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Prevalence Rate of Hepatitis B Virus Markers and Transmission among Sudanese High Risk Population

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Abstract

Hepatitis is an infectious disease of the liver caused by the hepatitis virus. The most common viruses are named for the first three letters of the alphabet, A, B and C. These viruses each cause a different type of infection with different symptoms and duration of illness. HBV and HCV are common nosocomial infections that cause higher rates of mortality and morbidity in blood recipient than other population. In addition to causing hepatitis, infection with HBV can lead to cirrhosis and hepatocellular carcinoma.[1]. Hepatitis B virus is classified as the type species of the Ortho-hepadnavirus, The genus is classified as part of the Hepadnaviridae family, hepadnavirus: hepa from hepatotropic (attracted to the liver) and dna because it is a DNA virus[2]
The virus particle, (virion) consists of an outer lipid envelope and an icosahedral nucleocapsid core composed of protein. These virions are 42 nM in diameter and are sometimes referred to as "Dane particles". The nucleocapsid encloses the viral DNA and a DNA polymerase that has reverse transcriptase activity. The outer envelope contains embedded proteins that are involved in viral binding of, and entry into, susceptible cells. The virus is one of the smallest enveloped animal viruses, but pleomorphic forms exist, including filamentous and spherical bodies lacking a core. These particles are composed of the lipid and protein that forms part of the surface of the virion, which is called the surface antigen (HBsAg), and is produced in excess during the life cycle of the virus [3] Acute infection with hepatitis B virus is associated with acute viral hepatitis – an illness that begins with general ill-health, loss of appetite, nausea, vomiting, body aches, mild fever, and dark urine, and then progresses to development of jaundice. It has been noted that itchy skin has been an indication as a possible symptom of all hepatitis virus types. The illness lasts for a few weeks and then gradually improves in most affected people. A few people may have more severe liver disease (fulminant hepatic failure), and may die as a result. The infection may be entirely asymptomatic and may go unrecognized.[3] Approximately 300 million individuals are chronically infected with hepatitis B virus in the world. Enzyme linked immune sorbent assay (ELISA)is still a main detection method for HBV infection, but ELISA result can neither efficiently reflect serum viral load or hepatitis activity nor monitor the efficacy of antiviral treatments. Currently, polymerase chain reaction (PCR) assay has been widely used for monitoring HBV load. HBV DNA monitoring has become an important tool to identify individuals with high viral replication, to monitor patients on therapy, and to predict whether antiviral therapy is successful. For example, with the introduction of new antiviral agents like

lamivudine, close monitoring of patients has become increasingly important due to the occurrence of antiviral drug-resistant virus strains or the presence of flares after withdrawal of antiviral therapy.[4] It is not possible, on clinical grounds, to differentiate hepatitis B from hepatitis caused by other viral agents and, hence, laboratory confirmation of the diagnosis is essential. A number of blood tests are available to diagnose and monitor people with hepatitis B. They can be used to distinguish acute and chronic infections.[5]Acute HBV infection is characterized by the presence of HBsAg and immunoglobulin M (IgM) antibody to the core antigen, HBcAg. During the initial phase of infection, patients are also seropositive for hepatitis B e antigen (HBeAg). HBeAg is usually a marker of high levels of replication of the virus. The presence of HBeAg indicates that the blood and body fluids of the infected individual are highly contagious[5]

Occult Hepatitis Virus Definition: Occult HBV is defined as positive HBV DNA in the blood in the absence of hepatitis B surface antigen in the serum and is classified into seropositive and seronegative infections depending on positivity for anti-core (HBc) and anti-HBs antibodies [6 7]. However, the presence of a serologic profile of anti-HBc/anti- HBs does not exclude OBI, and detectable HBV DNA has also been reported in those patients who are negative for both serologic markers [8,9]. The diagnosis of OBI requires a sensitive HBV DNA PCR assay because the level of HBV DNA in the sera of these patients is usually less than 10 4 copies/ml [10, 11]. However, the term occult Hepatitis B infection was initially described by Cacciola et al. in 1999 [12], who observed undetectable HBsAg in previously known HBV patients who were concurrently infected with HCV. However, they were able to demonstrate the presence of HBV DNA via PCR technique, hence the term occult hepatitis B infection [13].

