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Serological Markers *HBsAg* and *HBeAg* in Chronic Hepatitis B Carriers and their Correlation with Viral DNA by Polymerase Chain Reaction

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Abstract

Hepatitis B is the most widespread and important type of viral hepatitis which may become chronic and lead to complications like cirrhosis and hepatocellular carcinoma. India is an intermediate group (4-7%) among carriers with about 45 million infected individuals. This study was undertaken to assess serological markers *HBsAg* and *HBeAg* in chronic carriers of Hepatitis B virus infection and compare with viral load as determined by PCR. A cross-sectional study was conducted with 30 patients (*HBsAg* positive status for at least 6 months) attending the OPD and IPD of Shri BM Patil Medical College. 5 ml blood was drawn with full aseptic precautions from the patients after detailed history and written consent. *HBsAg* by ELISA was done for confirming the carrier status and then, serum was sent for *HBeAg* detection by chemiluminescence immunoassay and for assessment of hepatitis B viral DNA by Real-Time Polymerase Chain Reaction Assay. Majority of patients (46.7%) belonged to age-group 20-30 years. Therapeutic injections (36.7%) were the most common risk factor. All 30 cases were positive for *HBsAg*. Majority (63.3%) had HBV DNA below detectable levels while 10% were super carriers (>20,000 IU/ml). *HBeAg* positivity was seen in 23.3% patients. HBV DNA was detectable in all of these cases ranging from 66 IU/ml to 64,291,972 IU/ml. (log 10^7) Viral DNA levels were negative in 83% of the patients who were negative for *HBeAg*. Remaining 17% had detectable HBV DNA levels ranging from 50 IU/ML to 5544 IU/ml (log 10^3). Majority (63.3%) were chronic inactive carriers who may have total HBsAg clearance later. 20% were super carriers who are known to be highly infectious.

Keywords: Chronic carrier, *HBsAg*, *HBeAg*, HBV DNA.

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INTRODUCTION

Hepatitis B is the most widespread and the most important type of viral hepatitis.[1]According to WHO, 4 billion individuals are chronically infected by this virus and one-fourth of them run the risk of death from its fatal complications like chronic liver disease and hepatocellular carcinoma.[2]In our country, India, the carrier rate is 4-7% and there are 45 million people infected with this virus.[3]In early 4000 B.C. spreading jaundice was recognized by Hippocrates. Australia antigen was discovered by Dr. Baruch Blumberg for which he was honored with Nobel prize in Physiology and Medicine. In 1978 cloning as well as sequencing of HBV DNA was done by Pierre Tiollais, William Rutler and Kenneth Murray.[4] Molecular biology and PCR revolutionized the study of this virus and now its role in this field is of paramount importance. It is used for diagnosis, in management, for therapeutic decisions and also in identification of drug resistant strains.

Epidemiology

HBV is heterogeneous in its prevalence across the world and our Asia-Pacific region has intermediate to high prevalence being home to three-quarters of HBV positive chronic individuals worldwide.[5]India is defined in intermediate group. The general population has 2%-4% prevalence but high risk populations like professional blood donors, patients on hemodialysis and health care workers have a higher prevalence.[6]

Routes of transmission

Route for transmission for virus can be horizontal and vertical. Horizontal transmission includes blood transfusion, unsterile injections, surgical equipment, needle prick as well as sexual transmission. Vertical transmission constitutes of mother to fetal transmission in utero. Vertical transmission during intra-partum period and horizontal transmission during perinatal period and early childhood are important in India, however, the latter is believed to be more common.[7] Unsafe injection practices are rampant in developing countries and are responsible for 20 million new Hepatitis B infections.[8]Effective means of transmission of virus is sexual contact. The risk factors include unprotected sex with an unvaccinated MSM or a heterosexual individual, multiple sex partners or contact with sex workers. HBV genotype A is most commonly found in MSM candidates.[9] Breaches in infection control measures are most important means for patient to patient viral transmission.[10] Health care professionals are at a high risk during exposure prone procedures. They may become carriers in 5-10% cases and pose a risk to the patient. Transmission rate from a *HBeAg* positive doctor to patient is 5.5%-13% during high risk procedures.[4] The recipient is at the risk of HBV infection by *HBsAg* negative HBV positive carrier during blood donation or liver transplantation.[11] Risk factors for transmission of HBV infection through transfusion are occult HBV infection as well as window period infections.[12]

