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Original Research Article

Study on occurrence of Hepatitis B virus Genotypes at a tertiary care hospital in Jaipur

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ABSTRACT

Introduction: The hepatitis B virus's (HBV) capability to mutate genetically poses a threat to the sensitivity of serological and genomic diagnostics. As a result the knowledge about the distribution of HBV genotypes (GENs) and mutation has a significant impact on treatment plans, immunisation campaigns, treatment, and prophylaxis of HBV.

Aim and Objectives: The objective of our study was to identify HBV Genotypes in HBsAg-positive individuals.

Materials and Methods: The study was conducted on 117 HBsAg-positive patients who showed the presence of the S gene were subjected to DNA extraction. The extracted DNA was amplified by Nested PCR (Polymerase Chain Reaction) followed by RFLP (Restriction fragment length polymorphism).

Results: Out of 117 patients, 84% were genotype D and 16 % were genotype A. More patients of Genotype A had a fever and Jaundice (15.7%; 21 vs 5%; 13.2%) as compared to Genotype D. In this study 19.6% of patients were in the immune clearance phase, 18% in the inactive carrier, 6% in immune tolerance phase.

Conclusion: A better understanding of the prevalence, diagnostic, and serologic patterns of hepatitis B in this region can aid practitioners in recommending appropriate antivirals by genetic data.

1. Introduction

Hepatitis B Virus (HBV) infection is a major public health problem in India causing both chronic and acute hepatitis. HBV replicates by reverse transcription of an RNA intermediate in a living cell. This makes this hepatitis virus prone to mutation so that the genome of HBV exhibits high genetic variability with an estimated nucleotide replacement rate of $1.4 – 3.2 \times 10^5$ nucleotide substitutions per site per year. This resulted in so many subtypes and genotypes of the virus.[1]

There is an estimation by WHO that 296 million people are living with chronic hepatitis B infection in 2019 and 1.5 million new infections each year which leads to an estimated 820 000 deaths, mostly from cirrhosis and hepatocellular carcinoma.[2]

Hepatitis B virus has been classified into various genotypes from A to I based on an inter-group divergence of 8% or more in the complete genomic sequence, each genotype having a distinct geographical distribution.[3]

In all of the genotypes, Genotype A can be most commonly found in Northern Europe, North America and Central Africa, while Genotype B predominates in Asia.[3] another genotype, Genotype C is found in the Far East in Korea, China, Japan and Vietnam as well as the Pacific rim and Island Countries, while genotype D, which is also more or less pandemic, is found in

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the Mediterranean countries, the Middle East extending to India, North America and parts of the Asia-Pacific region. [1,4]

There are very few studies about the genotype of HBV from India. Genotypes influence the HBeAg sero-conversion rate, mutational patterns in the pre-core and core promoter regions, and the severity of liver disease. [1]

However, HBV genotyping remains a research tool and further studies are needed before it is included in clinical evaluation and treatment of Chronic Hepatitis B (CHB) patients. Though direct sequencing is the gold standard but the process is time-consuming, expensive and cumbersome. Moreover, sequencing has to be done on a large number of samples in batches. In this study, we used Restriction fragment length polymorphism(RFLP) which is done on conserved S gene and has been found to give comparable results with sequencing for genotyping but is much cheaper and easier to perform as compared to sequencing. [3,4]

The RFLP analysis methods have provided powerful newer approaches for eliciting the genetic differences among species, subspecies, various strains and genotypes in patients of chronic hepatitis patients. In our study, we have used RFLP for genotyping. Only limited data is available from India about HBV genotypes and none at all from Rajasthan. We studied the occurrence of HBV genotypes in known HBsAg-positive patients.

2. Materials and Methods

This retrospective study was conducted at the Advance Basic Science & Clinical Research laboratory, Department of Microbiology & Immunology of a tertiary care hospital in Jaipur for one year. All the Hepatitis B surface antigen positive (HBsAg) patients with positive PCR of the S gene were included in this study. Patients with other co-infections like HIV and HCV and known HIV positives were excluded. Ethical approval and consent of participation had been taken.

