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Full Length Research Paper

Identification of different stages of hepatitis B infection with enzyme linked immunosorbant assay (ELISA) and polymerase chain reaction (PCR) assay

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Despite the existence of hepatitis B vaccination, hepatitis B virus (HBV) infection is still prevalent worldwide and accounts for significant morbidity and mortality. In chronic infection with hepatitis virus B the fact that HBeAg becomes negative does not always means suppression of viral replication. It is encouraging that majority of patients do recover from the acute infection, however, those that progress to chronic disease state is at great risk of developing complications such as hepatocellular carcinoma, cirrhosis and liver failure. The selection of patients was those who had elevated levels of liver enzyme SGPT (ALT), The HB s Ag and anti HCV n = 1890 by ELISA were performed to identify the positive cases with HB s Ag. Furthermore, in this study we performed HB e Ag by ELISA for identifying the infectious and the carrier state. The PCR was performed for the confirmation of infectious status, carrier state and the late sero conversation state. HB s Ag were n = 1890; out of which n = 1242 are negative cases and 648 were positive cases. On further analysis of hepatitis B virus variant were carried out and HB e Ag, HB e Ab, HB c Core Ig M, and anti-HB c Core was also performed on n = 648 cases to put patients in different status of infection. During this study we also identified the chronic carrier status with No sero conversion and later sero conversion state. It is encouraging that we easily identify different stages of hepatitis B by Serological markers, DNA extraction, determination of HBV – DNA by PCR. Present study helps in understanding the nature of HBV infection and delivering better care for patients, it was very important to screen the different stages of the infection.

Key words: Hepatitis B, stages of HBs Ag, HBV infection.

INTRODUCTION

Hepatitis B virus (HBV) infection is well recognized and major health problem leading to significant morbidity and mortality worldwide especially in the developing countries. Approximately, 2 billion people in the world have been infected by HBV (Zuckerman and Zuckerman, 2000) 400 million of who are chronic carriers (Lee, 1997). Primary HBV infection in susceptible individuals can be either symptomatic or asymptomatic, the latter being often the case, especially in young individuals; but rarely, fulminant hepatitis can develop during acute phase. Most primary infections are self-limited with clearance of virus and development of immunity. Monitoring of hepatitis B virus (HBV) DNA in serum has become the standard method of assessing the replicative activity of HBV. The clinical importance of this method has been reported for the assesment, management, and antiviral treatment of patients with chronic HBV infection. The virus causes acute hepatitis of varying severity (Calvin and Zhang, 2005)

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Table 1. Active HB s Ag infection in ELISA positive male and female patients selected from different parts of Pakistan (n=1890).

S/No.	Sex	No. of samples	Positive	Percentage
1	Male	1098	432	39.34
2	Female	792	216	27.27
3	Total	1890	648	34.28

Table 2. HB e Ag was performed by ELISA positive male and female HBs Ag patients (n = 648), patients were in infectious state and in no sero conversion state (Chronic carrier).

S/No.	Sex	No. of samples	Positive	Percentage
1	Male	432	206	47.68
2	Female	216	156	72.22
3	Total	648	362	55.86

and persists in 95% of children and 2 to 10% of adult patients leading to chronic liver disease, cirrhosis, hepatocellular carcinoma (Calvin and Zhang, 2005; Bowyer and Sim, 2006; Abe et al., 2005) and even fulminant hepatitis. In Pakistan, HBV infection rate is increasing day by day. The reason may be the lack of proper health facilities or poor economical status and less public awareness about the transmission of major communicable diseases like hepatitis B virus, Hepatitis C virus and Human Immunodeficiency Virus (HIV) (Alam et al., 2007; Passos, 1993; El-Sayed, 1997; Chowdhury et al., 1999; Andre, 2000) The multiple studies have been conducted regarding prevalence rate of HBV infection based on various population groups in Pakistan. According to various study groups, the HBV prevalence rate has been reported as 2 to 10% among healthy blood donors, 5 to 9% among health care personnel, 3.6 to 18.66% among the general population, 3.16% among the pregnant women, 10 to 20% in patients with provisional diagnosis of hepatitis and 3.16 to 10.4% among professional blood donors (Alam et al., 2007) These reports highlight the lack of a country wide epidemiological studies that can present the overall disease status in the whole country.