Natural history of disease

Chronic hepatitis B infection has three phases in natural history which are the result of dynamic interplay between the HBV and its host. The important host factors include gender, alcohol consumption, infection with other viruses and the immune status of the individual. Phase 1: Immune tolerant Phase: There is *HBsAg*, *HBeAg* in circulation along with high levels of HBV DNA. The immune response against the virus is minimal and so serum aminotransferases are elevated little and the inflammation in the liver is also very less. Phase 2: Phase of symptomatic acute hepatitis B: During this phase, there is an increase in immunity which is accompanied by increased aminotransferases and inflammation in the liver. There is decrease in HBV DNA level. This augmentation of immunity causes hepatocyte destruction. Symptomatic acute hepatitis B manifests during this period. Phase 3: Phase of seroconversion: During this phase, there is conversion from HBe antigenemia to anti HBe. This is followed by reduction in virus replication and a decrease in aminotransferases. This phase is called as inactive carrier stage because both *HBsAg* and HBV DNA levels are low. In absence of cirrhosis, prognosis for healthy carriers is generally good. These patients if immunosuppressed can have a reactivation of replication.[13] Major proportion of recovered patients retain HBV genome in their liver. When T cell immunity breaks down, there may be reactivation of HBV replication. In reactivation of virus independent risk factors are male sex as well as genotype C.[14]

Emergence of variants

Variants of HBV may emerge in the chronic infection process. They facilitate immune

evasion. Pre-core and/or in the basic core promoter region mutation is present in the *HBeAg* negative mutants. When such mutations occur, infection is characterized by presence of *HBsAg*,absence of *HBeAg* and fluctuating HBV DNA levels usually > 2000 IU/ml. Such form of chronic hepatitis is associated with a high risk of liver fibrosis, cirrhosis and HCC. In such cases, treatment end points are difficult to define as *HBeAg* seroconversion cannot be used as a surrogate marker for cessation of virus replication. Many types of selections combine leading to complex combinations of mutations. Heavily mutated variants are found in HCC or late stage infection.[13] Synthesis of *HBeAg* is abolished by a point mutation at nucleotide 1896(A1896) ranging from G to A which leads to creation of a stop codon 28. Pre-core and core mutations occur in both, *HBeAg* positive as well as *HBeAg* negative hepatitis and the clinical significance has to be defined in the perspective of antiviral therapy.[15]

Pregnancy and HBV

In our country, sero-prevalance of HBV is found to be 0.9% among pregnant women. The risk of vertical transmission increases drastically when both high viral DNA level along with *HBeAg* positive status are present. Major route of transmission in our country are Vertical and horizontal transmission during early childhood and pregnancy.[12] Apart from other factors like DNA level, HBV structure, immune status of mother, placental barrier, genetic makeup of the new born, HLA-DRB1*07 is associated with susceptibility to intrauterine infection with HBV in infants.[16] An infant whose mother is both *HBsAg* and *HBeAg* positive and is unimmunized, has 70-90% chance of being *HBsAg* positive and upto 90% possibility of becoming a chronic carrier.[7]

Diagnosis of hepatitis B by serology. It is an important tool by which, the immunoassay of viral antigens and its corresponding antibodies is done.

Molecular methods

Backbone of hepatitis B diagnosis and its further management is detection and quantification of HBV DNA. Polymerase chain reaction is most preferred of all the types of molecular assays. It consists of extraction and purification of DNA which is amplified and then quantified. Evaluation of the relationship between HBV DNA level and hepatic pathology is the current hot spot in the in the diagnosis and treatment of HBV.[17] It is most important in the diagnosis of *HBeAg* negative variants. It is useful in deciding end point of treatment of CHB. However, for the diagnosis of drug resistant variants, if only serum DNA level is used for diagnosis, the selection of rtA181T/sW172* masks the diagnosis of resistance and hence HBV polymerase sequencing or line probe assay is additionally required to diagnose drug resistance in such cases.[18]

MATERIAL AND METHODS

After taking ethical clearance from the institution, a cross sectional study of 30 patients attending the OPD and IPD of Sri B M Patil Medical College Hospital and Research center in the period of January 2016 to June 2017 was undertaken. Both male as well as female patients with *HBsAg* positive status were included in study. Exclusion criteria were patients with co-existent HIV, HAV or HCV, patients with decompensated hepatic status, cirrhosis, hepatocellular carcinoma, those with impaired renal clearance, S creatinine>4mg/dl, those with severe malnutrition, auto-immune liver diseases and those who did not give consent. 5 ml of blood was withdrawn after informed written consent with full aseptic precautions.2ml was put in purple capped EDTA vial and 3 ml in red capped plain tube. After centrifugation, serum from the red capped vacutainer was tested for *HBsAg* by ELISA. After the carrier status was confirmed by this test, *HBeAg* was obtained by Automated Bi directionally Interphased Chemi Luminescent Immunoassay. Real Time Polymerase Chain Reaction was utilized for detection of HBV DNA. Results were expressed in IU/ml.

RESULTS

Study population included 30 chronic carriers of HBV who tested positive for *HBsAg* twice at an interval of minimum six months.

