

A molecular approach for the detection of Hepatitis B virus Genotyping it's clinical importance by using PCR in a Teaching Hospital.

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Abstract

Objectives and Aim

We performed a study on Seroprevalance, risk factors and HBV genotypes among the patients attending a tertiary care hospital in India.

Background:

Globally, Hepatitis B is one of the most common infectious diseases. It is ranked by the WHO as one of the top ten killers. The virus is responsible for approximately 1.5 million deaths worldwide in each year, two thirds of which are attributable to primary Hepatocellular carcinoma following HBV infection. About 360 million people are estimated to be chronically infected with HBV.

Method

284 samples were positive for HBsAg by ELISA. PCR amplification was done for detection of S' gene and these samples were subjected to further Genotyping

Results- The seroprevalence of Hepatitis B infection in our study was 6%, associated with blood transfusion as the major risk factor.

The most predominant genotype was C-66 (50.8%)

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I. Introduction

✓ Viral hepatitis due to Hepatitis B virus (HBV) is a major public health problem throughout the world affecting several hundreds of millions of people. It is a cause of considerable morbidity and mortality in human population from both acute infections and chronic sequelae which include acute infection, chronic active hepatitis, cirrhosis and primary liver cancer.¹

✓ Hepatitis B virus was recognized originally as the cause of serum hepatitis, the most common form of parenterally transmitted viral hepatitis, and an important cause of acute and chronic infection of the liver in many countries. More than one third of the world's population has been infected with HBV and WHO estimates that it results in 1 to 2 million deaths every year. The incubation period of hepatitis B is variable with a range of between 1 and 6 months. Acute hepatitis B infection is anicteric and asymptomatic most of the time, although a severe illness with jaundice can occur and acute liver failure may develop. The virus persists in about 10% of infected immunocompetent adults and in as many as 90% of infants infected perinatally, depending on the ethnic group of the mother. About 350 million people worldwide are persistent carriers of HBV. Liver damage is mediated by the cellular immune response of the host to the infected hepatocytes. Approximately 25% of all patients with chronic hepatitis will progress to cirrhosis and about 20 % of those with cirrhosis will develop Hepatocellular carcinoma.²

✓ Hepatocellular Carcinoma is one of the most common cancers worldwide. However the significance and magnitude of the problem vary from country to country.³

✓ As per WHO guidelines, Countries are classified on the basis of endemicity of hepatitis-B virus (HBV) infection into high (8% or more), intermediate (2-7%), or low (less than 2%) incidence countries. The prevalence of chronic HBV infection in India ranges from 2% to 10% as shown in different studies. India therefore comes under the intermediate to high endemicity category. India has approximately HBV carrier rate of 3.0% with a high prevalence rate in the tribal population.³ With a population of more than 1.25 billion, India has more than 37 million HBV carriers and contributes a large proportion of the global HBV burden. While

horizontal transmission in childhood appears to be a major route of transmission, the role of vertical transmission is probably underestimated⁴. Blood transfusion and unsafe therapeutic injections continue to be important modes of transmission of HBV.^{3,4,5}

II. Materials And Methods

- ✓ **Source of Data**
- ✓ **Collection of samples**
- ✓ **Sample processing**
 - Extraction of HBV genomic DNA
 - Separation of HBV genomic DNA by Agarose gel electrophoresis
 - PCR amplification of HBV DNA
 - Development of PCR products by Agarose gel electrophoresis
 - PCR amplification of HBV genotypes
 - Development of PCR products for identification of HBV genotypes by Agarose gel electrophoresis
- ✓ **Results and interpretation**
 - HBV genomic DNA
 - PCR amplification of HBV DNA
 - PCR amplification of HBV genotypes

a. Source of data

Total number of samples received during the study period of one year in the Department of Microbiology, JSS Hospital, Mysuru for HBV screening were 4648 out of which HBsAg was detected in 284 (6.2%) by ELISA.

b.INCLUSION CRITERIA

- All patients suspected of Hepatitis and in whom HBsAg is detected by ELISA
- Asymptomatic patients for whom serum samples are submitted to the laboratory for routine investigation and are HBsAg positive, in spite of the clinical presentation.

c.EXCLUSION CRITERIA

- Patients who are treated with Antiviral therapy are excluded from the study

d.Collection of samples

2ml of venous blood sample was collected in EDTA vacutainer from patients who tested positive for anti HBV antibody by 3rd generation ELISA (QUALISA).

Demographic data and clinical history and clinical examination of the patients who were seropositive was recorded in the Performa and risk factors for the infection were taken note of, haematological tests (PT, Platelet count) and liver function tests(LFT) were done for these patients. Liver biopsy, ultrasonography, CT abdomen and fibroscan were also done for these patients.

