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Detection of Hepatitis B Genotypes in HBsAg & HBeAg Carriers

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ABSTRACT:

Objective: Hepatitis B is noteworthy medical issues that may include the late continuation of liver cirrhosis and hepatocellular carcinoma. The present study aimed for the detection and diffrentiation of Hepatitis B virus HBsAg inactive non-replicative carriers, HBeAg-positive inactive replicative carriers, active carriers & HBeAg-negative chronic hepatitis B by Real Time PCR and their genotyping

Methods: This research conducted on 245 positive for HBsAg, 118 (48.16 %) were male and 127 (51.84%) were female patients, which was performed in central research station labortory of Microbiology at netaji subhash Chandra Bose subharti Medical College and Hospital, Meerut Between November 2016 to April 2018. The sera were separated and screened for HBsAg by ELISA kit. Positive samples for HBsAg were tested for HBeAg ELISA kit and DNA Viral load then sequenced for genotying

Results: Of the 245 HBsAg Positive case 55 (1.12%) were HBeAg positive. In 16 PCR positive and HBV genotyping, In HBsAg inactive Non-Replicative 37.5% (n=6) genotype-B and 6.25% (n=1) genotype-A, In HBeAg inactive Replicative 12.5% (n=2) genotype-B and 12.5% (n=2) genotype-A and In HBeAg Active Chronic Hepatitis B 18.75% (n=3) genotype-B and 12.5% (n=2) genotype-A were detected

Conclusions: Management strategy, using HBsAg, HBeAg and HBV DNA viral load, seems adequate for the confirmation and diffrentiation of Hepatitis B virus inactive, active carriers & HBeAg-negative chronic hepatitis B patients and genotype B was more prevalent in comparission to genotype A. Distribution of carriers & genotypes, help physicians to prescribe proper antiviral/interferon therapy according to current genotyping pattern in this region

Keywords: Hepatitis B virus, Carrier State, HBsAg, HBeAg, RT-PCR

INTRODUCTION

The human hepatitis B virus (HBV) is a small-enveloped DNA virus causing acute and chronic hepatitis. Notwithstanding the availability of a secure and safe vaccine, HBVIstill represents a first-rate global fitness burden, with approximately 350-400 million human beings chronically infected global and about 6 lakh deaths in step with 12 months

because of HBV-associated liver pathologies **[1]**. Between 15 and 40% of chronically infected people may increase intense liver disease and hepatocellular carcinoma (HCC), whilst the closing turn out to be inactive carriers. **[2]**. Hepatitis B carriers are about 5% of the total world's population, defined as being positive for HBsAg. HBV is endemic in lots of areas of the arena, which consist of Asia, Micronesia, and sub-Saharan Africa in addition to in a few populations in Australia, New Zealand, South the United States, the center east and the Arctic. An anticipated 1.25 million human beings in the US are HBsAg Positive (HBsAg-P). Fifteen % to 40 % of those carriers may expand hepatitis B-related sequelae of their lifetimes **[3-5]**.

HBV is present in blood, semen, saliva, vaginal secretions and menstrual blood of infected people and without issue transmitted thru touch with infected body fluids **[6]**. Perinatal vertical transmission is the most common mode of transmission worldwide **[7]**. In families of a chronically infected man or woman, HBV infection (HBVI) can arise via man or woman-to-man or woman, nonsexual contact **[8]**.Patients with HBeAg chronic hepatitis B (CHB) generally present inside the 1/3 or fourth decade of lifestyles. Liver damage levels from moderate (24 to 42%) to slight or extreme chronic hepatitis (50 to 65 %) or active cirrhosis (10 to 24%) **[9-12]**. CHB has an inclination to be milder in youngsters. Notwithstanding the reality that, intense liver disease which includes cirrhosis may also rise up in a small percentage of patients at some point of early life **[13]**. A key occasion within the natural history of HBeAg CHB is HBeAg seroconversion. Numerous studies have tested that seroconversion with marked reduction of HBV replication is related to biochemical and histologic remission of inflammatory pastime in most people of sufferers. Regression of fibrosis happens step by step months to years after HBeAg seroconversion. In longitudinal studies the determined threat of clearing HBeAg modified into about 50% and 70% within five and 10 years of analysis, respectively **[14-16]**. maximum research have discovered that the mean annual rate of spontaneous HBeAg seroconversion levels from 9 to 16 % in children or adults with increased ALT. amongst Asian, most of whom have regular ALT, spontaneous HBeAg seroconversion takes place at a completely low rate, much less than 2% inside the route of the number one to three years of age and 4 to 5 % in children older than 3 years **[17]**. Numerous determinants for HBeAg seroconversion had been recommended, along side gender, age, ALT stage, and greater currently HBV genotypes. Older patients and ladies are much more likely to clean HBeAg **[18]**. After HBeAg seroclearance, disorder states, no longer always static, are feasible: a few patients continue to be in an inactive carriers (IC) state, defined via the use of the European Association for the Study of the Liver (EASL) as fulfilling the following standards: 1) HBeAg negativity; 2) anti-HBe positivity; 3) consistently regular ALT ranges (<40 IU/mL, with measurements at the least every 3-4 months during 1 year); 4) serum HBV DNA tiers <2000 IU/mL.