Reason of OBI: The exact mechanisms of OBI are not well understood yet, it seem to be more complicated due to many factors related to both, host and virus cause viral replication suppression and infection control[20,21].

The reasons for the lack of circulating HBsAg in HBsAg-negative patients are unknown. Recent observations have suggested that the lack of HBsAg may be due to rearrangements in the HBV genome that interfere with gene expression or lead to the production of an antigenically modified S protein. [14, 15,16].

Viral Related factors (Virus Mutation):

Viral factors also have some effects on development of OBI. A large number of mutations which can reduce HBsAg expression were detected, help in virus obscuring and decrease immune recognition of the virus, and hence impair HBV covering. Some other studies demonstrate several possible mechanisms which explain low viral replication rate in OBI. One of these finding, is mutations of the X region of HBV which reduce the ability of the X protein to trans-activate host cellular proteins that are essential for viral replication, which led to the suppression of replication and expression of HBV DNA, and resulted in negative seropositivity for HBsAg[17]. Escape mutation of the S region was another possible viral factor associated with OBI, which also decreases reactivity in HBsAg detection assays [18].

In addition, however, cautious interpretation is necessary, as most of these studies lacked a control group or mutations appeared not only in patients with OBI but also those with overt HBV infections. Further studies should be conducted [19].

Host factors: OBI is characterized a low rate of HBV replication *in vivo*; however, occult HBV

strains are replication-competent *in vitro*. This suggests that host, rather than viral, factors are more responsible for OBI[34]. Similarly, many clinical observations indicate that OBI reactivation sometimes occurs under immunosuppressive conditions, such as during cancer chemotherapy treatment, HIV infection, or hematopoietic stem cell transplantation [20, 22].
This can be explained by a break in the balance between the host's immune system and the virus that occurs during occult infection caused by change in immune system function, resulting in reactivation of OBI. These findings strongly indicate the critical role of the host's immune system in development of OBI.[21].
This study aimed to investigate the occurrence of potential OBI in a population at high risk of infection such as hemodialysis patients, or a population whom might be at risk of transferring the disease to blood recipient and a low immune neonates.

Materials and methods

This study was carried out during the period between 2013 and 2015. 432 blood samples were included; 168 hemodialysis samples, 111 blood donors and 153 samples from pregnant ladies in different centers in Khartoum (Sudan).
Personal and socio-demographic data, information about risk factors were included in the study; all samples were retested for HBsAg, anti-HBcAb using ELISA, plasma samples found to be HBsAg negative samples were tested for HBV-DNA by PCR.
Population: The aim of this study was to investigate the prevalence of potential OBI in a population at high risk of infection. A total of 365 hepatitis B surface antigen negative sample were included in this study.
168 sample ranged between 23 to 78 years old were collected from patients with end-stage renal disease on regular hemodialysis out of which 165 were HBsAg negative (3 HBsAg positive were excluded), 111 ranged between 17 to 60 years old were obtained from family blood donors out of which 75 were HBsAg negative (36 HBsAg positive were excluded) and 153 were collected from pregnant ladies ranged between 19 to 42 years, out of which 149 were HBsAg negative (4 HBsAg positive were excluded) Patients and donors were selected from the hemodialysis unit, blood bank and antenatal clinics, Informed consent was obtained from all population involved in the study.
A questionnaire was designed to evaluate the effects of Gender, age, HBV vaccination history, duration of hemodialysis, previous infection with HBV, past blood transfusion and transplantation on the result obtained. All HBsAg negative samples were tested for the presence of HBV DNA. The study population consists of blood donations, hemodialysis patients and pregnant ladies from the state of Khartoum
Laboratory tests: Hepatitis B surface antigen as markers of previous or chronic HBV infection was detected by enzyme linked immune sorbent assay ELISA. For specimens that were hepatitis B surface antigen-negative by one assay, the results were confirmed by testing on other commercial hepatitis B surface antigen immunoassays. All procedures were performed according to the manufacturers' instructions.
Enzyme linked immune sorbent assay: Blood sample was drawn from all enrolled subjects, sera were taken and stored at -80°C. The serological markers of hepatitis B (HBsAg and anti-HBc/anti-body) were assayed using ELISA (Fortress, Spain).

