Section: Miscellaneous

Original Research Article

A STUDY OF VIRAL LOAD & GENOTYPES OF HEPATITIS B VIRUS AMONG SERO-POSITIVE PATIENTS AT TERTIARY CARE HOSPITAL OF SOUTH WEST BIHAR, INDIA

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Received *: 04/09/2024*
Received in revised form *: 26/10/2024*
Accepted *: 11/11/2024*

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DOI: 10.70034/ijmedph.2024.4.114

Source of Support: Nil,
Conflict of Interest: None declared

Int J Med Pub Health
2024; 14 (4); 604-609

ABSTRACT

Background: HBV genotyping is essential for understanding disease progression and guiding antiviral treatment, yet molecular epidemiological data on HBV is limited in this region. This study aims to support diagnostic advancements and personalized treatments by evaluating various HBV genotypes and immune responses. **Aim & Objectives:** The study's findings will reveal genotype prevalence and associated clinical and demographic factors, aiding in targeted preventive measures

Material & Methods: A prospective, cross-sectional study was conducted over 18 months (February 2021 to July 2022) at Narayana Medical College and Hospital, Jamuhar, Sasaram, Bihar, in collaboration with RMRIMS (ICMR), Patna, India.

Results: During the research period, out of 926 patients, 50 patients screened positive by RDT out of which 31 were males & 19 females. Viral Load was > 20,000IU/ml in 26 patients with a positive viral load which is 52% of the total positive for the virus. Maximum samples were positive for genotype D.

Conclusion: The current investigation identified the existence of two unique HBV genotypes, D and B, among the HBV seropositive individuals in Bihar. Notably, genotype D was shown to be the most frequent.

Keywords: Hepatitis B Virus, Genotype, Viral Load, National Viral Hepatitis Control Program.

INTRODUCTION

More than 200 crores individuals are susceptible for the hepatitis-B virus, resulting in over one million fatalities each year. This illness presents a significant threat to worldwide public health.[1,2] Hepatitis-B virus is now the 9th leading cause of death globally and the 5th most impactful infectious pathogen.[3] Chronic Hepatitis-B Virus infection presents diverse clinical outcomes, ranging from asymptomatic carrier states, chronic hepatitis to fibrosis, liver cirrhosis, and hepato-cellular carcinoma (HCC).[4] Those with chronic HBV face a 100-fold higher HCC risk, worsened by comorbidities like diabetes.[5]

Hepatitis-B Virus (HBV) varies by over 8% in genetic sequence across its 10 genotypes (A–J).[6] The geographic distribution of these genotypes greatly impacts infection progression, making HBV genotyping essential for evaluating disease progression and guiding antiviral therapy.[6,7]

The prevalence of surface antigen for HBV varies among the Indian population. There is limited understanding of epidemiology of Hepatitis-B virus at gene level in the area, despite Bihar having a significant prevalence of HBV-related liver

disease.[8] Previous research investigations have demonstrated a significant incidence of HBV infection in the region.[9] The co-evolution and migration of microorganisms with human populations is a well-recognized phenomenon, as proven by HBV. Although it is well acknowledged that some genotypes are associated with more severe sickness, it is unclear whether this is solely due to viral causes or whether the host immune system also contributes to the development of the disease.[10] This research may be beneficial in evaluating potential diagnostic methods and offering tailored patient therapy for individuals with different HBV genotypes and host immune responses.

MATERIALS AND METHODS

Study Design: In collaboration with RMIRMS (ICMR), Patna, Bihar, a prospective, cross-sectional study was carried out on patients with laboratory-confirmed hepatitis B virus from February 2021 to July 2022 in the Microbiology Department of Narayana Medical College & Hospital Sasaram, Bihar, India.

Inclusion Criteria

Inclusion criteria include patients who were reactive to anti-HBV antibodies.

Exclusion Criteria

Studies in non-representative populations, e.g., Epilepsy, Other malignancy, Therapy involving cytotoxic and bone marrow depressant & HCV & HIV positive patients

Data collection was started after obtaining clearance from the Institutional Ethical Committee IEC/IRB- No. NMCH/ IEC/ 2021/ 36

Laboratory Assay & Interventions

Collection and Storage of sample: A total of 926 individuals suspected of having HBV were subjected to venipuncture with aseptic measures in order to collect 10 ml of blood from each individual. The samples were held at temperatures of -20°C and -80°C, as specified in the Standard Operating Procedure.