The sample size was estimated with the assumption of prevalence of HBV to be 3.7%.[2] (With 4% error and 95% confidence limit) 117HBsAg-positive patients were included after informed consent Clinical, biochemical and serological parameters were noted down.

Method of genotyping adopted from Zeng et al 2004. [4]

Blood was collected in an EDTA vial and plasma was separated. DNA extraction was done using a Roche High pure viral nucleic acid extraction kit.HBV Viral load was done by RT PCR (COBAS TaqMan 48 analyzer). Amplification of S gene sequence was done by nested PCR using inner (YS1 & YS2) and Outer primers (PrsS2 & SIR).After amplification, the products were checked on Gel electrophoresis. Gel-positive PCR products were digested by 5 enzymes Dpn I, StyI, HpaII, EaeI and BsrI RFLP pattern was studied by electrophoresis on 3% gel which determined the type of genotype from A to H

Association of genotypes with severity of disease. The association of the severity of liver disease with genotype was studied based on various criteria as given below. [5,6]

1. ALT level
   (a) ALT > 60 IU/ml [above 1.5 times the upper limit of normal]
   (b) ALT < 60 IU/ml [below 1.5 times the upper limit of normal]
2. HBeAg
   (a) HBeAg Positive
   (b) HBeAg Negative
3. HBV DNA level
   (a) HBV DNA $>10^4$ IU/ml
   (b) HBV DNA $<10^4$ IU/ml

2.1. Specimen collection and processing

Blood was collected in an EDTA vial. Plasma was separated and stored at -20°C. DNA extraction was done using a Roche High pure viral nucleic acid extraction kit. Before starting DNA extraction the working solution was made as follows;

The Real-Time PCR (Roche) was done to estimate viral load.50μl master mix solution added to 50ul extracted DNA and loaded onto RT PCR (COBAS TaqMan 48 analyzer) Linear range of test 6IU/ml to $1.1x10^8$IU/ml (The titer between 6IU/ml and 29IU/ml have a high degree of variability)

2.2. Genotyping

The extracted DNA was amplified by Nested PCR.

2.2.1. Nested PCR

Following primers were used to amplify the sequence between nt 203 to nt 787, yielding an Amplicons of 585bp.

The outer primers were;

PrsS2 (sense, nt 2 820-2 837, 5'-GGGACACCATATTCTTGG) and

S1R (antisense, nt 842-821, 5'-TTAGGGTTTAAATGTATACCCA).

The inner primers were;

YS1 (sense, nt 203-221, 5'-GCGGGGTTTTTCTTGTTGA) and

YS2 (antisense, nt 787-767, 5'-GGGACTCAAGATGTTGTACAG).

5 μL of the extracted DNA was added to a 13 μL amplification mixture (Ready PCR Mix- Bangalore genie) containing Taq polymerase buffer, deoxyribonucleotide triphosphates and Taq polymerase. 10 pmoL each of primers PrsS2 and S1R were added (total volume of 25 μL).The PCR cycling conditions were an initial 3 min denaturation

at 94^{o}C, followed by 35 cycles of amplification including denaturation for 45 s at 94^{o}C, annealing for 60 s at 53^{o}C, and extension for 90 s at 72^{o}C. Strand synthesis was completed at 72^{o}C for 6 min. A 2 μL of the first-round PCR products was then used for the second-round PCR under the same conditions but with the primers YS1 and YS2.

2.3. Restriction of amplicons

10 μL of the second-round PCR products were mixed with 0.5 μL (5U) of the chosen restriction enzyme (New England Biolabs), 1.5 μL of 10× buffer and 3.0 μL of water. Tubes were centrifuged for 30 seconds and incubated at 37^{o}C for 4 hr. After incubation, inactivate the enzymes by heat. After that the samples were electrophoresed on a 3% Agarose gel for 4 hr. 15 μL restricted product was loaded with 4 μL dye. A 10 μL of undigested second-round PCR products with 3 μL dye was run in parallel with the enzyme-digested samples. Gel electrophoresis was done at 50 volts for 4 hrs. The restriction patterns were analysed using the Gel Documentation System (BioRad). The numbers of bands were calculated by comparing them with a 100bp DNA ladder.