A further repetition for at least ten sample by the same ELISA technique was carried out as a control for accuracy.
HBsAg ELISA: Fortress HBsAg is an in vitro diagnostic kit for the detection of hepatitis B surface antigen (HBsAg) in human serum or plasma.
Principle of The Assay: The test is an enzyme-immunoassay based on a 'sandwich' principle. Polystyrene microtiter strip wells have been coated with monoclonal anti-HBs(antibody to HBsAg), and HBcAb which constitutes the solid-phase antibody.
The test sample is incubated in such a well; HBsAg and HBcAb, if present in the sample, will bind to the solid-phase antibody.
Subsequently a guinea-pig anti-HBs, which has been labeled with the enzyme horseradish peroxidase (HRP), is added. With a positive reaction this labeled antibody becomes bound to any solid-phase antibody HBsAg complex previously formed.
Incubation with enzyme substrate produces a blue color in the test-well, which turns yellow when the reaction is stopped with sulphuric acid. If the sample contains no HBsAg, the labeled antibody cannot be bound specifically and only a low background color develops.
Assay principle scheme: Using the double antibody sandwich ELISA scheme:
Ab(p) + Ag(s) + (Ab)ENZ→ [Ab(p)–Ag(s)–(Ab)ENZ] →blue-yellow
Ab(p) +(Ab)ENZ 7 [Ab(p)] → no color
Incubation: 60 min Immobilized Complex Coloring Results15min.
Ab(p)–pre-coated anti–HBs antibodies;
Ag(s)–HBsAg antigens in sample;
(Ab)ENZ–HRP conjugated anti-HBs;
Nucleic Acid Extraction: DNA was extracted from all samples using Vivantis GF-1 Nucleic acid extraction kit.
The purification columns in the kits were fixed with a specially-treated glass filter membrane that is uniquely designed to efficiently bind DNA in the presence of high salts. The kit applies the principle of a spin mini-column technology and the use of optimized buffers ensure that only DNA is isolated while cellular proteins, metabolites, salts and other impurities are removed during subsequent washing steps. Water or low salt buffers with the appropriate pH is then used to elute highly pure DNA, the eluted DNA was stored at -80°C.
Polymerase chain reaction amplification of HBV DNA
Extracted DNA from patients' sera was tested for HBV DNA using PCR techniques. using the Maxime PCR PreMix Kit (Invitek, Germany).
HBV DNA was amplified using a thermocycler (Perkin Elmer 9700), one set of primer pairs (HBV-F)
5-TCGGAAATACACCTCCTTTCCATGG-3-HBV-R
5-GCCTCAAGGTCGGTCGTTGACA-3-HBV-L [23]
The diagnosis of occult HBV infection will be made when PCR amplifiable HBV DNA in sera was seen for at least two different regions (surface, precore and core) in the absence of detectable HBsAg.. The round of PCR was performed the primer set for 35 cycles (1st pre-Denaturation 94°C 5min, Denaturation 94°C 1 min, annealing 62°C 1min, Extension 72°C, followed by the extension reaction. final extension 72°C 7min. [24]
PCR products was subjected to electrophoresis on a 3% agarose gel stained with ethidium

bromide, and DNA was observed under ultraviolet light (Kaneko S, *et al* 1989)
Negative Controls that contain everything but the template DNA were used for detecting contamination or non-specific amplification in the reaction. Positive Controls were used for the verification of negative amplification results and the positive control reaction run containing the same components as the sample but include a template that is certain to amplify if the reaction goes as planned. Statistical analysis of data was performed using SPSS version 15. Data were analyzed by $\chi 2$ and Fisher's exact test. A p value <0.05 was considered statistically significant. Continuous data were presented as mean (SD).