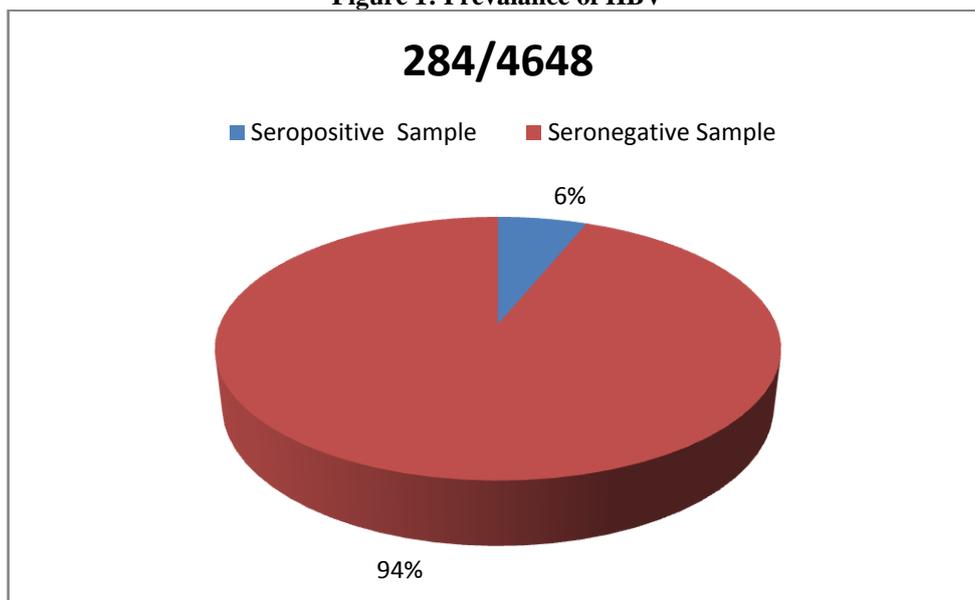
III. Result

a. Demographic characteristics

- ✓ Out of 4648 serum samples submitted to the Department of Microbiology at JSS Hospital Mysuru during the study period of one year , 284 samples were positive for HBsAg by ELISA (Figure 1).The patients for whom the samples were submitted formed the subjects of this study. Out of these 198 were Male (69.7%) and 86 were female (30.3%) (Table 2, Graph 3).The mean age of the patients was 39.426 ± 10.6 years.(Table 1, Graph 2). Majority of the samples for evaluation of the HBV infection were from the Department of Medicine (145/284[51.5%]),followed by Department of Surgery,OBG,Nephrology, Skin and from Dental also. (Table 3, Graph 4). The most predominant risk factors of HBV acquisition in our study was Blood transfusion (101/284[35.6%]),followed by tattooing (36/284[12.7%])and (34/284[12.0%]) patients had genital ulcers leading to a probable association with sexual transmission and exposure to major and minor surgical procedure i.e., abscess drainage (I and D) were found in [16/284(5.6%)] patients and oral /dental implant in (16/284[5.6%]). Other associated risk factors were Haemodialysis (12/284[4.2%]),Ear piercing (11/284[3.9%]), Nose piercing (10/284[3.5%]),while in (48/284[16.9%]) no specific history was elicitable.(Table 4, Graph 5).

✓ Genotype C (66/130[50.8%]) was the most predominant genotype followed by Genotype G (29/130[22.3%]), Genotype D (22/130[16.9%]), H (7/130[5.4%]), A (3/130[2.3%]), E (2/130[1.5%]) and F (1/130[0.8%]).(Table 5, Graph 6). Genotype B was not detected in any of the selected patients in our study

Figure 1: Prevalance of HBV



Demographic distribution

Table 1: Age distribution

AGE	FREQUENCY	PERCENTAGE
0-10 YEARS	-	-
11-20 YEARS	9	3.2
21-30 YEARS	63	22.2
31-40YEARS	109	38.4
41-50YEARS	66	23.2
51-60YEARS	29	10.2
61-70YEARS	8	2.8
TOTAL	284	100

MEAN =39.426
 SD=10.6
 MINIMUM=16.0
 MAXIMUM=70.0

Figure 2: Age distribution.