2.4. Identification of genotypes

Following the alignment of S gene sequences, five restriction enzymes, StyI, BsrI, DpnI, HpaII and EaeI were used to yield restriction patterns that would identify all possible HBV genotypes prevalent in our patients.

Genotyping was done on the basis of restriction patterns observed after digesting the 585 bp PCR product with different restriction enzymes.

2.5. Statistical analysis

Data analysis was carried out using the SPSS version 10.0 software packages for Windows.

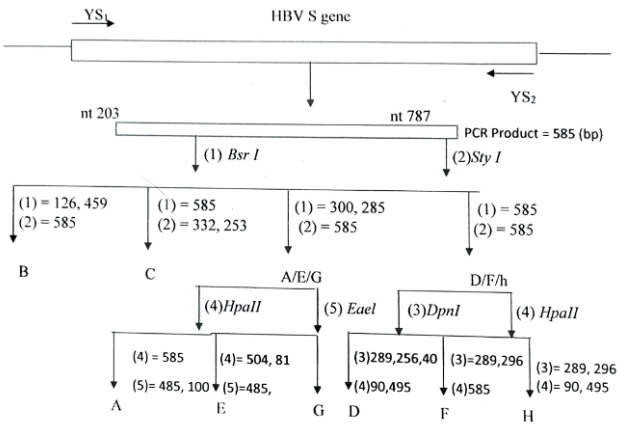

Figure 1: Following chart was used for the identification of genotypes

3. Result

The present study was conducted on patients reporting at the Advance research lab with varying clinical signs and symptoms for HBV viral load testing. Total of 117 patients were included in the study and processed for HBV genotyping.

In our study 93 were males and 24 were females belonging to various age groups in which 90 patients were young adults (16-50 yrs) and 20 patients were from elderly groups (more than 51 yrs).

On processing sample by different restriction enzymes, the band pattern obtained corresponding to different genotypes were as follows; in 98/ 117 (84%) samples band sizes corresponded to genotype D (BsrI -585 bp, StyI- 585 bp, DpnI- 289 bp, 256bp, 40bp, HpaII- 495bp, 90bp) and in 19/171(16%) samples bands corresponded to genotype A(BsrI- 300bp, 285bp , StyI – 585, HpaII- 585, EaeI- 485bp,100bp).

In this study 76% of patients were adults (age group 16-50 yrs) among genotypes D and 84% in genotype A .X^2 0.028 with 1df, P value > 0.055 not significant.(Table 2)

Out of 117 patients studied, 93 (80%) were males and 24 (20%) were females. Maximum patients were males in both genotypes. (80.6% and 73.6%) X^2 0.14 with 1df, P value (> 0.055).(Table 3)

Maximum patients were from Jaipur 64(54.7% %), followed by 10 (8.5%) patients from Bharatpur and 7(5.9%) from Alwar. Patients were from various districts of Rajasthan and nearby Rajasthan.(Table 4)

Maximum numbers of patients were asymptomatic among genotypes, A (73.6%) and D (72.4%). Higher numbers of patients in genotype A had jaundice (21%) and fever (15.7%) in comparison to genotype D (13.2%, and 5%). (Patients had more than one signs/symptoms).(Table 5)

No significant difference was found in ALT levels (which indicate the severity of liver disease) among both genotypes. X^2 =0.003 with 1df (P value > 0.055).(Table 6)

HBeAg positivity (indicates severity) was slightly higher in genotype A (37% vs 32.6%) but was not statistically significant. X^2 =0.008 with 1df P value >0.055.(Table 7)