Others evolve to HBeAg-negative (HBeAg-N) CHB, typically related to precore or basal core promoter mutant HBV, within which infectious agent replication and hepatic inflammation persist despite seroconversion to anti-HBe standing. it's characterised by unstable altitude of ALT and serum HBV deoxyribonucleic acid levels that may drop below IC cut-off levels, despite chronic infectious agent replication and hepatic inflammation. In the workshop 2000, management of HBV, and arbitrary level of 20,000 IU/mL serum HBV deoxyribonucleic acid cut-off was adopted to distinctive active and inactive chronic liver disease **[19]**. EASL told that there will be inactive HBV carriers with deoxyribonucleic acid levels between 2000 and 20,000 IU/mL. HBV deoxyribonucleic acid levels in HBeAg-N CHB can be fluctuate

from >2,000,000 IU/mL to undetectable **[20]** a few inactive groups once in a while have HBV DNA ranges between 2000 and 20,000 IU/mL, a HBV deoxyribonucleic acid stage amongst twenty00 and 20,000 IU/mL looks to be a ‘‘grey area’’ which may correspond to every determined CHB or inactive carriers. IC forms the largest group in CHBVI patients. around three hundred million humans are IC The inactive HBsAg carrier state is recognized with the aid of absence of HBeAg and presence of anti-HBe, undetectable or low levels of HBV DNA in PCR-based assays, repeatedly regular ALT tiers, and minimal or no necroinflammation, slight fibrosis, or maybe normal histology on biopsy[18,19] Phylogenetic analysis has led to the classification of HBV into eight genotypes, defined by an inter-group divergence of >8% in the complete genome sequence **[21,22]** and of >4% in the S gene **[23].** Since the first description of four genotypes (A-D) of HBV in 1988,**[22]** four more have been identified, designated E and F, G and H. Moreover, subgenotypes with distinctive sequence characteristics and a divergence in the complete genome of >4% have been found within genotypes A, B, C and F **[24,25].** There is some evidence that the long-term prognosis, the initial clinical picture, and the response to treatment May differ depending on which genotype has infected the patient .There is variation in geographic distribution of different genotype **[26]**. CHB or inactive carriers. subsequently, the purpose of this observe become to dettect HBsAg & HBeAg Carriers with the resource of RT- PCR and their genotypes in Meerut town as the HBV is growing in India.

2. Materials and methods

2.1. Study background and subjects

245 HBsAg case were taken. Blood sample were collected in clean, sterile, small test tube from HBV patients and prosses in central research station labortory of Microbiology, at netaji subhash Chandra Bose Subharti Medical College and Associated Hospital between November 2016 to April 2018

2.2. Pattern of sample collection and its processing

10 ml blood samples obtained in the Serology segment of branch of Microbiology from patients were analyzed. The blood sample were centrifuged and sera have been separated and screened for HBsAg and HBeAg by using ELISA kit.

DNA isolation from the serum samples turned into accomplished the use of the QIAamp DNA Mini kit” (Qiagen, Germany) following the producer’s recommendations **[27]**.

The isolated DNA was amplified through RT-PCR by artus HBV RG PCR kit (Qiagen, Germany) following the manufacturer’s instruction **[28].** 2 set of Primer was used (2 forward and 2 Reverse) for Pre-S gene and 1 set to detect the existence of HBV/B in a mixed population, a genotype B-specific PCR using HBV/B genotype-specific primers (PS-B1 and PS-B2) were performed **[Table 1]**

TRUGENE HBV kit was used for genotyping in Amplified HBV DNA **[29].**Sequencing of Amplified HBV DNA was performed by using cross-linking immunoprecipitation (CLIP) method and same primer were used as in PCR. Software assigned the viral genotype and the mutations and polymorphisms present in the sample. The TRUGENE HBV software module contains sequences that correspond to the S gene, and polymerase regions for genotype A through H reporting reference sequence for comparison.