Screening and Viral Load Detection of Samples: Screening of samples was done by Rapid card test (HEPACARD- Diagnostic Enterprises, Parwanoo, India).[11] Viral Load was detected by Viral Load Estimation by Truenat PCR (Molbio Diagnostics Pvt. Ltd., Goa, India).[12]

Total DNA extraction: Total viral DNA was extracted from whole blood, plasma, or serum using the QIAamp® DNA Mini and Blood Mini kits per manufacturer instructions.[13]

PCR of Products: Polymerase chain reaction was performed on a nested PCR machine (Veriti, Applied biosystems by Thermo Fischer Scientific) following protocol as in figure 1. Primers were synthesized from the Eurofins Genomics, Bengaluru, Karnataka, India. The primer sequences utilized for HBV genotyping through nested PCR Primer Sequencing, including their positions, specificity, and polarity, are detailed below [14]

First Gene Primer Sequence

P1b: 5" TCACCATATTCTTGGGAACAAGA 3" (nt 2823–2845, universal primer, sense codon)
S1-2: 5" CGAACCACTGAACAAATGGC 3" (nt 685–704, universal primer, antisense codon)

Second Gene Primer Sequence

Mix A

B2: 5" GGCTCMAGTTCMGGAACAGT 3" (nt 67–86, specific to types A to E, sense codon)
BA1R: 5" CTC GCG GAG ATT GAC GAG ATG T 3" (nt 113–134, specific to type A, antisense codon)
BB1R: 5" CAG GTT GGT GAG TGA CTG GAG A 3" (nt 324–345, specific to type B, antisense codon)
BC1R: 5" GGT CCT AGG AAT CCT GAT GTT G 3" (nt 165–186, specific to type C, antisense codon)

Mix B

BD1: 5" GCC AAC AAG GTA GGA GCT 3" (nt 2979–2996, specific to type D, sense codon)
BE1: 5" CAC CAG AAA TCC AGA TTG GGA CCA 3" (nt 2955–2978, specific to type E, sense codon)
BF1: 5" GYT ACG GTC CAG GGT TAC CA 3" (nt 3032–3051, specific to type F, sense codon)
B2R: 5" GGA GGC GGA TYT GCT GGC AA 3" (nt 3078–3097, specific to types D to F sense codon)

In the sequences, “M” represents a nucleotide that may be either A or C, while “Y” indicates a nucleotide that may be either C or T. "nt" refers to nucleotide.

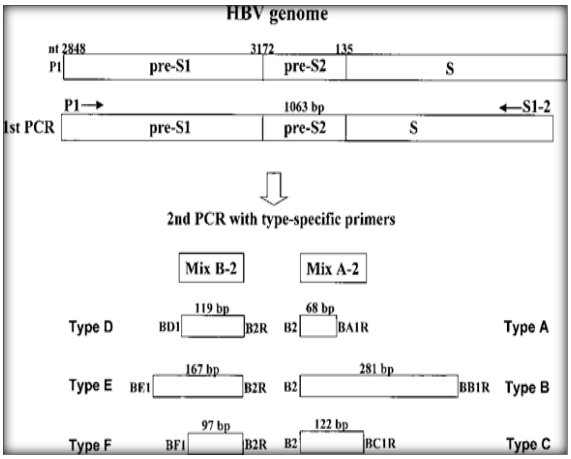

Figure 1: HBV genotyping protocol using type-specific PCR primers.[14]

Primers used were dissolved using sterilized double distilled water and TAE buffer, following the guidelines supplied by the manufacturer.[14]

The PCR cycle includes following steps as mentioned in the table 1: [Table 1]

Table 1: Steps of PCR Cycle

Step	Temperature	Duration
Initial Denaturation	95°C	3 minutes
Denaturation Cycle	95°C	30 seconds
Annealing Cycle	48°C	30 seconds
Extension Cycle	72°C	1 minute
Final Extension	72°C	5 minutes
Total Cycles	35 cycles	

Subsequently, the PCR result was subjected to electrophoresis using a 1.8% agarose gel supplemented with ethidium bromide. An unpurified PCR result was subjected to gel electrophoresis. The genotype was ascertained by measuring the size of the fragments using UV light in a gel documentation system manufactured by Bio-Rad.