HBV DNA was slightly higher in genotype A in comparison to genotype D but was not significant. X^2 0.089 with 1df,(P value >0.055).(Table 8)

66 patients could not be differentiated as reactivation or HBeAg negative hepatitis as details of the patients, and follow-up data was not available.(Table 9)

No significant difference was detected in various phases of HBV in correlation to genotypes A and D. X^2 0.323 with 3df (P value> 0.055)

4. Discussion

Genetic classification of HBV based on the comparison of complete HBV genomes has identified eight genotypes;

Table 1: List of enzymes

Restriction Enzyme	Recognition site	Inactivation
Sty I	5' C^CWWGG' 3 3' GGWWC^C' 5	Incubation at 65^0C for 20 min.
Bsr I	5' ACTGGN^' 3 3' TGAC^CN ' 5	Incubation at 80^0C for 20 min
Eae I	5' Y^GGCCR ' 3 3' RCCGG^Y ' 5	Incubation at 80^0C for 20 min
Dpn I	5' GA^TC ' 3 3' CT^AG ' 5	Incubation at 80^0C for 20 min
Hpa II	5' C^CGG ' 3 3' GGC^C ' 5	Incubation at 65^0C for 20 min.

Table 2: Age-wise distribution of genotypes in the study

Age group	Genotype D (84%) n=98	Genotype A (16%) (n=19)	Total (n=117)	P value
0-15 yr	7(7%)	-	7(6%)	
16-50 yr	74(76%)	16(84%)	90(77%)	0.867
>51 yr	17(17%)	3(16%)	20(17%)	
Total	98	19	117	

Table 3: Sex-wise distribution of genotypes

	Genotype D (n=98)	Genotype A (n=19)	Total (n=117)	P value
Male	79(80.6%)	14(73.6%)	93(80%)	
Female	19(19.4%)	5(26.3%)	24(20%)	0.708
Total	98	19	117	

Table 4: Distribution of genotype in different districts of Rajasthan

Place	Total (n=117)	Genotype D (n=98)	Genotype A (n=19)
Ajmer	3(2.5%)	2(2%)	1(5%)
Alwar	7(6%)	7(7%)	-
Bharatpur	10(8.5%)	8(8%)	2(10.5%)
Churu	2(1.7%)	2(2%)	-
Dholpur	2(1.7%)	2(2%)	
Dausa	1(0.8%)	1(1%)	
Gangapur city	4(3.4%)	3(3%)	1(5%)
Hanumangarh	1(0.8%)	1(1%)	
Jaipur	64(54.7%)	51(52%)	13(68%)
Jhalawar	2(1.7%)	2(2%)	
Jodhpur	2(1.7%)	2(2%)	
Karoli	1(0.8%)	1(1%)	
Kota	1(0.8%)	1(1%)	
SawaiMadhopur	3(2.5%)	3(3%)	
Nagaur	2(1.7%)	2(2%)	
Sikar	4(3.4%)	4(4%)	
Other	8(6.8%)	6(6%)	2(10.5%)
Total	117	98 (84%)	19 (16%)

Table 5: Signs and symptoms observed in both genotypes

Symptoms	D Genotype (n=98)	A Genotype (n=19)	Total (n=117)
Asymptomatic	71(72.4%)	14(73.6%)	85(73%)
Fever	5(5%)	3(15.7%)	6%)
Jaundice	13(13.2%)	4(21%)	16(14%)
Nausea, vomiting, malaise	19(19.3%)	3(15.8%)	22(19%)

Table 6: Association of alt levels with genotypes

	Genotype D (N=98)	Genotype A (N=19)	P
ALT >60	45(46%)	8(42%)	
ALT <60	53(54%)	11(58%)	0.957
Total	98	19	

Table 7: Association of HbeAg with genotypes

	Genotype D N=98	Genotype A N=19	P
HBeAg +	32(32.6%)	7(37%)	
HBeAg -	66(67.3%)	12(63%)	0.929
Total	98	19	