The sequence file was generated by GeneObject Software and analysed on NCBI website for HBV genotyping **[30]**

2.3. Statistical evaluation

Obtained information had been analyzed via the usage of the SPSS software program for windows version sixteen. The evaluation of

records in recognizes of age group and gender has been performed through Z- test. P < 0.05 was considering being statistically significant.

3. Results

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 2]**

All 245 HBsAg positive cases was tested for HBeAg and DNA amplification to detect HBsAg inactive non-repalicative carriers, HBeAg-positive inactive repalicative carriers, HBeAg-positive active chronic hepatitis B carriers & HBeAg-negative chronic hepatitis B carriers.

HBeAg positive were 55(1.12%), 20 HBeAg positive were male and 98 male were HBeAg-N and Highest HBeAg positive male were in 21- 30 age group. Out of 55 HBeAg casees, 35 were female and 92 female were negative for HBeAg, this was stastically significant (P< 0.039) by using Z test. **[Table 2]**

The PCR results showed that 84% (n=200) of patients were not detectaed HBV DNA, 9.24% (n=22) were between >Threshold (0.05 IU/ml) to < cutoff (10 IU/ml) and 6.72% (n=16) were ≥cutoff (10 IU/ml). Out of this 16 PCR positive patients, 7 (43.8%) were below 2000 IU/mL HBV DNA levels, 4 (25%) were between > 2000 IU/mL to 20000 IU/mL HBV DNA levels and 5(3.25%) were >20000 IU/mL HBV DNA levels

Out of this 16 PCR positive sample, 7(43.75%) were HBsAg inactive non-replicative carrier, 4(25%) HBeAg inactive replicative carrier, 5(31.25%) HBeAg active carrier and 0 were HBeAg-N chronic hepatitis B carrier. **[Table 3]**

All 16 PCR positive sample were sequenced for HBV genotyping. The prevalence of HBV genotype In HBsAg inactive Non-Replicative 37.5% (n=6) GEN-B and 6.25% (n=1) GEN-A, In HBeAg inactive Replicative 12.5% (n=2) GEN-B and 12.5% (n=2) GEN-A and In HBeAg Active Chronic Hepatitis B 18.75% (n=3) GEN-B and 12.5% (n=2) GEN-A were detected and over all prevalence of genotype- B and genotype- A were 68.8% (n=11) and 31.25% (n=6) respectively.When sequenced S gene. **[Table 4 &5]**

Tables for Detection of Hepatitis B Genotypes in HBsAg & HBeAg Carriers

Table 1: Real Time PCR primers for genotyping

S.N.	Primer	Nucleotide sequence (5' to 3')	Position (nt)	Polarity
Pre-S gene				
1	PS1	GGGTCACCTTATTCTTGGGA	2814–2833	Forward
2	PS2	CCCCGCCTGTAACACGAGCA	208–189	Reverse
3	PS3	TTGGGAACAAGATCTACAGC	2828–2847	Forward
4	PS4	GTCCTGATGCGATGTTCTCC	176–157	Reverse
5	PS-B1	ATTCAAAGCCAACTCAGAAA	2946–2965	Forward
6	PS-B2	ACAGTATTCTGAGCAGGGCTC	105–85	Reverse

Table 2: 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 (100%)	20 (36.36 %)	35 (63.64%)	55 (100%)

Table 3: Differentiation between HBsAg inactive non-replicative carriers, HBeAg inactive replicative carriers, HBeAg active chronic hepatitis B carriers & HBeAg-N chronic hepatitis B patients by Real Time PCR

Carrier types	HBsAg +ve	HBeAg		Viral Load IU/ml				Total
		- ve	+ve	NTC Threshold	<Threshold (0.05)	≥cutoff (10)	Total	
Inactive-Non-Replicative (Viral Load < 2000 IU/mL)	245	0	-	17	-	-	-	7
Inactive-Replicative (Viral Load 2000 to 20,000 IU/mL)		0	55		22	16	55	4
Active- chronic hepatitis B (Viral Load >20,000 IU/mL)								5
HBeAg-N chronic hepatitis B (Viral Load undetectable to >2,000,000 IU/mL)		190	0	183	-	-	183	0
Total	245	190	55	200	22	16	238	16