MATERIALS AND METHODS

All the patients with elevated liver enzyme, alanine transaminase (ALT), were selected in this study. The total number of blood samples (n = 1890) was collected from different parts of Karachi.

Serological markers

HB s Ag, hepatitis B e antigen (HB e Ag), hepatitis e antibody (HB e Ab), hepatitis Core IgM (HB c Ig M) and total core (anti HB c Core) were determined using an enzyme linked immunosorbant assay (ELISA) (DRG Diagnostic, Germany). The ELISA was performed on all the samples for detect the infectious state, chronic carrier state with no sero conversation and late sero conversation. The presence of anti-HCV was also determined by ELISA technique (DRG Diagnostic, Germany) to rule out the reason for elevated ALT. The ELISA test was performed on all the serological markers by following the kit protocol.

The PCR was performed for the confirmation of infectious status, carrier state and the late sero conversation state. This technique was also used to find out the quantity of HBV DNA present in the infectious state.

DNA extraction

The total number of (n = 362) serum samples from chronically infected patients was selected for the DNA extraction, which was further used for the detection of HBV-DNA and viral load was detected in the patients with real time PCR.

The HBV DNA was extracted from 200 µl serum samples using Gentra DNA extraction kit (Puregene DNA D-5000, Gentra Systems, Minnesota, USA). The extracted DNA pellet was resuspended in DNA hydration solution present in the kit.

Determination of HBV – DNA by PCR

The nested PCR was performed for HBV-DNA. The 40 cycles were performed and the condition used was 94°C for 45 s, 55°C for 45 s and 72°C for 1 min with extension for 10 min at 72°C. All precautions were carried out to avoid contamination during PCR as well as negative and positive control serum was also included in each run.

The quantification was carried with real time PCR using qaigen USA in such way that its specificity and sensitivity for HBV-DNA detection was less than 2 pg/ml.

RESULTS

HB s Ag were n = 1890; out of which n = 1242 are negative cases and 648 were positive cases as shown in Table 1. On further analysis of hepatitis B virus variant was carried out and HB e Ag, HB e Ab, HB c Core Ig M, and anti-HB c Core was also performed on n = 648 cases to put patients in different status of infection. During this study we also identified the chronic carrier status with no sero conversion and later sero conversion state.

In Table 2 shows that HB e Ag were reactive in 362

Table 3. Anti HB c Core done on ELISA positive male and female patients (n=648), patient were in infectious state, window phase immune state, no sero conversion and late sero conversion.

S/No	Sex	No. of samples	Positive	Percentage
1	Male	432	322	74.54
2	Female	216	187	86.57
3	Total	648	549	84.72

Table 4. Shows the different status of infection in patients (n = 648) during this study.

S/No	Status	No. of samples	Percentage
1	Infectious	362	55.86
2	Immunity	47	7.25
3	No sero conversion	54	8.33
4	Late sero conversion	185	28.55