Table 8: Association of HBV DNA levels with genotypes

	Genotype D N=98	Genotype A N=19	P
HBV DNA $>10^4$	50(51%)	11(57%)	
HBV DNA $<10^4$	48(49%)	8(42%)	0.766
Total	98	19	

Table 9: Various phases of HBV according to immune-pathogenesis

Phase	D N=98 (84%)	A N=19 (16%)	Total	P value
Immune tolerance HBeAg+, DNA$>10^3$,ALT<60IU/ml	6(6.1%)	1(5.2%)	7(6%)	
Immune clearance HBeAg+, DNA $>10^3$, ALT>60IU/ml	20(20.4%)	3(15.7%)	23(19.6%)	0.955
Inactive carrier HBeAg-,DNA$<10^3$, ALT<60IU/ml	17(17.3%)	4(21%)	21(18%)	
*Others (eg. reactivation/ HBeAg negative hepatitis,)	55(56%)	11(57.8%)	66(56.4%)	
Total	98	19	117	

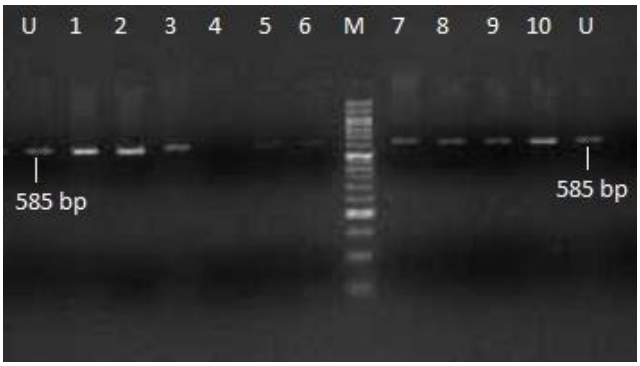

Figure 2: BsrI and Sty I digested 585 bp PCR product of HBV DNA. Lane 1-6= Bsr I digested 585 bp PCR product, Lane 7-10= Sty I digested 585 bp PCR product. For Genotype D M=molecular weight marker, U= undigested 585 bp PCR product.

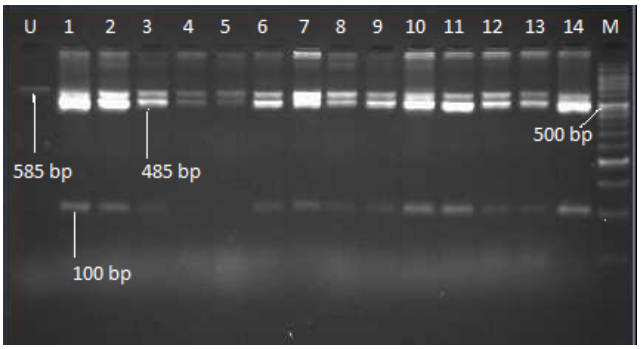

Figure 3: EaeI digested 585 bp PCR product of HBV DNA. Lane 1-14= partially digested 585 bp PCR product by RE Eae I Genotype D and A. molecular weight marker, U undigested 585 bp PCR product.

from A through H. Hepatitis B Virus genotypes have a distinct geographical distribution. The global distribution of HBV genotype is related to mode of transmission, racial or ethnic differences, environmental aspect or all of these factors.[7] There are many studies from various parts of the world about HBV genotypes. The distribution of HBV.

Genotypes of HBV vary geographically. Genotypes also influence the HBeAg sero-conversion rate, mutational patterns in the pre-core and core promoter regions, and the severity of liver disease.[1]

In our study out of 117 patients studied, 80% were males and 20% were females. The prevalence of HBV has been reported to be significantly higher in males than females, similar findings have been reported from India; 88% males and 12% females Delhi,[5] 77.8% males and 22% females from South India[6] Pakistan; 69.1% males and 30.9% females[7] and Afghanistan 78% males and 22% were female.[8]

This could be due to a higher prevalence of HBV infection in males as they are more exposed to the risk factors. In India, males are more educated and work outside their homes or in agricultural lands while women are mostly involved in household activities due to cultural preferences. Moreover, males are more commonly involved in practices leading to transmission of infection like barber visits and

multiple sex partners making them more prone to get the infection.

