

A Study Comparing Serological And Molecular Methods For Hepatitis B Virus Diagnosis

Mustafa Shaikh¹, Dr. Srinivas²

¹Research Scholar, KBN University, Department of Microbiology, Kalaburagi, India.
mustafa1248@gmail.com

²Professor, KBN University, Department of Microbiology, Kalaburagi, India.

ABSTRACT

The hepatitis B virus (HBV) causes hepatitis B, an infection of the liver that may be fatal if left untreated. On a worldwide scale, it poses a serious threat to public health. People with this condition are at increased risk for developing cirrhosis and liver cancer, and they may also have persistent infections. Between ten and fifteen percent of the world's HBV carriers live in India. Roughly 40 million people in India are thought to be HBV carriers. Cirrhosis, liver cancer, and early mortality are among the complications that around 15–25% of HBsAg carriers may face.

The hepadnavir family includes HBV. The DNA that makes it up is relaxed-circular DNA, which is half double-stranded. One strand is incomplete and does not code for anything, while the other is partly double-stranded and is known as the negative strand. The genome of the herpes simplex virus (HSV) has four primary overlapping reading frames: S, C, P, and X. Serological and virological indicators provide the basis of laboratory diagnosis.

Thirty to sixty days after HBV exposure, HBsAg may be found in the blood, and it stays there for different amounts of time. Molecular techniques are mostly used for the detection of nucleic acids.

Two patients had positive results for HBsAg when tested with the Meriscreen HBsAg card, and four patients had positive results for HBV DNA when tested with the PCR. People in the 21–30 age bracket were the most often impacted. It was men that made up 75% of the HBsAg positive cases. The method of dissemination that was greatest (15.2%) was blood transfusion. A total of 40.9% of the participants in this research were not immunised. A 66.7% sensitivity and a 100% specificity were achieved by the PCR.

Untreated hepatitis B infection is a serious public health concern in underdeveloped nations such as India. In order to reduce the infection rates, vaccination is quite important. In order to reduce mortality and morbidity, early diagnosis and treatment are crucial. It is possible to identify HBV infection using either serology or molecular techniques. The PCR assay has a diagnostic accuracy rate of 75%, a positive predictive value of 100%, and a specificity of 100% when it comes to detecting HBV DNA in blood or serum.

Keywords –hepatitis B virus, meriscreen HBsAg , PCR.

I. INTRODUCTION

When the liver becomes inflamed, it is known as hepatitis. The A, B, C, D, and E hepatotropic viruses are among those that cause it. One of the most frequent types of hepatotropic virus is hepatitis B virus (HBV), and the other is hepatitis C virus (HCV). Liver-related mortality and morbidity are significantly exacerbated by HBV and HCV. Hepatitis B is an illness of the liver that may be fatal if left untreated. On a worldwide scale, it poses a serious threat to public health. It causes a lot of death and illness, including cirrhosis and hepatocellular cancer.

257 million individuals were expected to be living with chronic hepatitis B infection in 2015, according to the World Health Organisation. This illness is characterised as hepatitis B surface antigen positive. The majority of the 8,87,000 fatalities in 2015 were attributable to cirrhosis and hepatocellular carcinoma, two forms of hepatitis B. From the 1980s to the early 2000s, when the vaccine was first introduced, the number of persons known to be chronically infected with hepatitis B decreased from around 5% to just under 1%. In 2016, 27 million people, or 10.5% of the total population believed to be living with hepatitis B, were aware of their infection.¹ Hepadnaviridae viruses, of which HBV is a member, are enveloped viruses with partly double-stranded DNA. ³ Hepatitis B virus (HBV) causes a range of diseases, including self-limited hepatitis, acute fulminant hepatitis, chronic hepatitis, and liver cirrhosis and hepatocellular cancer, among other problems.

II. REVIEW OF LITERATURE

Hippocrates, who lived in the fifth century B.C., was the first to describe epidemic jaundice. In 1983, German shipyard workers were given a smallpox vaccination that included human lymph, which led to the discovery of the first documented instances of Hepatitis B. In 1940, a bigger incidence of hepatitis was seen in troops who had received the yellow fever vaccination that included human serum, according to British doctor F.O. MacCallum.

His namesake hepatitis viruses—hepatitis A, which is disseminated by contaminated food and water, and hepatitis B, which is transmitted through contact with infected blood—were created by him. For his studies on protein polymorphism, Dr. Baruch Blumberg began collecting blood samples from people all around the globe in the late 1950s. Patients with haemophilia or leukaemia who had undergone many transfusions were studied by Dr. Baruch in 1963. Using a panel of 24 sera from healthy persons, these samples were examined for the presence of isoprecipitins. There was a distinct precipitin line created by two haemophilia sera and one panel sera from an aboriginal Australian, but no such line was seen with the other sera. We refer to this novel protein as the Australian antigen. In 1970, the whole Hepatitis B virus was found in blood samples by D.S. Dane using electron microscopy. This finding was the basis for Dr. Blumberg's 1976 Nobel Prize in medicine.