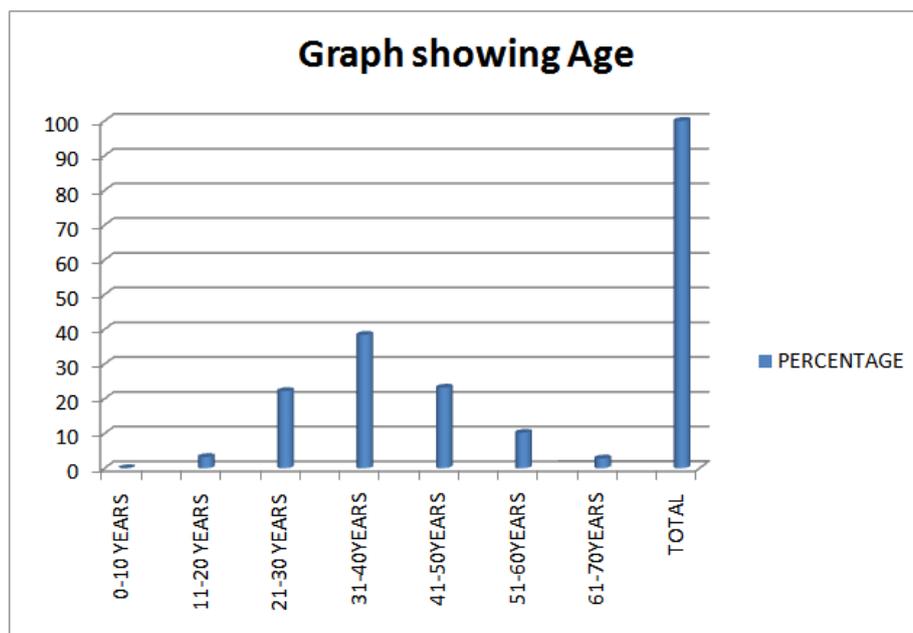


Table 2: Gender distribution

SEX	FREQUENCY	PERCENTAGE
MALE	198	69.7
FEMALE	86	30.3
TOTAL	284	100

Figure 3: Gender distribution

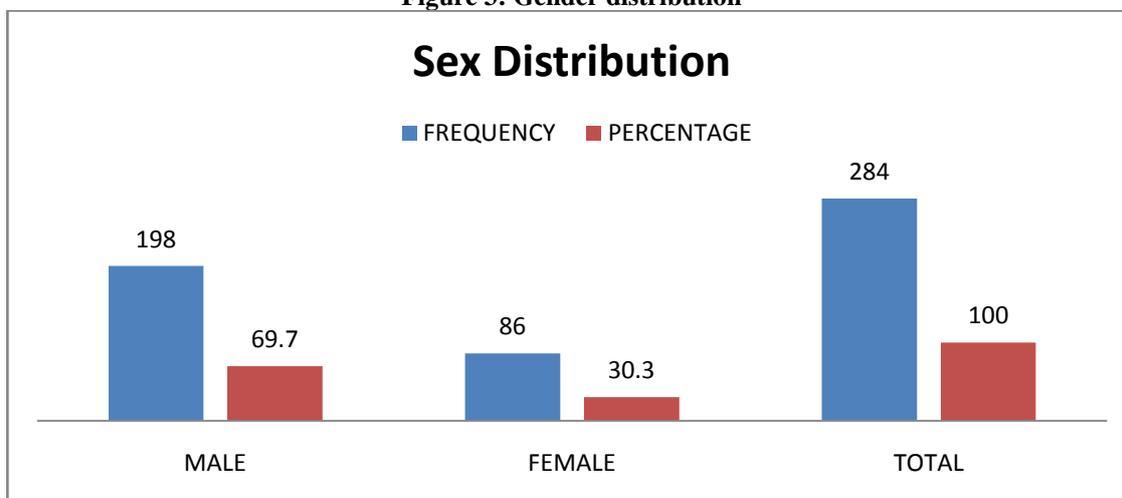


Table 3: Ward distribution

WARDS	FREQUENCY	PERCENTAGE
DENTAL	12	4.2
MEDICINE	145	51.5
NEPHRO	16	5.6
OBG	48	16.9
SKIN	28	9.9
SURGERY	35	12.3
TOTAL	284	100

Figure 4: Ward distribution

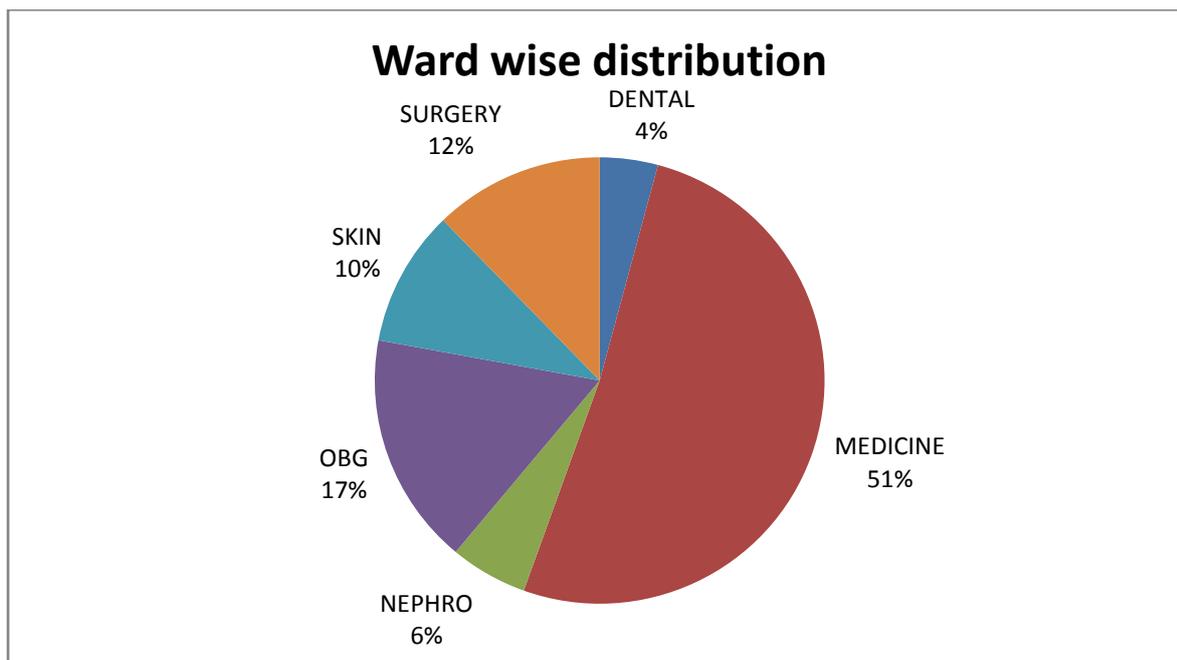


Table 4: RISK FACTORS

RISK FACTORS	FRQUENCY	PERCENTAGE
1.Surgery		
Abscess Drainage	16	5.6
2.Parenteral Route		
Blood transfusion	101	35.6
Ear piercing	11	3.9
Haemodialysis	12	4.2
Nose piercing	10	3.5
Tattoo	36	12.7
3.STD s		
Genital lesions and Ulcers	34	12.0
4.Unknown History		
	48	16.9
5.Others-Oral implant		
	16	5.6
Total	284	100