In our study, 77% of patients were in the 16-50 year age group. Similarly, various studies have reported that adult men or older were most commonly affected by HBV.[7–9] Maximum numbers of patients (54.7%) were from Jaipur district which could be because our lab was in Jaipur, moreover, the population of Jaipur is 5- 10 times more than that of other districts. In the present study, 117 samples were analysed for genotyping, and only two genotypes A & D were found in patients attending a tertiary care centre at Jaipur. Genotype D was the predominant type i.e. 84% and genotype A was 16%. Similar findings have been reported from other parts of India also, Saket et al from Delhi reported 80% genotype D and 17 % genotype A.[5]

Another investigator from Pune reported 91% Genotype D and 8% genotype A[40].[10] A study from Andaman Nicobar reported 91% type D and 8.6% type A.[11] While Thakur et al from Delhi reported a higher prevalence of genotype A than other studies (D63%, A37%.[12] On the other hand, study from the Andaman and Nicobar Islands, only genotype D was detected among three different primitive tribes (the Onge, the Andamanese, and the Nicobarese) and its introduction from the people of mainland India was suggested.[11] Similarly, other studies from India have reported a high prevalence of genotype A from other parts of India.

4.1. Global distribution of genotypes

In Bangladesh, the predominant genotypes are D and C, and Genotype D is considered to be the genotype originally present in the population of the Indian sub-continent and the high prevalence of genotype C in the Bangladeshi population. Genotype C is predominantly found in the Far East and Southeast Asia; their traditional links with the Mongolian ethnic group may explain this finding.[13]

Similar to Bangladesh, studies from Afghanistan and Middle Eastern Asia also reported genotypes D and C to be the most common.[14] Studies from Sri Lanka reported that the most common genotype was B(36%) followed by C(16%), D (12%), A(8%) and E (4%). Similarly, data from South India also report three genotypes D, A and C.[15]

Similar to our study, British Columbia also reported genotype D to be most common followed by genotype A but they also found some cases of genotype C, B and E.[16] Genotypes A and D are more common in Western Europe and North America. In the Mediterranean region, the Middle East and Central Asia, genotype D is dominant. HBV strains in India may therefore have originated from those in Europe or vice versa. Genetic studies of mitochondrial DNA and Y chromosome in the Indian population attest to the presence of significant European admixture, which is more marked in northern than in southern India. HBV genotypes have distinct geographical distributions. Genotype A prevails in North Western Europe, North America,

Sub-Saharan Africa, India and the United States,[17] while B and C are frequent in South Eastern Asia, China, Japan and Oceania.[18,19]

These findings are of great significance from the perspective of changing scenarios of HBV epidemiology. This raises the possibility that the Indian population originally had HBV genotype D, which has been partially replaced by genotype A, particularly in northern India, due to human migration from Europe over time. (Genotype A prevalent in Europe) HBV genotypes may be also influenced by racial background, and genotypes A and D are found in Caucasians and Europeans, but genotypes B and C occur in Asian HBV-infected patients.[20,21]

According to archaeological and anthropological findings, the ancestors of Caucasians (Arians) first colonized the North of the Caspian Sea. Because of difficulties in agriculture and climate change, they migrated in three directions: one group moved west towards Europe, another group moved south to Iran (and established the ancient Persian Empire) and the last group migrated to India. It might be that those people, who acquired the virus with the genotype D before their migration, then transmitted the virus generation by generation after their migration. This is why the dominant genotype in India, Iran and most of Europe is D.[22]

Epidemiological data about HBV genotypes in various Asian countries revealed the presence of all eight genotypes in Asia, particularly the predominance of genotype D.[23]