III. OBJECTIVES OF STUDY

- Determine the HBsAg seroprevalence in a hospital-based population across age categories.
- To determine the scope of the disease's spread in the community and the key variables contributing to it.
- This study aims to evaluate the HBsAg marker in patients and compare it with real-time PCR as a tool to identify active hepatitis B infection in patients suspected of having the virus. The goal is to detect the hepatitis B surface antigen using a quick card test and HBV DNA using RT-PCR.

IV. MATERIALS AND METHODS

Place of study: KHAJA BANDANAWAZ TEACHING AND GENERAL HOSPITAL, KALABURGI

Study period: 1 Year. August 2021 to July 2022.

Design of Study: A cross sectional hospital based study.

Ethical committee: Prior approval obtained from institutional ethics committee .

Informed consent: Taken from each patient who participated in the study.

Sample size: Calculated by using the formula

$$N = 4pq/l^2$$

$$= 4 \times 60 \times 40 / 144$$

$$= 66$$

Where: p= 60(prevalence)

$$q = 40(100-p)$$

$$l = 20\% \text{ of } p(\text{Allowable error})$$

V. RESULTS

Interpretation of results-

1. Meriscreen HBsAg kit-



FIGURE 1- Positive test



FIGURE 2-Negative test

2. AMPLIFICATION PLOTS OF PCR FOR HBV DNA POSITIVE SAMPLES

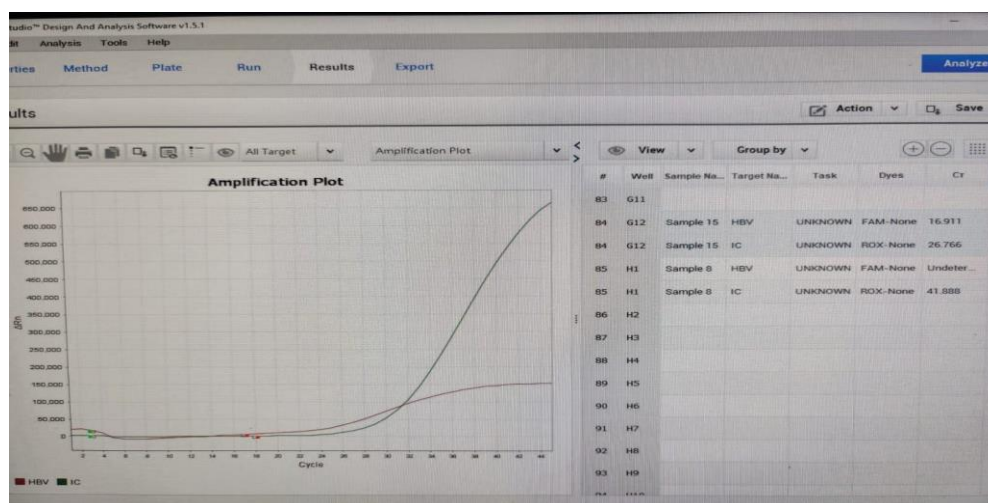


FIGURE 3- Amplification plot 1-showing cycle threshold at 18th cycle for sample no 15. Threshold of standard is 16.5.

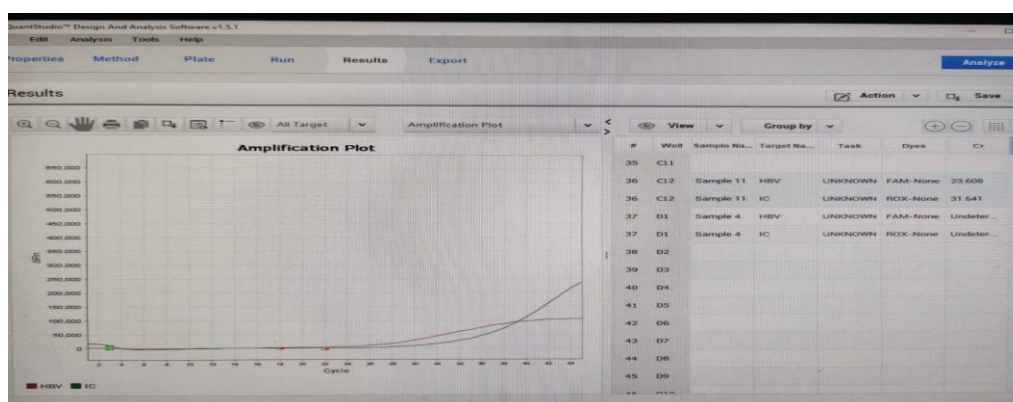


FIGURE 4- Amplification plot 2.- cycle threshold seen at 22nd cycle for sample no 11. Standard threshold value is 18.