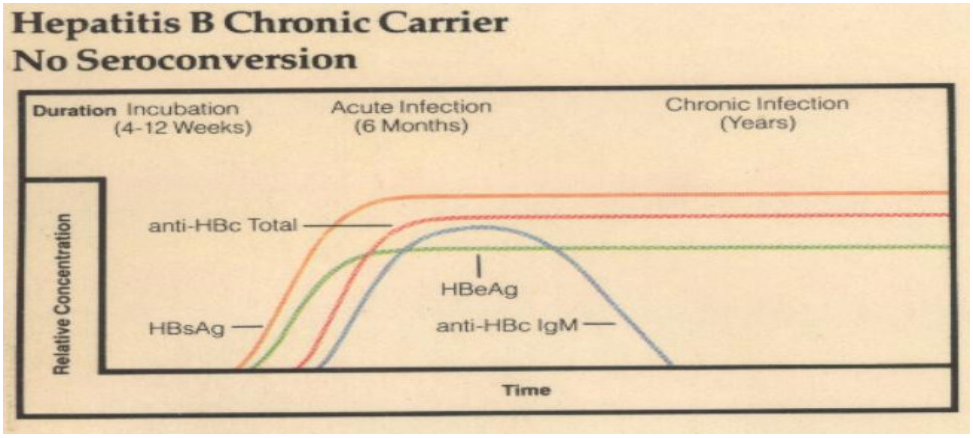

Figure 1: No Sero conversion: an individual has a high viral load and is considered highly infections. Anti HBe and anti HBs are not detectable

cases, as circulating HB e Ag were detected in most patients with acute HBV infection, and these patients readily transmit the infection. Further analysis was carried out to find the total number of patients with immunity and chronic state. It showed that HB core total was detected that n = 549 cases as shown in Table 3.

The HBV DNA detection with viral load was carried in n = 648 cases and was divided into three groups according to its viral load. The n = 362 with HB e Ag and HB core Ig M as shown in Table 2 cases, the viral load was found to be very high up to 430 pg/ml and these patients were places in group 1. Further detection was carried out on the cases to detect the HBV chronic carrier state with no sero conversion (Figure 1). The viral load was determined to be 198 pg/ml and these patients were selected as group 2. In group 3 only those cases were considered who had been treated and were having low titer of HBV-DNA viral load (3 to 5 pg/ml) and later on developed the immunity/status or HBV chronic late sero conversion. In these cases the HBe Ag was non reactive and anti HBe was developed, as well as the anti HB s and HB core total was reactive (Table 4).

DISCUSSION

There are three stages of HBV infection based on viral-host interaction, namely: The immune tolerant phase, the immune clearance phase, and the inactive carrier phase with or without reactivation (Figure 2). After acute infection of HBV, some patients may remain HBeAg positive with high levels of serum HBV DNA, little or no symptoms, normal ALT levels and minimal histological activity in the liver, this phenomenon is known as the immune tolerance phase. This phase is typical of infection in children and young adults. It usually lasts for 2 to 4 weeks, but can last for years in those who acquired the infection during the perinatal period (Merican et al.,

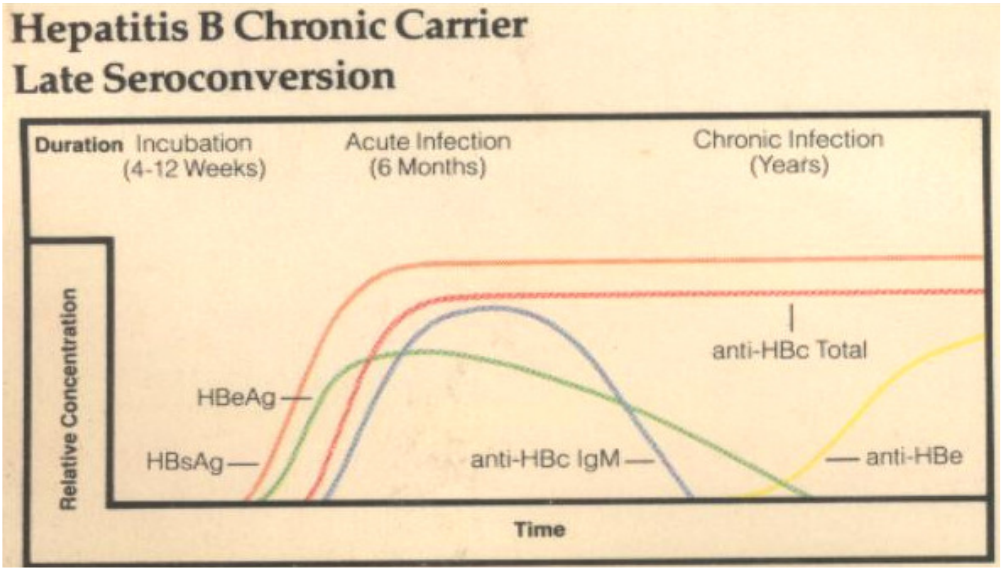

Figure 2. Late sero conversion: an individual has a lower viral load but still infectious. This individual eventually sero converts to anti HB e.