4.2. Association of genotype with disease severity

In the present study, patients were divided into groups based on ALT levels more than and less than 60, and the presence or absence of HBeAg and HBV DNA levels more than or less than 10^4 according to the severity of the disease. Higher HBe antigen positivity (37% vs 32.6%) and higher viral load (57% vs 51%) were found slightly more in Genotype A patients than in Genotype D but were not statistically significant. A higher number of patients with Genotype A patients had fever and Jaundice (15.7%; 21% vs 5%; 13.2%). However, ALT levels were slightly higher in Genotype D than in A (46 % vs 42%) patients but were not statistically significant.

On analysing the data as per HBV immuno-pathogenesis 19.6% of patients were in the immune clearance phase, 18% were inactive carriers, 6% were in immune tolerance and the rest of the patients could not categorised due to lack of follow-up data on Hbe Ag and ALT levels. In patients of immune clearance genotype D was predominant while in patients of immune tolerance and inactive carrier, both genotypes were equally represented. (P value not significant)

Similar to our findings, two studies from New Delhi also reported genotypes D and A with no significant

difference in clinical, biochemical, histological appearance and therapeutic response.[4,23]

A study from Lucknow reported that genotype A is more often associated with increased ALT, core antigen positivity - and cirrhosis of the liver as compared to genotype D.[13]

Similarly, the rate of HBsAg clearance was higher in genotype A than in genotype D patients and similar results were reported in a recent study from China. On correlating various epidemiological, virological and clinical variables and Hepatitis B genotypes in China it was observed that patients with genotype B were younger than those with genotype C and had a lower prevalence of HBeAg.[4]

Several other studies have reported a correlation between HBV genotype and HBeAg clearance. In these studies, all Asian patients found that the prevalence of HBeAg was higher in patients with genotype C compared to those with genotype B.[24,25]

Similarly, Sumi et al conducted a large cross-sectional study in Japan involving 585 patients with Hepatitis B and showed that the HBeAg-sero-conversion rate in patients with chronic liver disease was significantly higher in genotype B patients compared to genotype C.[26]

Many studies highlight differences in disease severity of genotypes B and C but variable reports are available about the correlation of disease severity in Genotype A and D. In our study genotypes B and C were not found so could not correlate them with disease severity**.** We found only Genotypes A and D while in another area where Genotypes C and B were prevalent investigator noticed different finding

There are some limitations of RFLP it requires a good quality sample, the procedure is

lengthy and laborious, and if DNA is not cut properly, it gives a nonspecific band. Moreover, it is based on only a part of, and not the entire, HBV genome. However, this RFLP method is reliable for HBV genotyping in comparison to sequencing and phylogenetic analysis for genotyping.[4]

Different techniques have been used by various authors, Line probe assay, PCR TFLP using different sets of enzymes, genotype-specific primer PCR RFLP, TSP PCR, ELISA etc. However, most of them have been confirmed with sequencing. However, the variety of techniques could cause some variations in results which should be kept in mind while interpreting the data worldwide.

Our study has some limitations. These include its selection bias as only those patients who reported at the Advance Research laboratory for viral load testing were included in the study as a result mainly patients from Jaipur were enrolled and low numbers from other districts. We used a method based on only a part of, and not the entire, HBV genome. A long term study should be done with proper follow-up in terms to ALT levels, HBeAg, and HBV viral load testing, and liver biopsy proper categorization and detecting seroconversion reactivation etc, for proper correlation of genotype with severity of liver disease.

5. Conclusion

In our study Genotype D (84%) was predominant followed by genotype type A (16%) with a higher number of patients with Genotype A having a fever and Jaundice (15.7%; 21 vs 5%; 13.2%) as compared to genotype D. This study has some limitations like the study population is hospital-based, the method used is based on only a part of the genome. Long-term study should be needed.

6. Ethical Approval

32352 Dated 5/9/21

7. Source of Funding

None.

8. Conflict of interest

None.

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