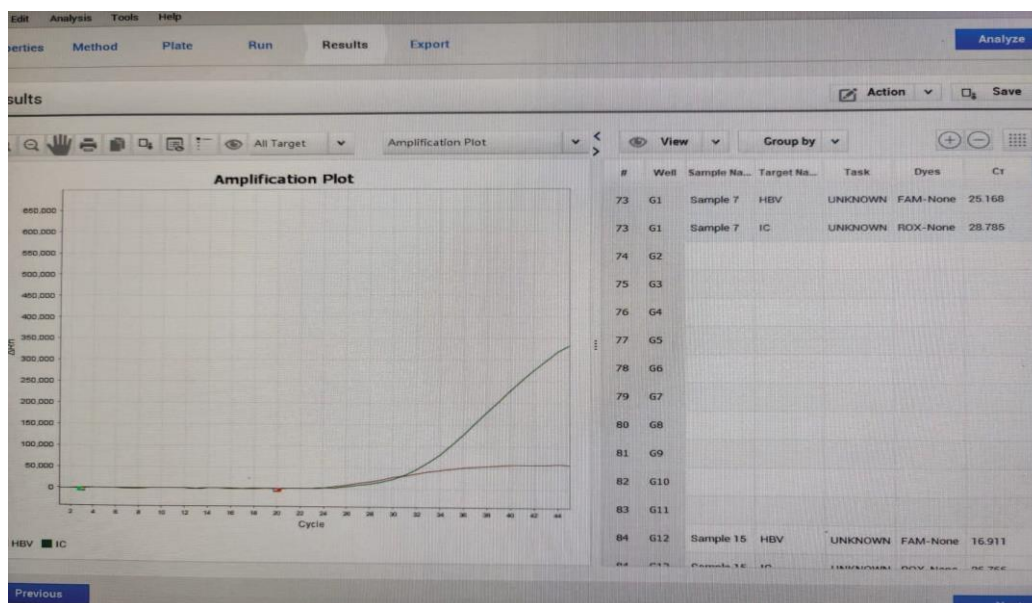


FIGURE 5- Amplification plot 3 -showing threshold value at 20th cycle for sample no 7.

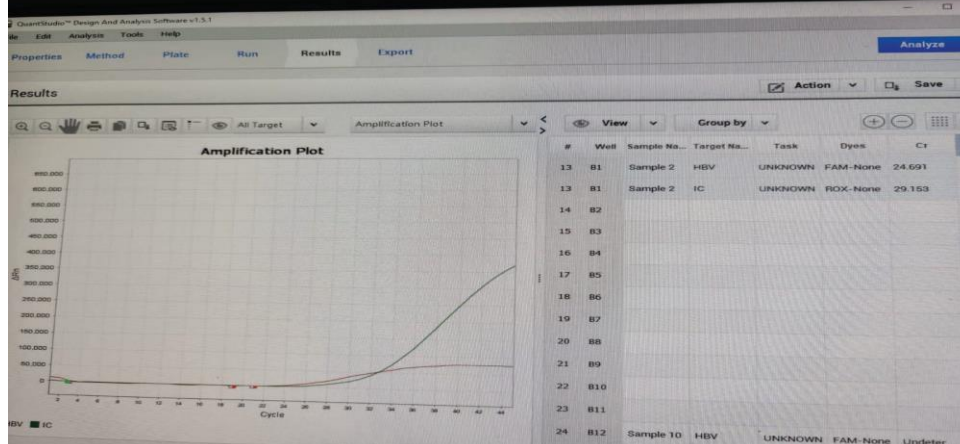


FIGURE 6- Amplification plot 4- showing threshold value at 21st cycle for sample no 2. Threshold value of standard is 19.

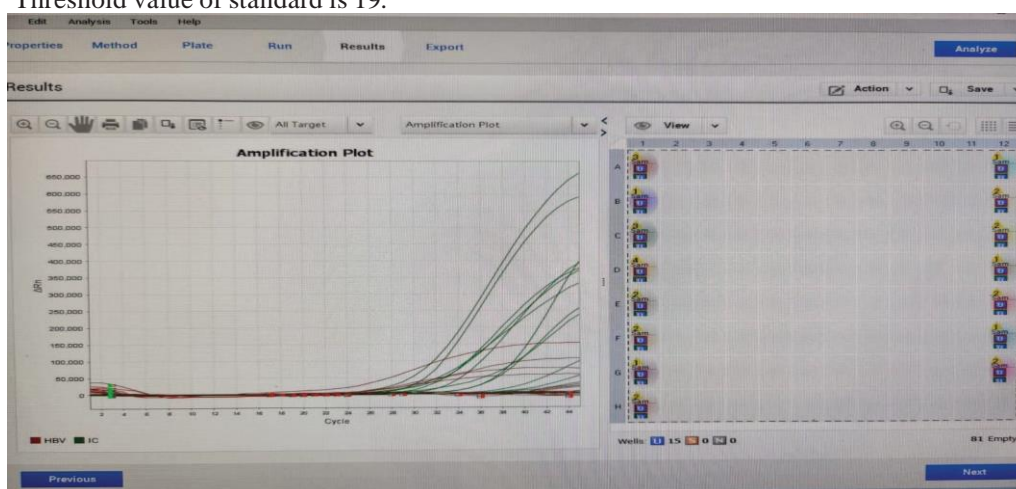


FIGURE 7- Amplification plot 5- . Threshold value of positive samples no's 15,11,7,2 is 18, 22,20,21 respectively.

OBSERVATIONS

TABLE 1 -Age wise distribution of patients.

Age in years	Number of patients	Percentage
<20 yrs	3	4.6
21-30 yrs	17	25.7
31-40 yrs	16	24.3
41-50 yrs	15	22.7
51-60 yrs	9	13.6
>60yrs	6	9.1
Total	66	100
Mean \pm SD	40.36 \pm 12.80	-----

Sixteen patients (or 25.7% of the total) were inside the 21–30 age bracket, sixteen patients (or 24.3% of the total) were in the 31–40 age bracket, and fifteen patients (or 22.7% of the total) were in the 41–50 age bracket. Patients' ages ranged from nineteen years old at the youngest to sixty-eight years old at the oldest. The patients' average age was 40.36 years.