2000).

Individuals in this group are highly contagious and can transmit HBV easily. When the tolerogenic effect is lost during the immune tolerant phase, immune-mediated lyses of infected hepatocytes become active and patients enter the second stage defined as immune clearance phase, the HBV DNA level decreases and ALT level increases. The duration of clearance phase lasts from months to years. This is followed by the carrier stage, in which sero conversion of HB e Ag to HB e Ab occurs, HBV DNA becomes non-detectable or at low level and ALT are usually normal, reflecting very low or no replication of HBV and mild or no hepatic injury. The inactive carrier stage may last for years or even lifetime. Patients in this stage can have spontaneous resolution of hepatitis B and develop HB s Ab, but a portion of them may undergo spontaneous or immuno suppression-induced reactivation of chronic hepatitis, featuring elevated ALT, high level of DNA, moderate to severe liver histological activity, and with or without HB e Ag sero conversion.

A proportion of patients who undergo HB e Ag sero conversion demonstrate a recurrence of high HBV DNA levels and intermittent or persistent ALT level elevations. These individuals have a naturally occurring mutant form of HBV that does not produce HB e Ag, due to a mutation in the precore or core promoter region. Most frequent precore mutation is a G-A change at nucleotide 1896 (G1896A) which creates a stop codon and results in loss of HBeAg synthesis, the most common core promoter mutation involves a 2 nucleotide substitution at nucleotide 1762 and 1764 (Bortolotti et al., 1990) HB e Ag-negative carriers are a heterogeneous group and most of them have low viral DNA levels, relatively normal levels of alanine aminotransferase, and a fair prognosis.

It has been reported that in Asia, Middle East, Mediterranean basin and southern Europe, about 15 to 20% of these carriers have elevated alanine aminotransferase and viral DNA (Bortolotti et al., 1990). HB e Ag-negative chronic hepatitis B (precore mutant) emerges as the predominant species during the course of typical HBV infection with wild-type virus and is selected during the immune clearance phase (HB e Ag sero conversion) (Funk and Rosenberg, 2002). The development of HB e Ag-negative chronic hepatitis B can occur either close to HB e Ag sero conversion or decades later. There are two main patterns of disease activity in this subgroup of patients: 30 to 40% of patients experience persistent elevated ALT levels (3 to 4 folds) and the remaining 60 to 70% patients can have erratic ALT patterns with frequent flares.

Sustained spontaneous remission is rare (6 to 15%) in these individuals, and spontaneous HB s Ag clearance is only about 0.5% per year (Keefe et al., 2004), hence, long-term prognosis is poorer among HB e Ag-negative individuals when compare with their counterparts who are HB e Ag-positive. Inactive HB s Ag carrier state is diagnosed by HB e Ag negative with anti-HB e positive, undetectable or low HBV DNA level, repeatedly normal ALT, and normal or minimal histological evidence of damage (Papatheodoridis et al., 2001) The prognosis of the inactive carrier is generally good and well supported by long-term follow-up studies (Lok and McMahon, 2001; Hsu et al., 2002; Franchis et al., 1993; Bellentani et al., 2002). An estimated 20 to 30% of HB s Ag carriers may develop reactivation of hepatitis B with elevation of biochemical levels, high serum DNA level with or without sero-reversion to HB e Ag. Recurrent episodes of reactivation or sustained reactivation can occur and

contribute to progressive liver disease and results in significant hepatic decompensation.

Conclusion

The study was carried out to have a clear picture of prevalence of HBV in different stages including the infection state, chronic carrier state and late sero-conversation state. Long-term infection increases risk of developing cirrhosis and HCC. It is encouraging that we easily identify different stages of hepatitis B by Serological markers, DNA extraction, determination of HBV – DNA by PCR.

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