Results

Blood samples were collected from 432 samples: 168 were collected from hemodialysis patients at Al Waledain Hemodialysis Centre, Salma Hemodialysis Centre, and Sudanese Association Kidney Transplantation Center. 111 were collected from blood donors visiting the National Centre for Blood Transfusion Services (Khartoum), and 153 samples were collected from pregnant ladies attending Omdurman Maternity Hospital.
Three procedures were followed for the purpose of detecting Hepatitis genes and antigens; namely ELISA for detection of HBsAg, ELISA for anti HBcAb and PCR.
ELISA was carried out firstly to detect, while PCR procedure was intended to discover the viral DNA among Hepatitis B surface antigen negative samples.
HBsAg test outcome reveal more cases as positive in blood donors group (29.7%) followed by pregnant ladies group (13.7%) and finally hemodialysis patients group (4.2%).
This pattern of association is found to be statistically significant (p-value =0.000) indicating that prevalence rate of HBsAg is differ according to the group of population.
Gender: Total number of study population were 432. Males were 223 (51.62%).
209 (48.37%) females were Samples from Hemodialysis patients were 168 (38.88% of total), whereas samples from Blood donors were 111 (25.69% of total). 153 (35.41% samples were collected from pregnant ladies.
Regarding the gender, the total number of subjects investigated were 432. Males were 223 (51.62%) and females were 209 (48.37%). The study disclosed the distribution of hepatitis B

Table (1): Distribution of HBsAg outcomes across the study group

Group	HBsAg test outcomes		
	Positive	Negative	Total
Hemodialysis patients	7 (4.2%)	161 (95.8%)	168 (100%)
Blood donors	33 (29.7%)	78 (70.3%)	111 (100%)
Pregnant ladies	21 (13.7%)	132 (86.3%)	153 (100%)
Total	61 (47.6%)	371 (252.4%)	432 (300%)

virus markers for both HBsAg and anti HBcAb was higher in males than females (p = 0.000) as shown in Table (1).
In this table, the results achieved to evaluate the prevalence rate of HBsAg among males and females in all subjects under this study showed a higher prevalence rate of HBsAg in males (15.25%) than in females (1,91%). This pattern of association was found statistically significant (p = 0.000).
The prevalence rate of HBcAb among males and females studied revealed a statistically insignificant pattern of association s (p = 0.332), i.e. the frequency rate of HBcAb was not associated with gender in the total study population.
No other socio-demographic or clinical characteristics were found significantly associated with HBsAg or anti- HCV seropositivity.
The results of this study suggest that HBsAg have more prevalence among vaccinated population while all HBcAb was detected among the population whom were not vaccinated, blood donation was a risk factor for hepatitis B virus transmission as most regular donors were represent seropositivity to both markers. Some of the characteristics of the study population are shown in Table (2).
The frequency rate of occult hepatitis B virus among study population was determined using PCR. The PCR was carried out on all 392 HBsAg negative samples to detect occult viral DNA.

Table (2): Distribution of HBcAb test outcomes across the study group

Group	HBcAb test outcomes		
	Positive	**Negative**	**Total**
Hemodialysis patients	3 (1.8%)	165 (98.2%)	168 (100%)
Blood donors	31 (27.9%)	80 (72.1%)	111 (100%)
Pregnant ladies	4 (2.6%)	149 (97.4%)	153 (100%))
Total	38 (32.3%)	394 (267.7%)	432 (300%)

Chi-square analysis was conducted to compare the positive PCR specimens detected among all hemodialysis, blood donors, and pregnant ladies cases.

Discussion

This is the first project focus on and compare between the prevalence of HBsAg, Anti-HBcAb

and occult hepatitis B virus infection among all study population.
The study population (432) had a mean age incidence of 34.73 ± 10.09 years and were mainly composed of males (51.62) and females (48.37%). Competitive ELISA was used to detect HBV core antibody (HBcAb) , the sandwich ELISA was used to detect HBsAg, and PCR was used to detect HBV.

Table (3): Frequency rate of HBsAg among males and females in the total study population

Group	**HBsAg o**utcome		
	Positive	Negative	Total
Males	189 (84.75%)	223 (100%)	412 (184.75%)
Females	205 (98.09%)	209 (100%)	414 (198.09%)
Total	38 (8.8%)	394 (91.2%)	432 (100%))

Table (4): Frequency rate of HBcAb among males and females in the total study population

Group	HBcAb test outcomes		
	Positive	Negative	Total
Males	35 (15.7.%)	188 (84.3%)	223 (100%)
Females	26 (12.44%)	183 (87.56%)	209 (100%)
Total	61 (14.12%)	432 (100%))	493 (100%))

DNA were used to analyze 392 plasma samples collected during the period of 2013 to 2015. The majority of Anti-HBcAb positive cases were males (63 %) while the same result was found associated with HBsAg where the majority of positive cases were also male (89%). No PCR test detected any HBV DNA, P value (Table 5.)
HBsAg among different population: The overall prevalence rate of HBsAg among the different study population under this study is 8.796%.(Table 5).