The dimensions of the product bands were determined based on the migration pattern of the DNA ladder. The anticipated band sizes, as shown in Figure 2, were as follows: for mix A genotype, A (68 bp), B (281 bp), and C (122 bp); for mix B genotype, D (119 bp), E (167 bp), and F (97 bp).[14]

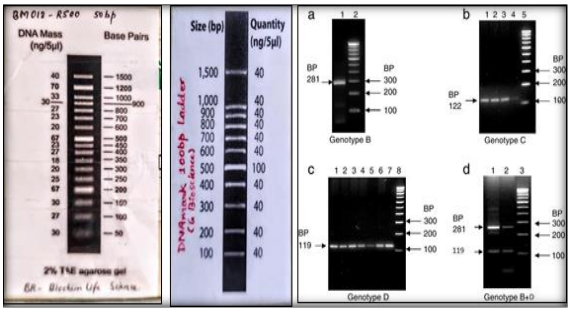

Figure 2: Agarose gel profiles of various HBV genotypes [14]

Statistical Analysis: Data was entered into an MS Excel spreadsheet, coded appropriately, and analyzed for errors using SPSS (Version 22.0). Tests were performed at a 95% significance level, considering a relationship significant if the p-value was below 0.05.

RESULTS

A total of 926 Patients samples, from various Departments, were analyzed in the Microbiology Department of Narayana Medical College & Hospital, Sasaram, Bihar, from February 2021 to July 2022. Out of these 926 samples, 50 (5.4%) samples screened positive for the Hepa Card test and 44 samples were confirmed positive for HBV DNA Viral-Load by Truenat PCR.

Table 2 shows the age & gender distribution of confirmed patients, out of a total of 44 confirmed patients, the majority 27 (61.37%) were males & out of those 5 (45.45%) were in the age group of 21-30 years, in females also majority 6 (54.55%) were in same age group. [Table 2]

Table 3 shows the HBV viral load distribution of confirmed patients. In a total of 50 patients, a maximum of 31 (62%) were males & out of those majority (18 =58%) had a viral load of >2x104 IU/ml.

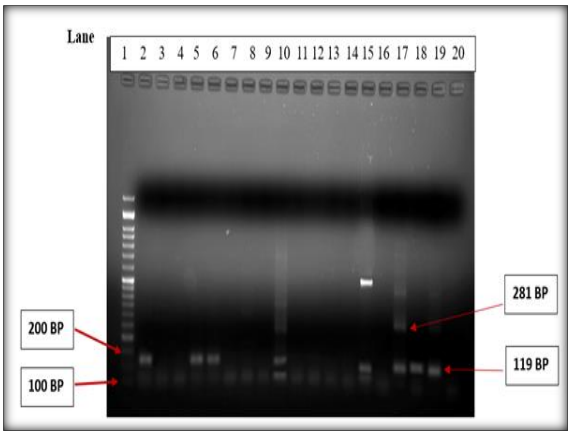

Figure 3: Profile of HBV genotypes on Agarose gel

A) Lane 1- 1 0bp DNA Ladder
B) Genotype D; lane 2,5,6 &18 (sample ID- HB1, HB4, HB5 & HB17)
C) Mix genotype- lane 10, 15, 17 & 19 (sample ID- HB9, HB14, HB16, HB18)

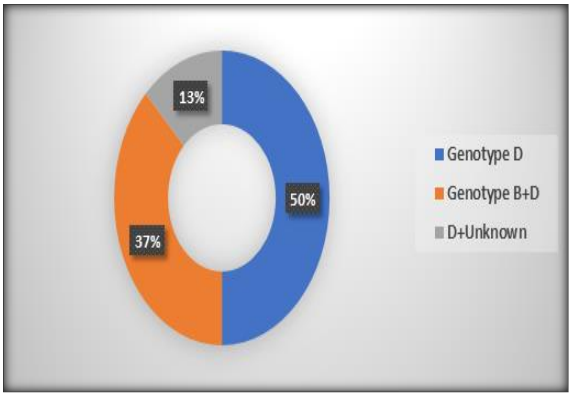

Figure 4 shows, major genotype isolated was Genotype D (50%) followed by Mixed genotype B+D (37%)