Figure 5: Risk factor

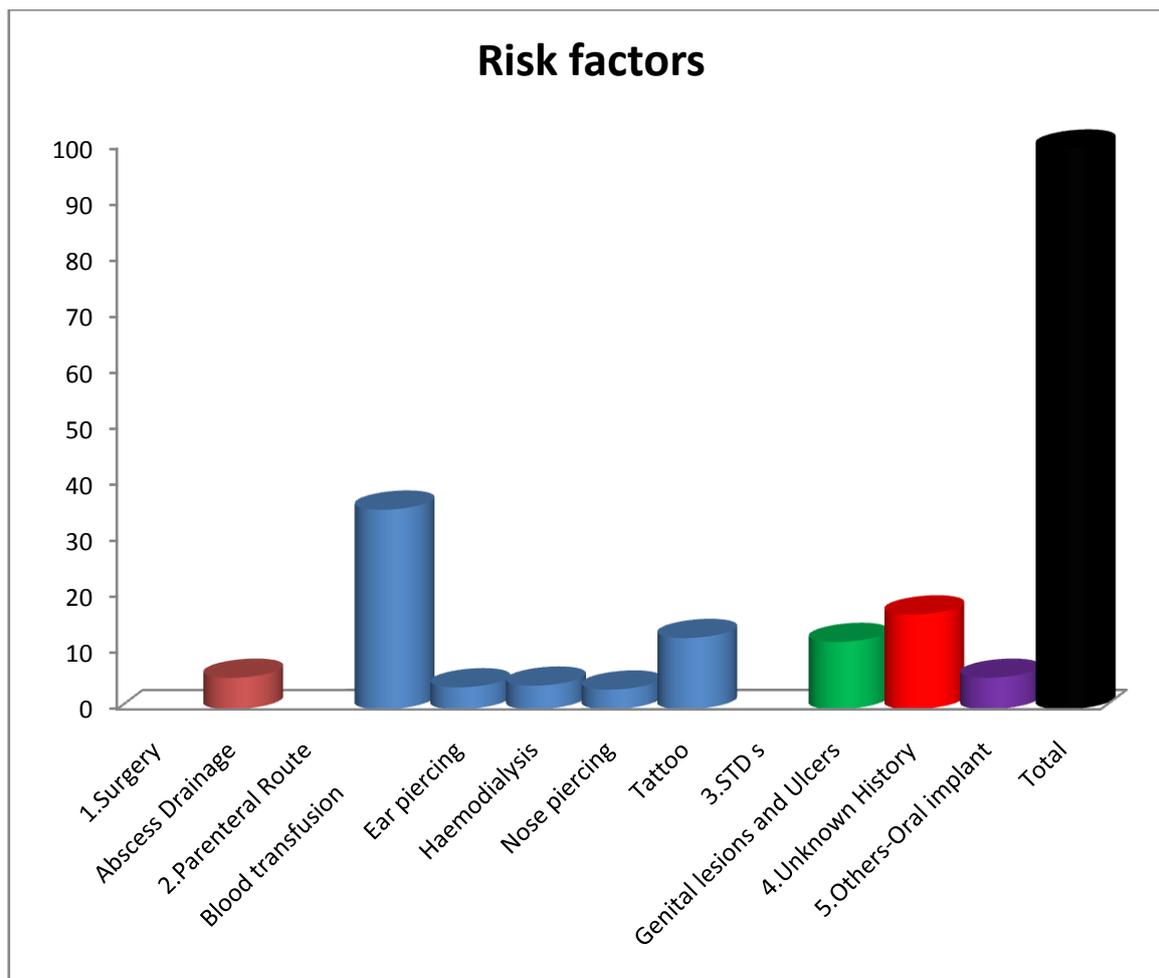
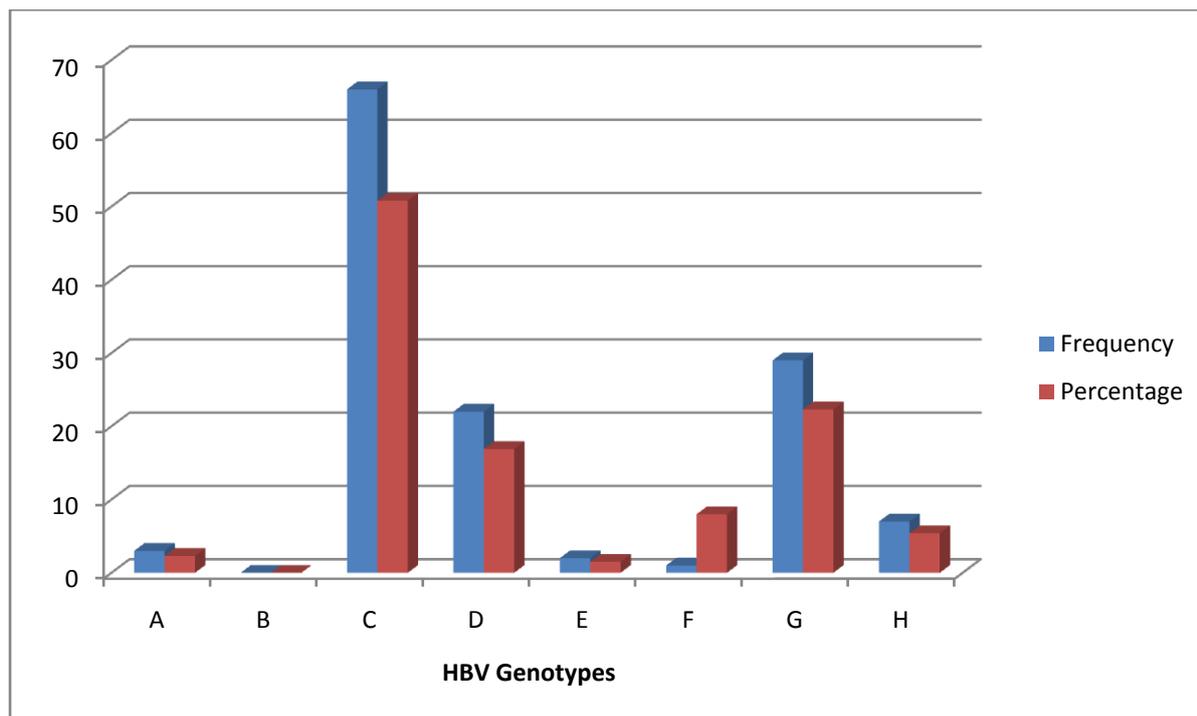