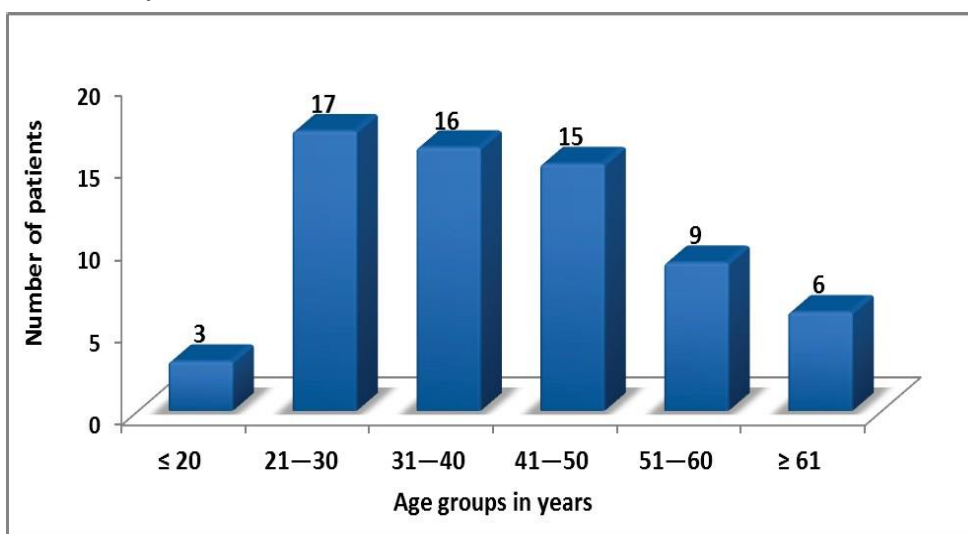


FIGURE 8 :Simple bar diagram represents age wise distribution of patients

Table 2: Gender wise distribution of patients

Gender	Number of patients	Percentage
Males	32	48.5
Females	34	51.5
Total	66	100

Study observed that, 34 (51.5%) of patients were females and 32 (48.5%) of patients were males. The male to female ratio in the study was **1:1.06**.

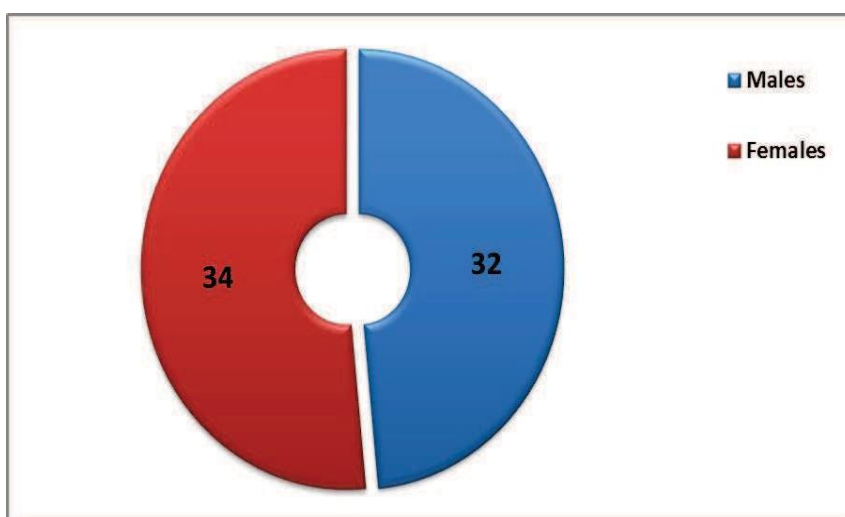


FIGURE 9 :Pie diagram represents gender wise distribution of patient

Table 3: Distribution of patients based on positive past history finding

Past history	Number of patients	Percentage
Blood transfusion	10	15.2
Drug abuse	6	9.1
History of NSI	3	4.5
Body piercing	1	1.5
Female sex worker	1	1.5
Old case of hepatitis	1	1.5
No significant history	44	66.7

Total	66	100
-------	----	-----

Out of 66 Hepatitis B patients; 44 (66.7%) of patients doesn't have any past history, 10 (15.6%) of patients had the past history of blood transfusion, followed by drug abuser 6 (9.1%) of patients and 3 (4.5%) patients had the past history of NSI.