Table 2: Age and Gender distribution of confirmed patients (N=44)

Age Group in years	Males	Females	Total
0-20	2 (66.67%)	1 (33.33%)	3 (6.82%)
21-30	**5 (45.45%)**	**6 (54.55%)**	**11 (25%)**
31-40	3 (37.50%)	5 (62.50%)	8 (18.14%)
41-50	4 (66.67%)	2 (33.33%)	6 (13.64%)

51-60	4 (80.0%)	1 (20.0%)	5 (11.37%)
61-70	4 (66.67%)	2 (33.33%)	6 (13.64%)
71-80	**5 (100%)**	0	5 (11.37%)
Total	27 (61.37%)	17 (38.63%)	44 (100%)

Table 3: HBV Viral Load Distribution of Confirmed Patients (N=44)

Gender	>2 x 10^4 IU/ml	$2x10^3 – 2x10^4$ IU/ml	Viral Load <$2x10^3$ IU/ml	Not Detected	Total
Male	18	3	6	4	31
Female	8	4	5	2	19
Total	26	7	11	6	50

Table 4: Relation between Viral Load and Genotypes

S. No	Patients ID	Genotype Detected	Viral Load
1	HB 1	D	$3.01x10^5$
2	HB 4	D	$3.05x10^6$
3	HB 5	D	$4.52x10^6$
4	HB 9	B+D	$6.2x10^7$
5	HB 14	B+D	$1.4x10^8$
6	HB 16	B+D	$3.41x10^7$
7	HB 17	D	$4.21x10^7$
8	HB 18	B+D	$1.36x10^9$

Table 4 shows, that samples with high viral load are positive for genotype D, and mixed genotype samples show high levels of viral load.

DISCUSSION

HBV genotypes show geographical distribution and prevalence variations. Studying the genotypes prevalent in specific areas can provide insights into disease epidemiology and help tailor treatment strategies based on regional genotype patterns. The prevalence of HBV in various populations exhibits significant variation, ranging from 0.1% in wealthy countries to 20% in impoverished ones.[15]

The results of our analysis showed that 50 out of 926 people in this location had an overall prevalence of 5.4% for the HBV. The predicted carrier rate of HBV in India in 2018 was 4.7% on average.[16] Research conducted in Tamil Nadu, including both urban and rural populations, found that 5.7% of the participants tested positive for HBsAg, which is consistent with the findings of our own investigation.[17] Conversely, research conducted by Ghosh S et al. in West Bengal[18] and by Kumar A et al. in Kanpur[19] shown that the incidence of HBsAg positivity was 2.97% (227/7653) and 2.25% (450/20000) respectively, which was lower compared to our study. Research conducted by Arjun L et al. in 2017 in Patna, Bihar, revealed a prevalence rate of 34%, which is higher than the rate seen in our study.[7]

By comparing the genotype distribution in South West Bihar with national and global patterns, the study highlighted regional variations in hepatitis B virus genotypes. This comparison can offer valuable information for developing targeted interventions and adapting treatment protocols based on genotype-specific considerations.

The study shows participants' average age was 35.88 years (±14.73). This aligns with Arankalle VA et al.'s findings, which reported an average age of 39 years (32 to 46 years).[20] In contrast, Kumar A et al. found a higher mean age of 43.22 ±13.57.[19] Studies by Thakur V et al. and Chauhan R et al. reported lower mean ages of 25 and 27 years, respectively. [21,22] Notably, all these studies indicated a higher prevalence of males among participants.