Table 5: Hepatitis B Genotyping Result

Genotypes	Frequency	Percentage(%)
A	3	2.3
B	-	-
C	66	50.8
D	22	16.9
E	2	1.5
F	1	8
G	29	22.3
H	7	5.4
TOTAL	130	100

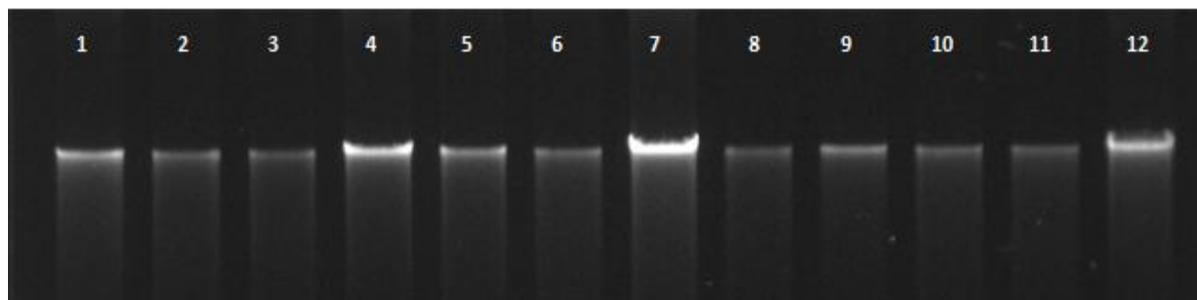
Graph 6: Hepatitis B Genotyping Result



Distribution of HBV genotypes

Out of 284 samples which were HBsAg positive, HBV DNA could be extracted from 130 samples (picture 1) on which PCR amplification was done for detection of ‘S’ gene (Picture 2) and these samples were subjected to further Genotyping. (Picture 3).

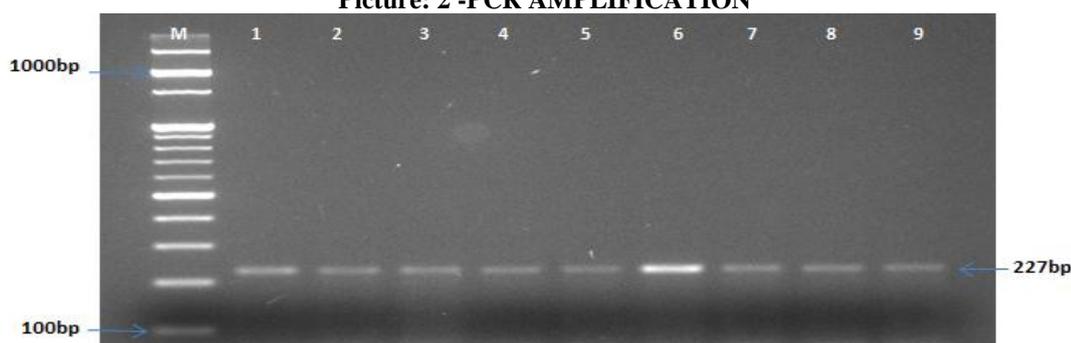
Picture: 1 –HBV GENOMIC DNA



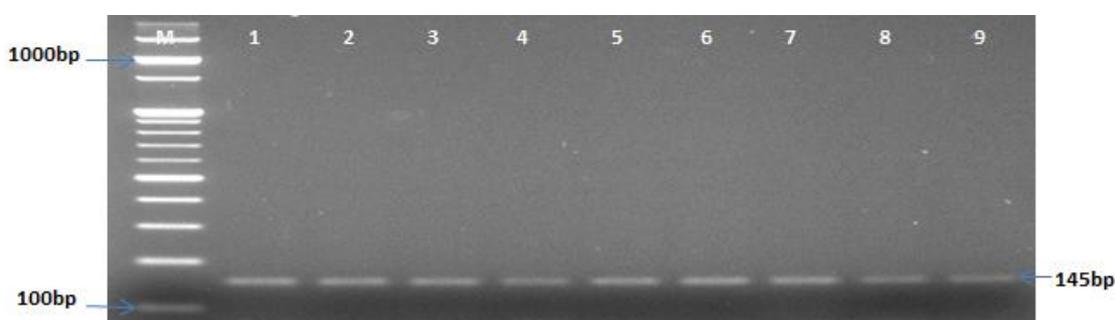
Fig; R1- HBV genomic DNA from seropositive sample separated in 1.2% AGE and image captured using Gel documentation unit.

✓ The result above showed successful isolation of DNA from the given seropositive samples using HeliniPureFast viral nucleic acid Minispin prep kit which is based on spin column technology. All bands are uniformly separated and intact without any sign of degradation and smearing which validate DNA sample for implementation in further downstream application.

Picture: 2 -PCR AMPLIFICATION



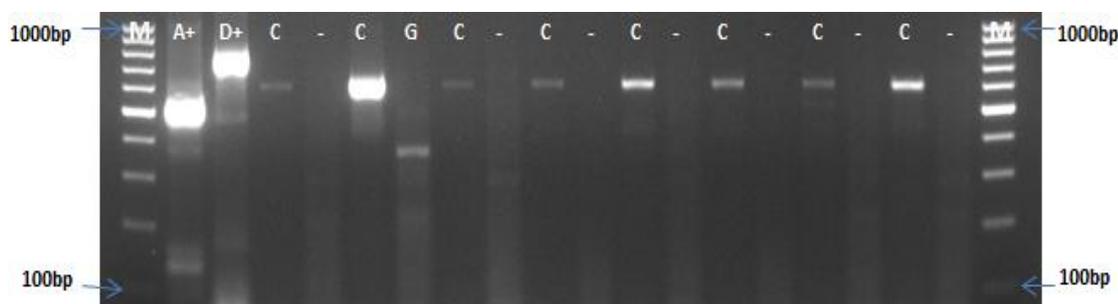
Fig; R2A: Fig; PCR Product from respective seropositive HBV-DNA sample amplified using Internal control GAPDH with product size of 227bp defined by 100bp DNA Marker (M) and separated in 3.0% AGE and image captured using Gel documentation unit.