Age & Sex distribution

Age wise distribution shows that majority of the patients belong to age-group 20-30 years (46. 7%). Range of age is 20-75 years. Mean age is 34.03 .Males were 40% (12) and females were 60% (18).

Risk Factor Analysis

The factors taken into account were blood transfusion, surgery, other invasive procedures,

family history, frequent injections, tattooing and promiscuous behavior (life-style). Most frequent risk factor identified was therapeutic injections (36.7%) followed by family history(20%).

Serological profile

All *HBsAg* positive cases were included in this study. *HBeAg* positivity was found in 23.3% (7) patients. Most of the *HBeAg* positive patients belong to less than 40years age group. However, p value was not significant. This could be because of the small sample size. No significant association was found between *HBeAg* positivity and gender.

Table 1. Analysis of Risk Factors

Risk Factors	Patients	Percentage
Injections	11	36.7%
Family History	6	20%
Blood transfusion	1	3.3%
Life-Style	1	3.3%
Unknown	11	36.7%

Therapeutic injections were found to be the most common (36.7%). The other risk factors like positive family history was found in 20% cases, promiscuous life-style in 3.3%, blood transfusion in 3.3% cases.

Table 2. *HBeAg* Status of Patients

HBeAg status	Patients	Percentage
Positive	7	23.3
Negative	23	76.7
Total	30	100.0

23.3% patients were found to be HBeAg positive and 76.6% were HBeAg negative.

Table 3. Viral DNA Load of Patients

Viral DNA	Patients	Percentage
<200	3	10
201-2000	3	10
2001-20000	2	6.7
>20000	3	10
Below detectable	19	63.3
Total	30	100.0

63.3% (19) people had viral DNA below detectable levels. 10% (3) had < 200IU/ml. 10% (3) had between 201-2000 IU/ml. 6.7% (2) between 2001-20,000 IU/ml. 10% (3) > 20,000 IU/ml super carriers

HBV viral load profile. 63.3% (19) people had viral DNA below detectable levels.13.3% (4) had < 200 IU/ml. 10% (3) had between 201-2000 IU/ml. 6.7% (2) were between 2001-20,000 IU/ml.6.7% (2) were > 20,000 IU/ml.

Association of HBV DNA load and *HBeAg*. Out of 7 *HBeAg* positive cases, 43% (3) people had DNA> 20,000 IU/ml. 14% (1) had between 2000-20,000 IU/ml. 14% (1) had between 201-2000 IU /ml. 29% (2) had less than 200 IU/ml. Significant association was found with p value 0.001 between *HBeAg* and viral load.

Categories of chronic HBV carriers according to their serological and virological profile *HBeAg* negative patients were characterized into chronic inactive group which is further divided into simple and healthy carriers on the basis of their viral DNA load being more or less than 2000 IU/ml. Chronic inactive carriers- 82.6% (19) Healthy carriers-17.4% (4) Simple carriers-nil.

Exclusively on the basis of viral DNA: DNA below detectable level-60% (18)Less than 2000 IU/ml- 16.67% (5) 2000 IU/ml to 20,000 IU/ml- 13.33% (4) 20,000 IU/ml- 10% (3).

DISCUSSION

In our present study, 30 chronic carriers were enrolled. The study includes asymptomatic subjects who were detected during routine screening of blood donors, family contacts of HBV positive patients, during ante natal checkup and preoperative evaluation of patients. The duration of study was from January 2016 to June 2017. Quantitative estimation of HBV DNA was done by PCR assay and *HBsAg* and *HBeAg* were obtained from the laboratory. The results obtained were subjected to appropriate statistical analysis.

Profile of the study group

Age and gender The age of the patients in the present study ranged from 20-75 years with a mean age of 34.03 years. Most (46.7%) people belong to 20-30years age group. In present study, males were 12 (40%) and females were 18(60%). Most of the *HBeAg* positive patients fall in the age group of 20-40 years. Similar findings were reported by Dixit *et al* that *HBeAg* positive patients tend to be younger than *HBeAg* negative patients.[19] However, a study by Shakeri *et al* found the lowest prevalence of *HBeAg* in 35-40years age group.[20]

Risk factors: The detailed history taken revealed certain risk factors for the HBV positive status. Among them, therapeutic injections were found to be the most common(36.7%). The other risk factors like positive family history was found in 20% cases, promiscuous life-style in 3.3%, blood transfusion in 3.3% cases. (As shown in table I) Our findings are consistent with Shanmugan *et al*. at Chennai.[21] Our study had 12 women diagnosed to be *HBsAg* positive during early pregnancy or before that and were *HBsAg* positive after delivery. They were evaluated for *HBeAg* and viral DNA in their post-natal period. This specific group had 58.3%(7) women *HBeAg* positive and 41.7% (5) who were *HBeAg* negative. All *HBeAg* positive women had significant load of viral DNA. The specific groups of health care providers and I/V drug abusers were not found to be risk factors in our study. This may be due to small sample size.