This study highlights a concerning link between HBV seropositivity and age. Among men, infection rates peaked in the 21-30 and over-70 age groups, while for women, the 21-30 age group was most affected. This suggests higher HBV vulnerability in younger individuals in this area. Similar findings have been reported in other Indian studies, though some also noted increasing HBsAg positivity with age.[19-22]

Previous studies suggest that elevated HBV infection rates in older populations may result from birth cohort effects or iatrogenic factors, including the use of non-sterile instruments in vaccinations. Increased hepatitis B immunization in recent years has likely reduced infection rates. The high prevalence of HBsAg among older adults may reflect their lack of immunization. This study observed declining seropositivity among individuals over 45, possibly due to self-selection from chronic HBV infection.

The majority of patients in this research were proven to have Genotype D, which aligns with the findings of several earlier studies conducted in India.[22,23,24,25] This research also demonstrates the co-infection of two genotypes in a small number of samples.[22,26] These samples also exhibit a much higher viral load compared to the samples containing just a single genotype. The majority of research likewise demonstrate the same outcome.[27,28]

Studies on HBV viral load and genotypes reveal their crucial impact on disease severity and treatment response.[28] High viral loads heighten liver damage risk and chronic hepatitis B progression, while certain genotypes increase liver cancer likelihood.[29] Recognizing these relationships aids in developing targeted treatments and improving outcomes, as

distinct HBV genotypes also correlate with varying clinical effects, notably elevated viral loads.[30]

In addition, research has also highlighted the significance of early detection and monitoring of Hepatitis-B Virus infection, as timely intervention can prevent complications and improve long-term prognosis. One key aspect of managing HBV infection is regularly monitoring viral load levels in patients. By closely tracking viral load, healthcare providers can assess the effectiveness of treatment and make adjustments as needed to achieve viral suppression. Monitoring viral load can identify patients at risk for complications like liver damage or liver cancer. Regular monitoring also allows for early detection of any changes in viral load, which can prompt timely intervention to prevent disease progression. Overall, maintaining low viral load levels through regular monitoring is essential for optimizing treatment outcomes and improving long-term prognosis for patients with HBV infection.

The molecular mechanism behind the elevated HBV viral load in patients with mixed genotypes has been described in in vitro models, demonstrating that the genotypes interact to enhance their replication. [31,32]

This study revealed a strong correlation between individuals with very high Hepatitis-B Virus DNA levels (>2x10^4 IU/mL) and those with genotype D or co-infection of genotypes B+D. Co-infected patients demonstrated a higher viral load than those with genotype D alone. Lindh et al. reported similar results, particularly among patients who were HBeAg-negative.[33] In the HBeAg-positive control group, patients with genotype D showed higher HBV DNA levels compared to those with other genotypes. However, Ni et al. found no significant differences in initial viral load between genotypes B and C in 460 children with HBV.[34]

CONCLUSION

Limitations: Considering the small sample size, clinical nature of this study's methodology, and lack of long-term follow-up data, it is possible that the findings may not provide an accurate representation of the entire state of Bihar. Additionally, the study did not account for potential confounding variables such as comorbidities or medication adherence, which could affect treatment responses.

Recommendations for future research:

Conducting larger, multi-center studies to increase the sample size and diversity of participants, as well as including detailed information on patient characteristics and treatment regimens. Future research should investigate the long-term effects of HBV treatment on liver function and quality of life. Additionally, examining genetic factors influencing treatment response and disease progression may offer valuable insights for personalized medicine strategies in managing HBV infection. Overall, addressing these research gaps will be crucial in advancing our understanding of HBV infection and improving patient outcomes in the future.

Acknowledgment: The Authors are grateful to the Administration of Narayana Medical College & Hospital, Sasaram, Bihar for providing the infrastructure to conduct the study in the Department of Microbiology and to the Director of RMRIMS (ICMR), Agamkuan, Patna, Bihar, for the support in the technical guidance for Genotyping of HBV in the Department of Biochemistry of his esteemed institute.

Disclosures

Human subjects: Consent was obtained or waived by all participants in this study. NMCH Institutional Ethical Committee issued approval NMCH/IEC/2021/36.

Animal subjects: All authors have confirmed that this study did not involve animal subjects or tissue.

Declaration of interests: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the s financial interests/personal relationships which may be considered as potential competing interests: Dr. Rajan Pathak reports equipment, drugs, or supplies was provided by Rajendra Memorial Research Institute of Medical Sciences.

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