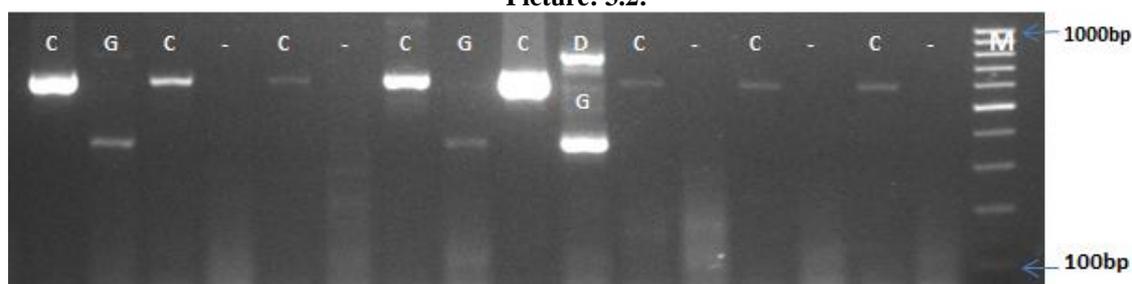
Fig; R2B: Fig; PCR Product from respective seropositive HBV-DNA sample amplified using HBV specific primer with product size of 145 bp defined by 100bp DNA Marker (M) and separated in 3.0% AGE and image captured using Gel documentation unit.

✓ The result above showed successful amplification of HBV DNA using Internal control (Fig;R3A) as well as HBV specific primer (Fig;R3B) provided in the kit which has been designed for S gene of HBV genome which is highly conserved region and shows 100% homology with a broad range of clinically relevant reference sequences. The expected product size of HBV primer is 145bp and that of internal control i.e. GAPDH is 227bp. DNA marker of 100bp was included to mark the PCR product based on molecular size (bp). All the PCR products formed from respective DNA samples are specific to the primer used which further validate the source of DNA samples which is of HBV origin and further can be implemented for identification of genotypes using Helini HBV genotyping PCR kit [Genotype A-H].

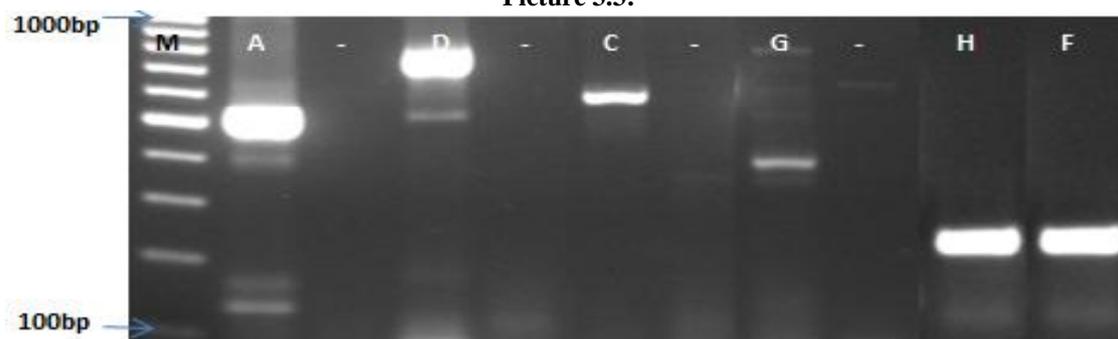
Picture: 3.1 –HBV GENOTYPING USING PCR



Picture: 3.2:



Picture 3.3:



Fig; R4 (A-upper, B-middle, C-lower): PCR Products of different HBV-genotypes from seropositive samples and Positive controls (Genotype A and D) along with 100bp DNA marker, separated in 3% AGE and Gel image captured by Gel Doc System.

✓ The above result shows successful amplification of HBV genotypes prevalent in seropositive samples under study. 2 primer mixes were used for single sample named Genotype Primer set-I and Genotype Primer set II. Primer set I amplify HBV- genotypes A, C, B & F and Primer set II amplify HBV-genotypes D, E, G & H. Genotype A positive control and Genotype B positive control were also included in PCR reaction and finally amplified products were separated by 3% Agarose gel electrophoresis as depicted in Fig R4A (1st and 2nd sample lane). The more prevalent genotype identified as Genotype C with product size of 607bp followed with Genotype G with product size of 366bp, then Genotype D with product size of 756bp. Other Genotypes that includes Genotype H, Genotype A, Genotype E and Genotype F are less frequent among the population under studied. Maximum number of sample showed single genotype which is C. Few samples also being reported with 2 genotypes in the combination of Genotype C and G. Fig R4C (Lower image) displays banding pattern of all the detected genotypes. DNA marker of 100bp was included to mark the PCR product based on molecular size (bp). All the PCR products formed from respective DNA samples are specific to the primer used without any non-specific amplification that successfully reported prevalent Genotypes from HBV seropositive samples.

IV. Discussion

✓ Hepatitis B virus is a global public health problem. The present study Titled“ Hepatitis B virus genotyping and its clinical importance by using PCR” was taken up with the objective of detecting the prevalence of HBV infection at J.S.S. Hospital, Mysuru, to find the association of probable risk factors with seropositivity and also determination of the genotypes in the study population.

✓ Inclusion criteria : All patients suspected of Hepatitis and in whom HBsAg is detected by ELISA. Asymptomatic patients for whom serum samples are submitted to the laboratory for routine investigation and are HBsAg positive, inspite of the clinical presentation.

