Detection of hepatitis B virus carriers by real time polymerase chain reaction

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ABSTRACT

INTRODUCTION: Hepatitis B is noteworthy medical issues that may include the late continuation of liver cirrhosis and hepatocellular carcinoma.

OBJECTIVE: The present study aimed for the detection and differentiation of hepatitis B virus inactive, active carriers and HBeAg-negative chronic hepatitis B by Real Time PCR.

METHODS: The study was conducted on 4927 patients in the city of Meerut, India, which was performed in central research Station Laboratory of Microbiology at Netaji Subash Chandra Bose, Medical College and Hospital, between January 2013 to April 2017. The sera were separated and screened for HBsAg by immune-chromatographic card test, then positive samples of HBsAg were tested again for HBsAg using ELISA kit. Positive samples for HBsAg were tested for HBeAg ELISA kit and DNA Viral load.

RESULTS: 245 positive sample for HBsAg, 118 (48.16 %) were males and 127 (51.84%) were females. Of the 245 HBsAg Positive cases, 55 (1.12%) were HBeAg positive; 20 were males while 35 cases were females and this was statistically significant (P< 0.039) by using Z test. The highest HBeAg positive males were found in age group of 21- 30 years. In RT-PCR, 4(1.63%) were inactive carrier, 5(2.04%) active carrier and 0 were HBeAg negative chronic hepatitis B carrier.

CONCLUSION: The management strategy, using HBsAg, HBeAg and HBV DNA viral load, seems adequate for the confirmation and differentiation of hepatitis B virus inactive, active carriers and HBeAg-negative chronic hepatitis B patients. Further studies are needed to standardize and improve the management of this group of patients.

Key words: Hepatitis B virus, Carrier State, Hepatitis B surface antigen, Hepatitis B e antigen, RT-PCR.

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INTRODUCTION

The human hepatitis B virus (HBV) is a small-enveloped DNA virus causing acute and chronic hepatitis. Despite the availability of a safe and effective vaccine, HBV infection still represents a major global health burden with about 350-400 million people chronically infected worldwide and approximately 600,000 deaths per year due to HBV-associated liver pathologies.[1] Between 15 and 40% of chronically infected individuals may develop severe liver disease and hepatocellular carcinoma (HCC), while the remaining become inactive carriers.[2]

Approximately, 5 % of the world's populations are carriers of HBV which is defined as being positive for hepatitis B surface antigen. HBV is endemic in many areas of the world, such as Asia, Micronesia, and sub-Saharan Africa as well as in certain populations in Australia, New Zealand, South America, the Middle East and the Arctic. An estimated 1.25 million people in the United States are positive for hepatitis B surface antigen. Fifteen percent to forty percent of these carriers may develop hepatitis B-related sequelae in their lifetimes.[3-5]

HBV is present in blood, saliva, semen, vaginal secretions and menstrual blood of infected individuals and easily transmitted through contact with infected body fluids.[6] Perinatal vertical transmission is the most common mode of transmission worldwide.[7] In households of a

chronically infected individual, HBV infection can occur via person-to-person, nonsexual contact.[8] Patients with HBeAg-positive chronic hepatitis B usually present in the third or fourth decade of life. Liver damage ranges from mild (24 to 42%) to moderate or severe chronic hepatitis (44 to 63%) or active cirrhosis (10 to 24%).[9-12] Chronic hepatitis B tends to be milder in children. Nevertheless, severe liver disease including cirrhosis may occur in a small proportion of patients during childhood.[13] A key event in the natural history of HBeAg positive chronic hepatitis is HBeAg seroconversion. Several studies have shown that seroconversion with marked reduction of HBV replication is associated with biochemical and histologic remission of inflammatory activity in the majority of patients. Regression of fibrosis occurs gradually months to years after HBeAg seroconversion. In longitudinal studies the observed probability of clearing HBeAg was about 50% and 70% within 5 and 10 years of diagnosis, respectively.[14-16] Most studies have found that the mean annual rate of spontaneous HBeAg seroconversion ranges from 8 to 15% in children or adults with elevated alanine aminotransferase (ALT). Among Asian, most of whom have normal ALT, spontaneous HBeAg seroconversion occurs at a very low rate, less than 2% during the first 3 years of age and 4 to 5% in children older than 3 years.[17] Several determinants for HBeAg seroconversion have been reported, including gender, age, ALT level, and more recently HBV genotypes. Older carriers and females are more likely to clear HBeAg.[18] After HBeAg seroclearance, two disease states, not necessarily static, are possible: Some patients remain in an inactive carrier (IC) state, defined by the European Association for the Study of the Liver (EASL) as fulfilling the following criteria: **(1)** HBeAg negativity; **(2)** anti-Hbe positivity; **(3)** persistently normal ALT (PN-ALT) levels (<40 IU/mL, with measurements at least every 3-4 months during 1 year); **(4)** serum HBV DNA levels <2000 IU/mL.