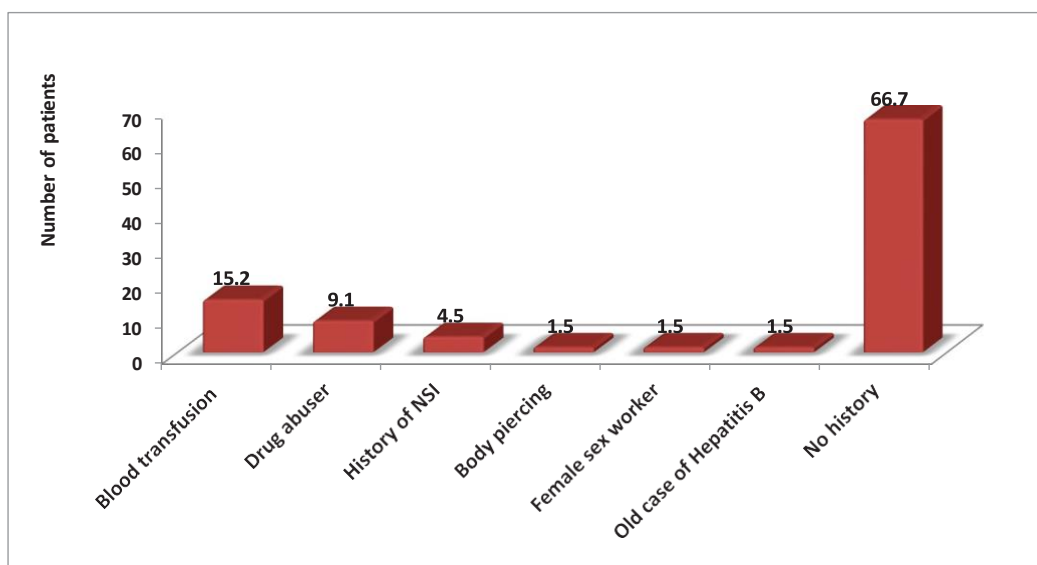


FIGURE 10 - Simple bar diagram represents past history distribution of patients

Table 4 : Distribution of patients according to history of vaccination

Vaccination status	Number of patients	Percentage
Vaccinated	26	39.4
Not vaccinated	27	40.9
Not known	13	19.7
Total	66	100

In the study out of 66 Hepatitis B patients; 26 (39.4%) of patients were vaccinated against hepatitis B, 27 (40.9%) of patients had not vaccinated and 13 (19.7%) patients had not known about vaccination.

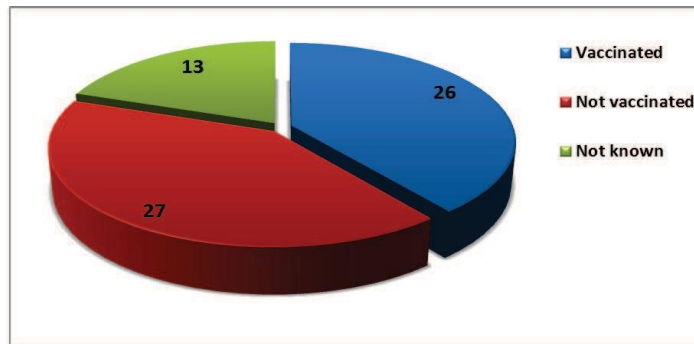


FIGURE 11-Pie diagram represents history of vaccination

Table 5: Incidence of Hepatitis B positive

RESULT	Number of patients	Percentage
Hepatitis B positive	4	6.1%
Hepatitis B negative	62	93.9%
Total	66	100%

In this study out of 66 patients; 4 (6.1%) of patients were HBsAg positive and 62 (93.9%) patients were HBsAg negative. **The incidence of HBsAg in the study was 6.1%.**

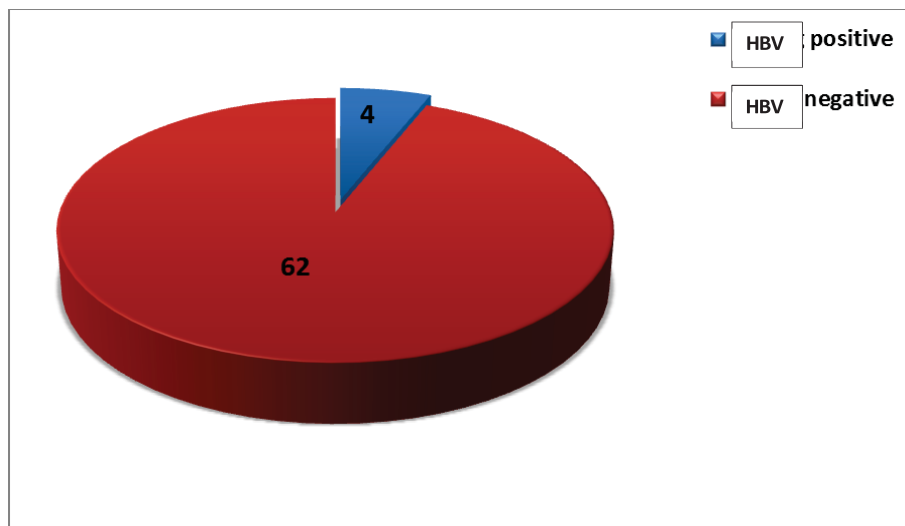


FIGURE 12-Pie diagram represents Incidence of Hepatitis B positive

Table 6: Age wise comparison of Hepatitis B positive patients

Age in years	Total	Hepatitis B positive patients		Hepatitis B negative patients	
		Number	Percentage	Number	Percentage
<20years	3	0	0.0	3	100.0
21-30 years	17	1	4.9	16	94.1
31-40 years	16	2	12.5	14	87.5
41-50 years	15	0	0.0	15	100.0
51-60 years	9	1	11.1	8	88.9
>61 years	6	0	0.0	6	100.0
Total	66	4	6.1	62	93.9
Fishers exact test		P=0.073, NS			

Out of 4 Hepatitis B positive patients; 2 (50.0%) of Hepatitis B positive patients is in the age group of 31—40 years followed by 1 (25.0%) patient is in the age group of 21—30 years and 1 (25.0%) patient is in the age group of 51—60 years. There was no statistical significant difference of distribution of Hepatitis B positive patients with respect to age groups ($P>0.05$).