✓ Exclusion criteria: Patients who are treated with Antiviral therapy are excluded from the study. Serum was separated from blood sample and subjected to ELISA for anti HBV antibody detection using 3rd generation HBV kit. From seropositive patients serum was collected and stored at -70°C for genotyping. Risk factors for the infection were noted. Haematological tests (Platelet count, Prothrombin Time) and liver function tests (LFT) were done.

✓ Viral DNA was extracted from serum samples of seropositive patients by using HeliniPureFast viral nucleic acid Minispin prep kit and Polymerase Chain Reaction (PCR) was performed for detection of S gene ,genotyping was done by using Helini Biomolecules kit cat no 9002.

a. Seropositivity

✓ The seroprevalence of Hepatitis B infection in our study was 6%. This may not necessarily represent the endemicity, magnitude in this part of the state, as ours is a tertiary care centre and significant no. of samples were from clinically suspected cases. The prevalence of Chronic HBV infection in different studies ranged from 2 to 10% (below 8%) as per literature survey. Therefore India has intermediate to high endemicity⁶

✓ Pragati Abhimanyu Bullee *et al*⁷ in Yavatmal (Maharashtra), India found that the Seroprevalence of HBsAg positive was 1.57% while in healthy donors it was 0.87%. The prevalence of HBsAg in the general population of Asia, Africa, Southern Europe and South America ranges from 2% to 20%. HBsAg seropositivity of 3-4% is reported in the Indian population.

✓ There is wide variation in the prevalence in different regions of the country with the highest prevalence in Andaman and Arunachal Pradesh⁸. Various studies across India reports HBsAg seroprevalence ranging from 1.6% to 5.7% in South India, 3-6% in North India, and 2.97% in West Bengal.⁹

✓ The HBV seropositivity in our study was high in male (69.7%) as compared to female (30.3%) similar results were observed in another study conducted by Atul Rukadikare *et al*, they found that seropositivity was high in male i.e., 73.39% than female 26.60%.¹⁰

✓ This could be due to greater exposure of male patients to the health care set up. Most of the patients infected with Hepatitis B were in the age group of 31 to 50 years in our study, similar results were seen in study conducted by Prasad Bhatte *et al*.¹¹

b. Risk Factors

✓ Parenteral route including blood transfusion and contact with infected persons is the significant risk factor for HBV transmission. Sujatha R¹² *et al* conducted a study in a tertiary care centre Kanpur and observed that history of contact with infected persons was 41% and Blood transfusion 14%. In our study conducted in a tertiary care hospital, the professional blood donors and unsafe injections practice constitutes major high risk group for HBV infection and also showed the predominance of blood transfusion 35.6%, probable sexual route of transmission 12.0% as high risk factors for HBV transmission.

✓ Recently increased tattooing trend in both urban and rural population has been commonly associated with increased risk of HBV transmission, it was shown in cross sectional study conducted by Prasad Bhatte *et al*, 17.6% patients were found to be infected due to tattooing, similar results were observed in our study i.e., 12.7%. This shows that Tattooing is one of the significant preventable risk factors of HBV transmission, it can be prevented by public awareness, health education.

✓ Body piercing including nose and ear piercing has become a universal fashion and in urban areas its more of fashion whereas in rural areas its a customary trend. But because of lack of sterilization of the instruments used it has contributed to the another most important risk factor for HBV transmission.

✓ In Eastern India, Bhagirathi Dwivedi *et al* found that out of 712 cases, 122 were HBsAg Positive i.e., (17.1%) with risk factors of body piercing including nose, ear piercing¹³

✓ India being a highly populated country, most of the people suffer from skin infections like abscess, boils, furuncles, cyst etc. due to lack of cleanliness and hygiene, close contact and these people visit quacks for the low cost of treatment and are prone for various minor surgical procedure related infections. Sidra *et al* observed that around 37% of persons were infected by minor surgical procedures whereas in our study we found that only 5.6% subjects were infected by minor surgical infections.¹⁴

✓ Haemodialysis is the most advised treatment for impaired renal function, due to chronic kidney disease, diabetic nephropathy. It is an expensive treatment modality and requires multiple visits and not affordable by all, patients opt for lower and cheaper centres for dialysis where they may contract HBV infection by usage of old needles or not changing of membrane used in Haemodialysis unit. Rubina Malhotra *et al* conducted a retrospective study and found that 1.5% patients were found to be positive for HBsAg in patient on dialysis¹⁵. Pankaj Puri *et al* concluded that the prevalence of HBV infection amongst dialysis patients in India varies from 5 to 13%¹⁶

c. HBV Genotype distribution

✓ In our study, it was found that the most predominant genotype was C-66(50.8%), followed by G-29(22.3%), D-22(16.9%), H-7(5.4%), A-3(2.3%), E-2(1.5%), F-1(0.8%).

✓ To identify the most prevalent genotypes in Pakistan Nazish Badar *et al* conducted a cross-sectional study and found that among (214) PCR positive samples only genotype C and D were identified in local population with 21 cases (9.81%) of genotype C and 195 (91.1%) of genotype D.¹⁷

✓ In Arunachal Pradesh a study was conducted by B.J. Borkakoty *et al*. They found that the predominant genotype was genotype A (41.6%) followed by genotypes C (27.8%) and D (11.1%)¹⁸