Others evolve to HBeAg-negative chronic hepatitis B (CHB), usually associated with precore or basal core promoter mutant HBV, in which viral replication and hepatic inflammation persist despite seroconversion to anti-HBe status. It is characterized by fluctuating ALT and serum HBV DNA levels that can drop below IC cut-off levels, despite persistent viral replication and hepatic inflammation.

At the Management of Hepatitis B workshop in 2000, an arbitrary level of 20,000 IU/mL was adopted as the serum HBV DNA cut-off level distinguishing active and inactive chronic hepatitis.[19] EASL acknowledges that there can be inactive HBV carriers with DNA levels between 2000 and 20,000 IU/mL. HBV DNA levels in HBeAg-negative chronic hepatitis B can fluctuate from undetectable to >2,000,000 IU/mL[20] some inactive carriers occasionally have HBV DNA levels between 2000 and 20,000 IU/mL, a single HBV DNA level between 2000 and 20,000 IU/mL appears to be a "gray area" which can correspond to both active CHB or inactive carriers. Thus, the aim of this study was to detect HBV carriers by Real Time PCR in Meerut city as the HBV is increasing in India.

METHODS

Study design and settings: This descriptive study has enrolled 4927 patients from city of Meerut in India. They were referred to the Central Research Laboratory of Microbiology at Netaji Subash Chandra Bose, Subharti Medical College and Hospital Between January 2013 to April 2017.

Ethical consideration: participants were asked to provide their written consent form after being prepared to understand the nature and the aims of the study.

Inclusion and exclusion criteria: This study has enrolled 4927 patients who were referred to the Central Research Station Laboratory of Microbiology at Netaji Subash Chandra Bose, Subharti Medical College and Hospital because they were suspected of having HBV infection and its sequelae from January 2013 to April 2017. We included only patients whom their age was between 21-65 years. We excluded those who had been immunized for HBV; having co-infection of HBV with HCV, HDV, or HIV; having liver infections due to other viruses, autoimmune liver diseases, diabetes, and history of alcohol consumption; or using immune modulation drugs like systemic steroid, interferon, interleukins, or cytokines.

Sample collection and processing: Blood sample from patients suspected of having HBV were collected in a clean and sterile test tube. Five millilitres of blood samples, received in the Serology Section of Department of Microbiol-

Table 1:- Sex and Age distribution among HBsAg & HBeAg positive patients

Age group (Years)	HBsAg		Total	HBeAg		Total
	Male	Female		Male	Female	
21 – 30	69	74	143 (58.37%)	8	19	27 (49.09%)
31 – 40	33	29	62 (25.31%)	7	13	20 (36.36%)
41 – 50	11	15	26 (10.61%)	4	3	07 (12.73%)
51 – 65	5	09	14 (5.71%)	1	-	1 (1.81%)
Total	118 (48.16 %)	127 (51.84%)	245 (4.97%)	20 (36.36 %)	35 (63.64%)	55 (1.12%)

ogy from patients suspected of acute hepatitis, were analyzed. The sera were separated and screened for HBsAg by Hepa Card (J. Mitra & Co. Pvt. Ltd. New Delhi, India) and positive serum was stored in frozen (-20 °C) until tested for the viral markers. The positive serum samples for HBsAg by Hepa Card were tested again for HBsAg using commercially available ELISA kit (ERBA Transasia Bio-medicals Ltd. Daman, India). Serum samples tested positive for HBsAg were tested for HBeAg by ELISA; Beijing Kewei Clinical Diagnostic Reagent Inc. Beijing, China.

DNA isolation from the serum samples was performed using the "QIAamp DNA Mini Kit" (Qiagen, Germany) following the manufacturer's recommendations.[21] The isolated DNA was amplified by Real Time PCR by using artus® HBV RG PCR Kit (Qiagen, Germany) following the manufacturer's recommendations.[22]

Statistical analysis: Obtained data were analysed by using the SPSS software for windows version 16. The comparison of data in respect of age groups and gender were performed by Z- test. P < 0.05 was consider to be statistically significant.

RESULTS

Of the 4927 serum sample, 2218 (45.01%) male and 2709 (54.98%) female were tested for HBsAg with age range of 21 – 65 years. In this study, it was observed that 245 were positive and 4682 were negative. In 245 positive cases 118 were male and 127 were female. In 4682 negative cases 2100 were male and 2582 were female. Highest positive case were found in the age group of 21- 30. **Table 1**

All 245 HBsAg positive cases was tested for HBeAg and DNA Viral load to detect HBeAg-positive inactive carriers, active carriers, and HBeAg-negative chronic hepatitis B carriers.