In the previous data, Sudan has been classified among countries with a high hepatitis B surface antigen (HBsAg) endemicity of more than 8.1% with a hepatitis B surface antigen (HBsAg) seroprevalence ranging from as low as 6.8% with a hepatitis B surface antigen (HBsAg) seroprevalence ranging from as low as 6.8% in central Sudan to as high as 26%.
Thus, the results are in accordance with literature data, except for slight variation between our results and the previous ones which can be described by many factors such as different epidemiology of hepatitis B virus among different populations, different sample size, laboratory methods used to detect the virus .
HBcAb among different population: The presence of anti-HBc indicates previous or ongoing infection with hepatitis B virus in an undefined time frame, the overall prevalence rate of HBcAb in this study was 14.35% (Table 4).
No data present the overall prevalence of HBcAb among this different population, in Sudan, several conditions may result in positive isolated anti- HBc in an apparently healthy blood donor. One possibility is recovered past HBV infection, which did not apply to our donors as they had no history of hepatitis.
Nevertheless, hepatitis B infections are sometimes asymptomatic and patients do not manifest jaundice or any other symptoms/signs.
Another possibility is false-positive results due to be poor specificity of the test [25]. In this study 10% of the test was repeated with the same method, to minimize false-positive results, and all reactive samples were positive in repeated measurements.
As the previous study conclude that HBV DNA can be detected when anti-HBcAb was positive, no HBV DNA was detected in the serum of HBsAg, anti-HBc positive blood donors, hemodialysis patients and pregnant women examined in this study, representing 0% of the total patient investigated.
Thus it has been suggested that the presence of anti-HBcAb does not rule out the presence of HBV DNA and possibility of HBV transmission.
The molecular properties of occult hepatitis B virus (HBV) have been poorly characterized in this study, this may be due to the extremely low virus concentration since patient may had latent HBV infection for long time without detectable viremia and symptoms of liver disease.
Hemodialysis patients: In the hemodialysis 165 samples (96%) were HBsAg negative (4% were positive) and 3 samples (2%) were positive for Antibody against – HBcAb, and 3(2%) were positive for HBsAg.
The incidence of occult HBV (HBV DNA) was similar to the results found by Nafeesa *et al* in Sudan, no patients with occult HBV were detected [26].
While conflicting results have been reported on the frequency of OHB in Sudan (Abdulhafez)[23], who reported the highest frequency rate (3.3%) of HBV DNA among heamodialysis patients, and another study by [27] detect HBV DNA in(11.5%) out of HBcAb negative hemodialysis patients samples.
The seroprevalence was low for both anti-HBcAb and HBsAg compared with that study of Gasim et al in Sudan who reported a prevalence of HBsAg (4.5%) and anti-HBcAb (39.4%) other African countries, for example, 8% in Kenya [28] (Table 5).
As there is a discrepancy in results of occult hepatitis B infection through the study carried in the last years, to isolate and identify OHB in hemodialysis patients, it is clear that more studies

are needed to entirely clarify the incidence of OHB in Sudan.

Table (5): Demographic, serologic and virologic data of positive HBsAg and Anti-HBcAb

Population	Hemodialysis cases (168)		Blood Donors (111)		Pregnant women (153)	
Variables	Pos. HBsAg (3)	Pos. HBcAb (7)	Pos. HBsAg (31)	Pos. Anti-HBc (33)	Pos. HBsAg (4)	Pos. Anti-HBc (21)
Mean Age	46.02 (years)		26.27 (years)		38.54(years)	
		3	2	3	2	0
Females	0	5	0	0	21	4
Post-blood donation	0	1	31	32	0	0
Post-transplants	0	0	0	0	0	0
Post-HBV infection	0	0	0	0	0	0
Vaccinated HBV	2	0	3	1	2	0
No HBV vaccine	1	9	28	32	2	0
Post-bilharisiasis	0	0	0	0	0	0