Note: 7 samples was cancel in PCR due to low volume/ spoilage of sample

Table 4: Detection of HBV Genotypes & Viral Load in HBV carriers

Carrier Types	Viral Load (IU/ml)	Genotype	Total 16
HBsAg Inactive-Non-Replicative	2.6×10^1	B-AB073846	**7**
	5.375×10^1	B-D00329	
	5.725×10^1	B-D00329	
	3.25×10^2	A- AF 090842	
	4.35×10^1	B-AB073846	
	1×10^1	B-D00329	
	1.125×10^1	B-D00329	
HBeAg Inactive Replicative	2.75525×10^3	B-D00329	**4**
	4.2315×10^3	A- AF 090842	
	7.7855×10^3	B-D00329	
	3.54425×10^3	A- AF 090842	
HBeAg Active Chronic hepatitis B	1.00413×10^6	B-D00329	**5**
	3.78439×10^5	A- AF 090842	
	9.0689×10^5	B-D00329	
	7.36287×10^5	B-D00329	
	8.39305×10^5	A- AF 090842	

Table 5: Prevalence of HBV genotypes in HBsAg & HBeAg carriers (n=16)

S.N.	Carrier Type	Genotype		Prevalence No. (%)
		B	A	
1	**HBsAg Inactive-Non-Replicative**	**6**	-	6 (37.5%)
		-	**1**	1(6.25%)
2	**HBeAg Inactive Replicative**	**2**	-	02 (12.5%)
		-	**2**	02 (12.5%)
3	**HBeAg Active Chronic hepatitis B**	**3**	-	3(18.75%)
		-	**2**	02 (12.5%)
4	**Total Prevalence**	11 (68.8%)	05 (31.25%)	16 (100%)

4. Discussion

This take a look at become carried out on HBV inactive, active carriers and HBeAg- negative CHB. The suitable identification of the inactive carriers, active carriers and HBeAg- Negative CHB has critical diagnostic implications, seeing that those patients survival is corresponding to that of non-infected population, at least in Western research. In keeping with global guidelines, an accurate approaches to chronic HBVIrequires a correct differential analysis among chronic hepatitis and the inactive carriers thru HBV DNA for as a minimum 12 months. A few facts advise that using HBV DNA and ALT by alone to define inactive carriers, without resort to liver biopsy, might not detect considerable histological disease in approximately 10% of patientss **[31]**. Considering liver biopsy is seldom recommended in those patients, there are few confirmations of these findings and liver histologic modifications on this group are difficult to evaluate. As liver biopsy is an invasive method of evaluating liver fibrosis, and contains small risk of complications, we don't forget appropriate to now not routinely carry out biopsy in that asymptomatic organization of patients. Despite a few limitations, brief elastography seems a great approach for figuring out liver fibrosis in all inactive carriers **[32]**. Current research on HBeAg stage in distinctive populace of HBV chronic infection have proven that HBeAg is lowest in the inactive carrier patient and HBV DNA quantification may also assist to distinguish among active, inactive and CHB carrier state. The mixture of serum HBeAg and HBV DNA tiers indicates promising effects as a single-factor measurement, instead of quarterly laboratorial monitoring over a year, for identifying active, inactive and HBeAg-negative CHB carrier state inactive carriers **[31-37]**.

In the Western countries, the reactivation is uncommon and when it occurs is due mainly to the presence of cofactors which include alcohol or drugs. Big predictors of mortality in HBeAg-Positive carriers are the presence of clinical comorbidities, older age at analysis, and abnormal GGT tiers **[2,38-40]**. Any other research concluded that most people of inactive carriers have a low level of viral replication **[41]**. HBV reactivation came about in ten of our sufferers, that's in step with posted records that estimate that reactivation occurs in 10-34% of these sufferers **[42,43]**.