HBeAg positive were 55 (1.12%), 20 male were positive for HBeAg and 98 male were negative for HBeAg and the highest HBeAg positive males was found in the age group of 21- 30 years. Out of 55 HBeAg cases, 35 were female and 92 female were negative for HBeAg, this was statistically significant (P< 0.039) by using Z test. **Table 1**

In PCR, 4 (1.63%) were inactive carrier, 5 (2.04%) active carrier and 0 were HBeAg negative chronic hepatitis B carrier. **Table 2**

Table 2:- Differentiation between inactive carriers, active carriers & HBeAg negative chronic hepatitis B patients by Real Time PCR

Carrier types	HBsAg +ve	HBeAg		Viral Load IU/ml				HBV carriers by PCR
		- ve	+ve	NTC Threshold (PCR negative)	>Threshold (0.05)	≥ Cutoff (10)	Total	
Inactive- HBeAg Positive (Viral Load 2000 to 20 000 IU/mL.)	245	-	55	17	22	16	55	4
Active-HBeAg Positive chronic hepatitis B (Viral Load >20 000 IU/mL)								5
HBeAg negative chronic hepatitis B (Viral Load undetectable to >2,000,000 IU/mL)		190	-	183	-	-	183	-
Total	245	190	55	200	22	16	238	9
Note:- 7 samples was cancel in PCR due to low volume/ spoilage of sample								

DISCUSSION

This study was performed on HBV inactive, active carriers and HBeAg-Negative CHB. The correct identification of the inactive carriers, active carriers and HBeAg-Negative CHB has important prognostic implications, since the survival of these patients is comparable to that of non-infected population, at least in Western studies. According to international guidelines, a correct approach to chronic HBV infection requires an accurate differential diagnosis between chronic hepatitis and the inactive carriers through HBV DNA for at least one year. Some data suggest that the use of HBV DNA and ALT alone to define an inactive carrier, without resort to liver biopsy, may not detect significant histological disease in about 10% of patients.[23] Based on this, liver biopsy was performed in only 12 patients. We acknowledge this as limitation, as it is a retrospective study, although the results are similar to other studies that consider there is minimal or no necro-inflammatory activity in the inactive carrier state. Since liver biopsy is seldom recommended in these patients, there are few confirmations of these findings and liver histologic changes in this group are difficult to assess. As liver biopsy is an invasive method of evaluating liver fibrosis, and carries small risk of complications, we consider it appropriate not to routinely perform biopsy in these asymptomatic group of patients.[24] Despite some limitations, transient elastography appears an ideal approach for identifying liver fibrosis in all inactive carriers.[24] Recent studies on HBeAg level in different population of chronic HBV infection have shown that HBeAg level is lowest in the inactive carrier patient and HBV DNA quantitation may help to differentiate between active, inactive and CHB carrier state. The combination of serum HBeAg and HBV DNA levels shows promising results as a single-point measurement, instead of quarterly laboratorial monitoring over a year, for identifying active, inactive and HBeAg- negative CHB carrier state inactive carriers.[23-29]

The results showed that 16 % of the patients were PCR positive for serum HBV DNA, In 84 % the level was undetectable. Out of this 16% PCR positive patients, The level of HBV DNA was < 2000 IU/mL in 12.18, > 2000 to 20000 IU/mL in 1.7 %, and > 20000 IU/mL in 2.1 %. In the Western countries, the reactivation is rare and it occurs mainly due to the presence of co-factors such as alcohol or drugs. Significant predictors of mortality in HBeAg-positive carriers are the presence of medical co-morbidities, older age at diagnosis, and abnormal GGT levels.[2,30-32] Another study concluded that the majority of inactive carriers have a low level of viral replication.[33] HBV reactivation occurred in ten of our patients, which is in line with the published data that have estimated the reactivation to occur in 10-34% of these patients.[34,35]

Because HBV DNA levels in HBeAg-negative chronic hepatitis B (CHB), can fluctuate from undetectable to >2,000,000 IU/mL[20] and some inactive carriers occasionally have HBV DNA levels between 2000 and 20,000 IU/mL, a single HBV DNA level between 2000 and 20,000 IU/mL appears to be a "gray area" which can correspond to both HBeAg-negative chronic hepatitis B or inactive carriers. Also HBV DNA level > 20,000 IU/mL appears to be a "red area" which can correspond to both HBeAg-negative chronic hepatitis B or active carriers, for the confirmation of this red area patients, detection of HBeAg and HBV DNA viral load should be perform. It is thus important for the clinician to be aware of the importance of serial HBV DNA measurements and status of HBeAg and life-long follow-up to confirm that inactive carrier state is maintained.

CONCLUSION

The management strategy, using HBsAg, HBeAg and HBV DNA viral load, seems adequate for the confirmation and differentiation of Hepatitis B virus inactive, active carriers and HBeAg-negative chronic hepatitis B patients. Further studies are needed to standardize and improve the management of this group of patients.

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Abbreviation list: Alanine aminotransferase (**ALT**), Chronic hepatitis B (**CHB**), Deoxyribonucleic Acid (**DNA**), Enzyme Linked Immunosorbent Assay (**ELISA**), European Association for the Study of the Liver (**EASL**), Gamma-Glutamyl Transferase (**GGT**), Hepatitis B virus (**HBV**), Hepatocellular carcinoma (**HCC**), Human Immunodeficiency Virus (**HIV**), Inactive carrier (**IC**), International unit (**IU**), Millilitre (**mL**), Polymerase Chaise Reaction (**PCR**), Statistical Package for the Social Sciences (**SPSS**)

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