V. Conclusion

- The present cross sectional study titled “Hepatitis B virus genotyping and its clinical importance by using PCR” was undertaken in the Department of Microbiology, spanning over one year duration (January 2015 to December 2015) showed a seropositivity of 6% in patients attending this tertiary care centre.
- Predominant risk factor was found to be Blood transfusion followed by, patients having genital ulcers leading to a probable association with sexual transmission. The other risk factors were exposure to major and minor surgical procedure, haemodialysis, tattoo, oral implant, Nose and Ear piercing. Prevention strategies must be targeted towards this population of patients.
- Stringent blood banking laws need to be introduced. It is important to screen the blood donors to control the spread of HBV infection. The latest specific measure is the introduction of viral nucleic acid amplification test (NAAT). NAAT screening is currently in use in most of the developed countries but not yet mandatory in India. It detects viral genes rather than antibodies or antigens. NAAT will enable earlier detection of HBV.
- HBV genotype D,A and C are widespread in this region of southern India according to literature survey,^{19,20}. But we found in our study Genotype C to be most predominant followed by Genotype G and D.
- The complexity and uncertainty related to the geographical distribution of HBV infection and chronic hepatitis B, determination of its associated risk factors, and evaluation of co-factors that accelerate its progression, underscore the difficulties in global prevention and control of HBV. Because there is lack of awareness and health education, negligence, the focus of primary prevention efforts should be safer blood supply in the developing world, safe injection practices in health care and other settings, and decreasing the number of people who initiate injection drug use.
- Further studies need to be done on genotyping of large number of sample in order to determine the association with disease severity, progression and risk factor.

References

- [1]. Bennett JE, Dolin R, Blaser MJ. Mandell, Douglas, and Bennett's Principles and practice of infectious diseases. Elsevier Health Sciences; 2014 Aug 28.
- [2]. Tille P. Bailey & Scott's diagnostic microbiology. Elsevier Health Sciences; 2013 Aug 13.
- [3]. Kumar P, Clark ML. Kumar and Clark's clinical medicine. Elsevier Health Sciences; 2016 Jun 29.
- [4]. Jazayeri SM, Alavian SM, Carman WF. Hepatitis B virus: origin and evolution. Journal of viral hepatitis. 2010 Apr 1;17(4):229-35.
- [5]. K George Mathew and Praveen agarwal. Medicine Prep manual for undergraduates. 5th edition. Elseviers. 2015.
- [6]. WHO guidelines of Infectious Diseases. 2014, August.
- [7]. Bulle PA, Tekam S, Gedam DS, Gujar V, Deshmukh D. Prevalence of Hepatitis B Surface Antigen (HBsAg) Positivity among General Population in Yavatmal (Maharashtra), India. Int. J. Curr. Microbiol. App. Sci. 2016;5(7):513-7.
- [8]. Borkakoty BJ, Mahanta J, Biswas D. Circulating genotypes of hepatitis B virus in Arunachal Pradesh. Indian Journal of Medical Research. 2008 Jan 1;127(1):65.
- [9]. Rashmi KS, Misbah-Ul-Khair S, Ravikumar KL. Profile of Hepatitis B ‘e’Antigen and Antibodies to Hepatitis B ‘e’Antigen in Hepatitis B Seropositive Patients at a Tertiary Care Hospital in Bengaluru, India.
- [10]. Chowdhury A., Santra A., Pal S., Chakravarty R., *et al.* Community based epidemiology study of Hepatitis B virus infection (HBV). *Indian J. Gastroenterol.* 200120(2): A2.
- [11]. Bhate P, Saraf N, Parikh P, Ingle M, Phadke A, Sawant P. Cross Sectional Study Of Prevalence And Risk Factors Of Hepatitis B And Hepatitis C Infection In A Rural Village Of India. *Arquivos De Gastroenterologia.* 2015 Dec;52(4):321-4.
- [12]. *Sujatha.R, Nidhi Pal, Arunagiri.D&Narendran. D.* Seroprevalence and Risk Factors of Hepatitis-B Infection in Patients attending a Tertiary CareCenter in Kanpur [UP],India.*International Journal of Current Medical And Applied Sciences*, vol.4. Issue 3, November: 2014. PP: 98-103
- [13]. Dwibedi B, Sabat J, Ho LM, Singh SP, Sahu P, Arora R, Kar SK. Molecular epidemiology of hepatitis B virus in primitive tribes of Odisha, eastern India. *Pathogens and global health.* 2014 Dec 1;108(8):362-8.
- [14]. Shaheen S, Nouroz F, Khan S, Noreen S. A Review on Hepatitis B Virus, its Genotypes and Risk Factors. *Middle-East Journal of Scientific Research.* 2015;23(6):1152-7.
- [15]. Malhotra R, Soin D, Grover P, Galhotra S, Khutan H, Kaur N. Hepatitis B virus and hepatitis C virus co-infection in hemodialysis patients: A retrospective study from a tertiary care hospital of North India. *Journal of natural science, biology, and medicine.* 2016 Jan;7(1):72.
- [16]. Puri P. Tackling the hepatitis B disease burden in India. *Journal of clinical and experimental hepatology.* 2014 Dec 31;4(4):312-9.
- [17]. Nazish B, Umer F, Shafaqat A, Nadia N, Muhammad A, Javed Anwar Q. A Molecular Approach for Genotyping of Hepatitis B Virus Using Restriction Pattern Analysis of S Amplicon in Pakistan. *Open Journal of Medical Microbiology.* 2012 Mar 28;2012.
- [18]. Borkakoty BJ, Mahanta J, Biswas D. Circulating genotypes of hepatitis B virus in Arunachal Pradesh. *Indian Journal of Medical Research.* 2008 Jan 1;127(1):65.
- [19]. Hepatitis B genotype *Virology Journal* 2008;5:156
- [20]. Vivekanandan P, Abraham P, Sridharan G, Chandy G, Daniel D, Raghuraman S, Daniel HD, Subramaniam T. Distribution of hepatitis B virus genotypes in blood donors and chronically infected patients in a tertiary care hospital in southern India. *Clinical Infectious Diseases.* 2004 May 1;38(9):e81-6.

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