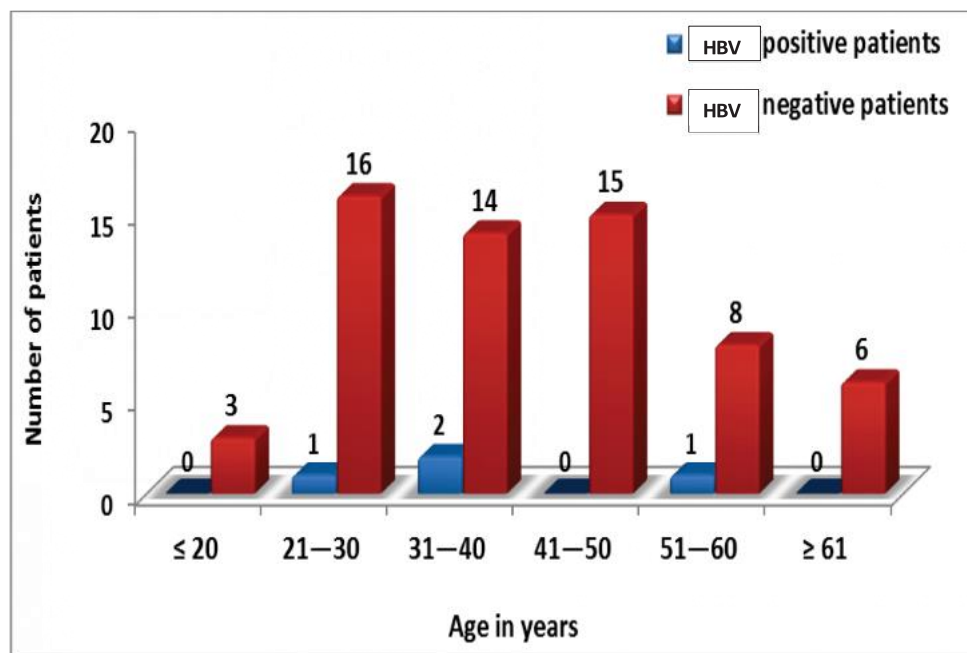


FIGURE 13:Multiple bar diagram represents age wise comparison of Hepatitis B positive patients

Table 7- Gender wise comparison of Hepatitis B positive patients

Gender	Total	Hepatitis B positive patients		Hepatitis B negative patients	
		Number	Percentage	Number	Percentage

Males	32	3	9.4	29	90.6
Females	34	1	2.9	33	97.1
Total	66	4	6.1	62	93.9
Fishers exact test	P= 0.205, NS				

Out of 4 HBsAg positive patients; 3 (75.0%) of Hepatitis B positive patients were males and 1 (25.0%) Hepatitis B positive patient was female. Percentage of Hepatitis B positive patients of males were predominant as compare to females, but not statistical significant ($P>0.05$).

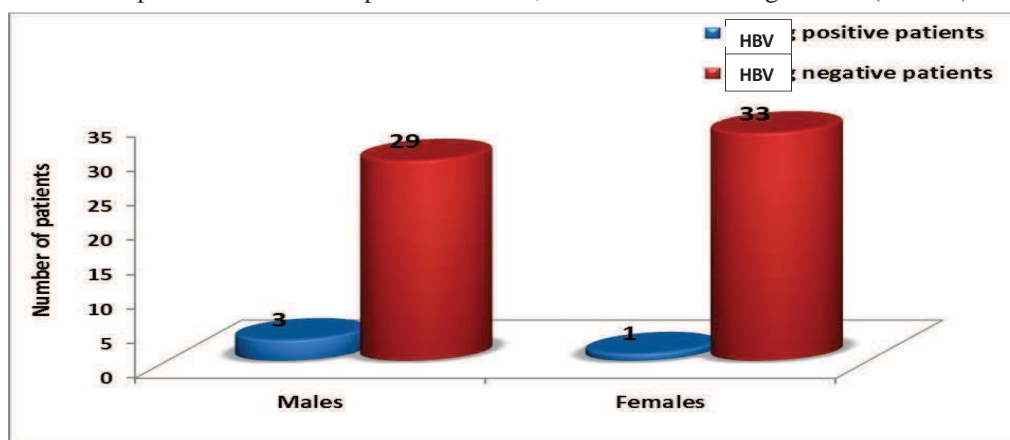


FIGURE 14-Multiple bar diagram represents gender wise comparison of Hepatitis B positive patients

Table 8: Correlate and discordant of patients with PCR

SL NO.	RESULT	HBsAg	PCR	NO. OF CASES	PERCENTAGE
1	POSITIVE	POSITIVE	CORRELATE	2	50.0
2	NEGATIVE	NEGATIVE	DISCORDANT	2	50.0
	TOTAL	----		4	100

In the study; Out of 4 positive patients 2 (50.0%) of patients were correlate with PCR and 2 (50.0%) were discordant with PCR.