Because HBV DNA stages in HBeAg-negative CHB patients, can differ from >2,000,000 IU/mL to undetectable **[20]** and some inactive carriers sometimes have HBV DNA tiers amongst 2000 and 20,000 IU/mL, a HBV DNA stage between 2000 and 20,000 IU/mL seems to be a ‘‘gray region’’ that would correspond to each HBeAg-negative CHB or inactive carriers. additionally HBV DNA level > 20,000 IU/mL seems to be a ‘‘red area’’ which could correspond to every HBeAg-negative CHB patients or Active carriers, for the affirmation of this red vicinity patients, detection of HBeAg and HBV DNA viral load have to be carry out. In this Research the prevalence of HBV genotype In HBsAg inactive Non-Replicative 37.5% (n=6) GEN-B and 6.25% (n=1) GEN-A, In HBeAg inactive Replicative 12.5% (n=2) GEN-B and 12.5% (n=2) GEN-A and In HBeAg Active Chronic Hepatitis B 18.75% (n=3) GEN-B and 12.5% (n=2) GEN-A. In deferent research in India. The prevalence of GEN-B and GEN-A become 68.8% and 31.25% respectively from islated HBV DNA. GEN-B was predominant in our isolate and the variable consequences become discovered in one-of-a-kind research performed in distinct a part of india; a take a look at performed via Saket Chattopadhyay et al in new delhi, most effective GEN-A and D have been found and GEN-D turned into dominant, GEN-A changed into 16% and GEN-D 84% **[44]**. A look at conducted with the aid of Perumal Vivekanandan et al from Southern part of India, GEN-D was detected in 57.3%, GEN-A was detected in 18%, and GEN-C became detected in 11.5% **[45]**. A examine conducted by means of Swati S. et al from weatern a part of India, detected most effective two GEN, GEN-D become the primary (91.93%), GEN-A become regularly occurring in 8% **[46]**. A have a look at changed into accomplished in post graduate institute chandigarh and they've got GEN-D,A,B,C and in one pattern GEN-B & C was mixed **[47]**. Numerous take a look at carried out out side of India proven exceptional incidence, GEN-C was predominant in Bangladesh and Taiwan **[48, 49]**. GEN-D is fundamental in Turkey and Iran **[50,51]**. Predominance of the HBV GEN-E changed into detected in Niger **[52]**. Recent studies counseled that Acute hepatitis B virus infection with GEN-A also can increase the risk of development to CHB. In Japan, the patience of HBVI after Acute Hepatitis B changed into higher in patients with GEN-A (23%) than people with GEN-B (11%) or C (7%) infections. **[53]** Of specific discussion, an incresaed of Acute hepatitis B virus infection with GEN-A may bring about a redistribution of HBV GENs amongst sufferers with CHB in any nations in which hepatitis B vaccination has not but been released. As an example, in a nation-extensive study, Matsuura et al. discovered that the prevalence of HBV GEN-A in CHB patients in Japan grown from 1.7% for the duration of 2000 to 3 .5% in 2006 **[54].** GEN-F turned into the regularly occurring GEN amongst the acute symptomatic infections in Buenos Aires city, Argentina and GEN-F showed an inclination to reason an adverse disorder final results some of the chronic infection **[55].** Certain HBV GENs and sub-GENs C, B2-5 and F1 regarded to be associated with a higher hazard of growing HCC, and others GENs B1, B6, and A2 appeared to be associated with a decrease danger of complications of HBV. Patients with GEN-C were much more likely to have HCC. Waitlist death rate become highest amongst patients with GEN-D, even as post-transplant mortality was highest among sufferers with GEN-C **[56].** In China, HBV co-infections with 2 or 3 GENs had been associated with higher viral load and development to chronic diseases. GEN-B infection was related to HCC recurrence and GEN-C was determined more in HCC patients **[57].** In different take a look at from China, HBV GENs B and C have been associated with specific patterns of last-stage liver illnesses that required transplantation and GEN-C can also carry a more danger and severity of recurrence because of lamivudine-resistant mutants **[58].** In Northern India, GEN-A

modified into extra frequently related to ALT elevation, HBeAg-P, absence of anti-HBe and among aged 25 years and above, cirrhosis of liver, than GEN-D **[59].** sufferers with HCC with GEN-C had a greater tumor recurrence possibilities after restoration resection of HCC as compared with humans with GEN-B **[60].**In a have a look at from Spain that include each interferon-treated and untreated patients, the ones infected with GEN-F had been pronounced to have lower cumulative opportunity of sustained biochemical remission and HBV DNA loss and a considerably higher cumulative liver-related demise rate than those infected with GEN-D or A **[61].** A study from India stated that GEN-D turned into related to extra extreme liver sickness and HCC in more youthful sufferers than GEN-A **[62].** In Taiwan, GEN-C became associated with more excessive liver disorder and GEN-B turned into related to the improvement of HCC in young non-cirrhotic patients. In assessment, GEN-B had a rather decent prognosis in Japan and China and become hardly ever related to the development of HCC. In addition, GEN-D was associated with more excessive liver sickness than GEN-A in India and can are expecting occurrence of HCC in young patients **[63]** it is as a result essential for the clinician to be aware of the importance of serial HBV DNA measurements & status of HBeAg and existence-lengthy observe-up to verify that IC state is maintained. GEN-B and GEN-A were detected by sequencer and GEN-B was more prevalent in comparission to GEN-A.

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