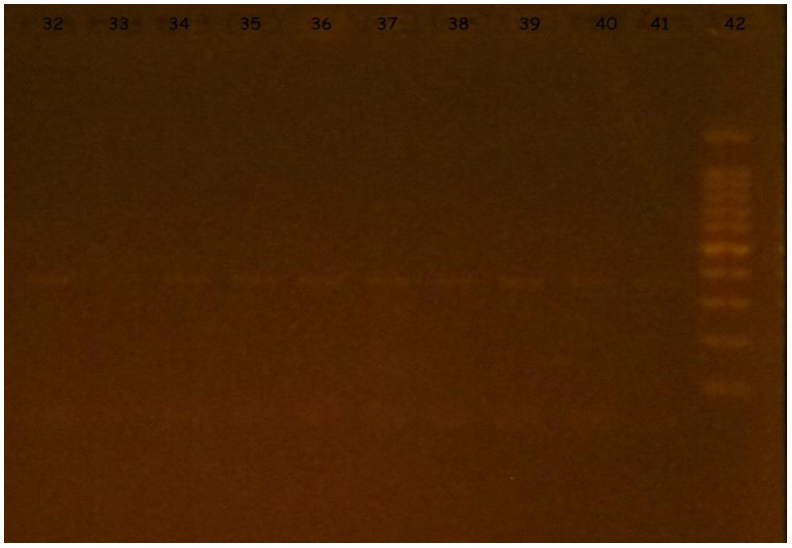
41=ladder (100pb)

42,43,44,45,46 = the rest of the negative cases.



(fig:6): PCR results for suspected population:

- 20 = ladder(100pb).
- 2,13,12,21,26,29,28,30,31= negative suspected cases.
- The rest of the results are positive suspected cases.



(fig:7): The rest of the suspected result

- The rest of the positive suspected samples.

CHAPTER FIVE

5. Discussion

Viral hepatitis is a major public health problem affecting several hundred million people worldwide. It causes considerable morbidity and mortality from both acute infection and chronic sequelae including chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) (Hatim ,2008).

It remains a public health burden in the world, mostly in developing countries including Sudan. The current study presented the prevalence of HBV infection among rheumatoid arthritis patients to demonstrate the infections within them in both gender.

Out of 120 samples, 34 (28.3%) were positive to HBV infection, 3 (2.5%) were confirmed cases, and 31 (25.8%) were suspected with rheumatoid according to their signs and symptoms. The obtained results indicate that there is a significant relationship between HBV infection and rheumatoid arthritis. Relying on the statistical analysis,31 out of 40 suspected patients were positive to HBV when tested by PCR, this indicates that hepatitis B virus has a link with rheumatoid arthritis with a p.value (0.00). The previous results agree with case report study carried by Pease and Keat (1985) in three cases using their history, joints symptoms and laboratory tests (AST, Alkaline phospataes, bilirubin, ESR and HBsAg in the serum). They found that HBV associated arthritis always precedes the onset of jaundice, sometimes by several weeks, and may be the only manifestation of infection. Furthermore, it confirmed that the suspected population have the highest infection rate with HBV.

According to Ching-sheng *et al.,* (2009*),* the prevalence of HBV infection in the Rheumatoid arthritis cohort was generally higher than that in the non-Rheumatoid arthritis cohort (69.9 %,60.1% cases per 1000 subjects, respectively) This result agrees with ours, the authors collected there data from Taiwan's National Health Insurance Research database between 1999 and 2009 since they end up saying rheumatoid arthritis patients characterized by an increased risk of Hepatitis B virus infection than non-rheumatoid arthritis patients and supporting Hepatitis B virus linked to systemic autoimmune diseases.

In contrast, Hanne *et al.,* (1990) found an evidence of previous hepatitis B exposure in 16.8% of patients with polymyalgia rheumatic, and their results do not support the concept that current or previous hepatitis B virus infection plays any role in the pathogenesis of the majority of cases of polymyalgia rheumatica.

According to this study, the most affected age group from rheumatoid arthritis was between 21-40 year. The group constituted 46.7% of the total patients, this result was similar to case report done by

Pease and Keat (1985) since the three cases in their study their ages were (28, 35, 30) years old. It is also agree with Abd Elrahman *et al.*, (2018), their results revealed HBsAg was highest among the 21-24years age group. This result also agree with Eke *et al.,* (1989*)* who found HBsAg was highest among women of 20-24 years age group in Nigeria. Relying on our results 6 (5%) of males and 28 (23.3%) females were positive to hepatitis B virus males were more susceptible compared to females with HBV infection, this can be due to their attitudes (sharing needles, illegal relationships and the barber tools etc…). Since females get autoimmune diseases in far greater number than males, it is thought that the females immune system is stronger and more reactive . Second, it appears that females hormones affect rheumatoid risk and flares. So not all the females whom affected by arthritis necessary they have it triggered by HBV(Intremountain Healthcare, 2016). However, in Sudan Elduma *etal* (2007), conducted a study using ELISA to determine the seropositivity of HBV infection, found that 6.5% males were positive and 4.2% females were also positive and this agree with results showing that males are more infected than females .

The recognition of hepatitis B virus as a cause of acute arthritis is important both for patient management and for the safety and protection of clinical and laboratory staff. There may be no history of contact with a likely hepatitis virus carrier. This condition may also represent a valuable clinical model of virus-induced arthritis in which research into pathogenetic mechanisms is facilitated by the presence of a known environmental agent.

Conclusion and limitations

- The result reveled that there is a relation between HBV infection and rheumatoid arthritis.
- The positive results in the suspected cases were more than the diagnosed cases.

Recommendations

- Based on this study it's recommended to do HBV screening test for patients with rheumatoid symptoms.
- It's better to be more researches about viral infections that trigger autoimmune diseases.
- A vaccination policy for HBV should be implemented and be available for all.
- However, the study had a few limitations which could affect the interpretation of these results. Sample size was not quite enough, so I recommended to increase the sample size in the next researches.

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