Table 9: Statistical Analysis of RT-PCR test.

1	Sensitivity	66.7%
2	Specificity	100.0%
3	Positive predictive value	100.0%
4	Negative predictive value	50.0%
5	Diagnostic accuracy test	75.0%

DISCUSSION

A major issue in public health across the world, hepatitis B is a major concern. Blood and blood products are the primary vectors for the transmission of HBV infections. There are two billion HBV infections and 350 million persons who carry the virus chronically over the world. The locations are categorised as having a high prevalence (>8%), an intermediate prevalence (2-7%) or a low prevalence (<2%) of HBV infections.⁵³ Reducing the hepatitis B carrier status, the World Health Organisation (WHO) advocated for universal hepatitis B vaccination.⁵⁴ Cirrhosis of the liver and hepatocellular cancer may be triggered by this. In order to gauge the scope and spread of diseases in a community, seroprevalence studies are crucial.

This research comprised sixty-six individuals with probable hepatitis B who visited the outpatient and inpatient departments of Khaja Bandanawaz Teaching and General Hospital in Kalaburagi. Below, we explain the results that were acquired after assembling the data and comparing them with comparable research.

Prevalence of Hepatitis B:

One sixth of the participants in this trial tested positive for hepatitis B.

Given that 3% to 8% of the world's population has hepatitis B, this estimate is nearly reasonable.

The 2009 research by Sameen Afzal Junejo et al. found a similar prevalence rate of 4.6%. The seroprevalence of hepatitis B was determined to be 4.5% in studies conducted in 2008 by Hakim et al. and Noorali et al.

This study's results are at odds with those of Wang C et al.(2009) and Yan YX et al. (2010), which found a much higher incidence of 11.9% and 12.5%, respectively.

About 3-4.2 percent of the Indian population has hepatitis B. According to the majority of research, the total rate of HBsAg positive falls anywhere between two percent and eight percent.

Batham A et al. demonstrated that, according to a meta-analysis of population weights, the estimated positive prevalence is 3.1% in non-tribal groups and 11.85% in tribal communities.

Environmental, societal, and genetic variations may explain why this research found a greater incidence.

Age wise distribution of patients:

The participants' ages ranged from eighteen to seventy-eight, with a mean of forty-three years. Among those aged 31–40, the frequency was 24.3%, as shown in Table 4. Khan F et al. found a frequency of 23.83% in the 31–40 age range, which is in line with our results.

Rabyang et al. found a rate of 6.10 percent for the 31–40 age range in a comparable research in northern India, although this finding contradicts the current findings.

In line with previous research by Sangramsinghpatel et al.⁵⁸ and Khakhkharvipul M et al., we found a prevalence rate of 25% in the age groups of 21–30 and 51–60.⁵

Contrasting with the current research, a comparable one conducted in northern India by Rabyang et al. found a rate of 5.34% for the age groups of 21–30 and 51–60 years. In this particular investigation, we did not find any statistically significant differences across the age groups.

Gender wise distribution of patients :

Within the scope of this investigation, 32(48.5%) of the patients were male and 34(51.5%) were female. Rabyang et al. and Abdul Malik et al.⁷⁷ came to similar conclusions.

Results were more favourable for men (9.4%) than females (2.9%). Consistent with Rabyang et al.'s results, nevertheless, it is not statistically significant.

The greatest frequency was seen among males(10.3%) rather than females(3.7%), according to Patil SS et al.⁶⁰ and Nafees et al.

Males had a greater frequency (84% vs. 17%), according to studies conducted in Sudan and Iran.⁷⁹th percentile

Contrary to other research, including the current one, a Ghanaian study by V.E. Senoo-Dogbey and D.A. Wuaku observed a female majority (73.8% vs. 26.2% of the participants).

The prevalence of females is greater (67.2%) than men (33.8%), according to another cross-sectional research by B.Mangkara et al.

Distribution of patients based on significant Past history.

There was no prior history of hepatitis B in 44 (66.7% of the total) individuals. Ten patients, or 15.6% of the total, had previously had blood transfusions.

A total of 2.4% of the patients in the Rabyang et al. research had a transfusion history.⁷⁷ These findings are inconsistent and do not align with the current investigation.

Measures such as (1) donor education, (2) stringent donor selection criteria, (3) enhanced serological screening processes, and (4) improved blood collection and transfusion techniques may have contributed to the drop in seropositivity.

Six patients (9.1% of the total) in this research had a history of intravenous drug misuse. Sandesh K, Varghese T, Harikumar R, et al. also discovered a comparable rate of around 8% across risk categories in north Kerala.

However, 19.8% of those surveyed had a history of drug using, according to research by Jamie et al.

The results were fairly similar to those of Ali et al. (6%), with 3 patients (4.5%) having a history of NSI.

This research contradicts Rabyang et al., who found a much higher number of patients with NSI (39.7 percent).