Serological profile: The present study consists of chronic carriers of Hepatitis B virus. The serological marker *HBeAg* was obtained in all these patients. In this study, 23.3% patients were found to be *HBeAg* positive and 76.6% were *HBeAg* negative. (Table 2) Our findings are in consonance with those observed in the study by Shammugam *et al.*[21] Their study reported replicative carriers to be 23.4%.

Quantitative detection of HBV DNA: This was done in the plasma of all 30 patients by Polymerase Chain Reaction Assay. In the present study, the analysis of HBV DNA load showed that most(63.3%) were negative for viral DNA in their plasma, it was detected in varying amounts in the remaining 36.7%. In the study conducted by Hasan N K *et al*.[22] and Rabbi *et al*.[23] the results showed HBV DNA positivity in 44.8% and 40.2% respectively. Our findings are also in accordance with these studies. However, the results of studies by Behnava *et al.* [24] showed increased number which is in discordance with our results. In HBV DNA positive cases, the value ranged in between 50 IU/ml and 64,291,972.91 IU/ml(log 10^7).

Serological and virological profile

The level of HBV DNA was compared with serological profile *HBeAg* positive and *HBeAg* negative in our study. The results showed that 100% of *HBeAg* positive cases were also positive for HBV DNA and the value ranged from 66 IU / ml to 64,291,972.91 IU/ml and most of them had high viremic levels > 20,000 IU/ml. This is in accordance with the studies by Widita H *et al.*[25]done in Indonesia. Viral DNA level was negative in 83% of the patients with negative *HBeAg*. 17% had detectable level of DNA ranging from 50 IU/ml to 5544 IU/ml which is in the low viremic range. The results of various studies by Rabbi *et al.*[23] Hasan *et al.*[22] and Shammugham *et al.*[21] showed that the *HBeAg* negative group had DNA positive status in 31.5%, 7.6% and 7% respectively. There is association between *HBeAg* and viral DNA (p=0.001) in our study. Shamima Akhter *et al.*[26] observed a positive correlation among *HBeAg* and HBV DNA in chronic carriers, however some discordance was observed. Hence assessment of HBV DNA was also indicated. However, in a study by X Liu *et al* [17] *HBeAg* and HBV DNA were not associated (k=0.29) and in this study, 40.04% of the *HBeAg* negative patients showed HBV DNA replication. This could be related to the mutation in pre-C region. Thus, *HBeAg* is useful in diagnosis and treatment of Hepatitis B infection but it cannot replace HBV DNA specially when we consider *HBeAg* negative CHB patients. It can be used as a complementary test. HBV DNA quantitation by q PCR is a reliable, accurate & reproducible test which can be used to diagnose, understand the natural history and progression or regression of the disease and also actively guide and monitor the therapy.

CONCLUSION

Our country has a large pool of Hepatitis B patients and asymptomatic carriers are the main reservoir responsible for the transmission of infection in the community. This study was aimed at detecting *HBeAg* in these patients and correlating the information with their viral load. The purpose was to highlight that the seemingly benign carrier state may progress and has to be diagnosed, monitored and councelled regarding blood, semen and organ donation at the same time alleviating any undue anxiety caused by misinformation. Treatment is also to be started promptly when required. Our study revealed that majority were chronic inactive carriers who were healthy and had low risk of being infective as the viral load was below detectable level. However, it is not wise to consider them as potential donors for blood, semen or solid organs. The role of HBV

DNA PCR cannot be overemphasized. Monitoring and measurement of HBV DNA level of such individual is necessary. Pregnant women and lactating mothers are to be actively monitored and immunization for this group along with inclusion of Hepatitis B vaccination as Universal immunization is mandatory and it helps in reducing disease prevalence in community. The limitations of this study are that it is a single centric study with a small sample size. The markers that reflect the behavior of virus in the host are studied but the whole range of markers which reflect and quantitate the host response have not been included. Clinical parameters ALT,AST are not considered and there is lack of genotyping. We recommend a multi centric vertical study with large sample size.

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CONFLICT OF INTEREST

The authors declares that there is no conflict of interest.

AUTHORS' CONTRIBUTION

RB and PRS collected the cases after informed written consent. Designed the research methodology, analyzed the data wrote the manuscript.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in manuscript.

ETHICS STATEMENT

Institute Ethics Committee approved the study. The nature, methodology involved in the study were explained to the patient and informed consent was obtained. All information collected was kept confidential and patient was given full freedom to withdraw at any point during the study.

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