Distribution of patients on past history of Vaccination -

This research's results are in line with those of A.S. Saddik, A.A. Alzailayee, and M.B. Foda, where 41% of 66 hepatitis B patients received a vaccine. Vaccination rates in the current study were 26 (39.4%).⁶⁷

Vaccination rates were 53.5% in the research by V.E. Senoo-Dogbey and D.A. Wauku. This result is marginally more than what the current research found.

Among the patients surveyed for this research, 40.9% had not had vaccinations and 19.7% were unaware of whether or not they had been vaccinated.

Indirect evidence that vaccination may successfully prevent persons from HBV infection may come from a study by Yiwei guo et al., which found that vaccinated individuals faced practically zero-level common risk factors of HBV infection compared to those who were not vaccinated. This result agrees with what we found in our current investigation..

Serologic versus RT-PCR Technique-

Two of the sixty-six samples were positive for HBsAg by RDT, while four samples tested positive for HBV DNA by RT-PCR. Two instances (50%) match the results of RT-PCR, whereas two cases (50%) do not.

Occult hepatitis, in which there is no HBsAg but HBV DNA in the blood, may explain the contradictory PCR findings shown in this investigation.

This conclusion is in line with what Maria Belopolskaya et al. found in their investigation.⁶⁵

There was a perfect match between serological and RT-PCR results for Hepatitis B viral infection in the

research conducted by A.S. Sadak, A.A. Alzailayee and M.B. Foda and Hudu.S.A et al.

A research conducted in June 2022 by Iker Falces-Romero et al. found 99% concordance with PCR and a mere 1% result that did not match.No.

In a separate research from 2010, Al Shaban Z.O. et al. found that RT-PCR detected a positive result in 14.58% of the total samples, compared to 6.25% of the samples that tested positive for serology.

Statistical Analysis:

SENSITIVITY-

Consistent with the results of D. Bulent et al. (74.3%), the current investigation found that RT-PCR had a sensitivity of 66.7%.

C. Daniel et al. found that a multiplex RT-PCR test could identify hepatitis B, hepatitis C, and human immunodeficiency virus type 1 with a sensitivity of 95%. No. 87 Compared to the 66.7% found in the current research, this figure is quite high.

Another research that contradicts the current one, by S.Priyanka et al., found a diagnostic sensitivity of 97.4 percent.

The sensitivity level was found to be 90% in the research conducted by V. Alicia et al.

SPECIFICITY-

This study's RT-PCR specificity of 100% is in agreement with previous research by V. Alicia et al. 89, D. Buulent et al., and C. Daniel et al.

There was complete specificity in the research by P. Shantanu, J. Amita, and J. Bhawana. In contrast to the current study, the lone research conducted by S.Priyanka et al. demonstrated a much lower specificity of 99.4 percent.

POSITIVE PREDICTIVE VALUE-

A perfect 100% positive prediction value was achieved in this investigation.

Consistent with the current research, S.Priyanka et al. demonstrated a lower positive predictive value of 97.4%.

NEGATIVE PREDICTIVE VALUE-

In contrast to the 99.4 percent found in the research by S. Priyanka et al., the current investigation reveals a negative predictive value of 50%.

In terms of negative predictive value, no other studies corroborated the results of the current investigation.

DIAGNOSTIC ACCURACY –

The current research found that the test had a diagnosis accuracy of 75%. Neither these results nor any studies that found them to be inconsistent were located.

Table 10: COMPARISION OF STATISTICAL ANALYSIS OF DIFFERENT STUDIES

	PRESENT STUDY	D.Bulient et al	C.Daniel et al	S. Priyanka et al	V.Alicia et al
SENSITIVITY	66.7%	74.3%	95%	97.45	90%

SPECIFICITY	100%	100%	100%	99.4%	100%
POSITIVE PREDICTIVE VALUE	100%	-	-	97.4%	-
NEGATIVE PREDICTIVE VALUE	50%	-	-	99.4%	-
DIAGNOSTIC ACCURACY	75%	-	-	-	-

VI. SUMMARY AND CONCLUSION

CONCLUSION

Hepatitis B prevalence was evaluated in patients visiting tertiary care hospitals in this research. Hepatitis B was more common in men than in women, with a prevalence of 6.1%.

Among those aged 31–40, the frequency was 24.3%, which was the highest of any age group. In this investigation, we did not find any statistically significant differences across the age groups. Disease transmission patterns and size are estimated using prevalence studies.

A very little medical history was present in 66.7% of individuals. There was a 15.6% prevalence of transfusion history, a 9.1% prevalence of intravenous drug misuse, and a 4.5% prevalence of needle stick injuries.

Out of the total number of patients, 27 (or 40.9%) had not had a Hepatitis B vaccine, whereas 26 (or 39.4%) had. Only thirteen patients (19.7%) knew if they had been vaccinated.

A simple, inexpensive, and quick way to detect HBsAg is using a rapid card test.

Since real-time PCR is highly specific for HBV DNA, it is considered the gold standard. Treatment plans benefit greatly from early identification and diagnosis. Additionally, it aids in transfusion blood screening, which in turn reduces the transmission of hepatitis B.

Two of the sixty-six samples were positive for HBsAg by RDT, while four samples tested positive for HBV DNA by RT-PCR. Two instances (50%) match the results of RT-PCR, whereas two cases (50%) do not.

The current investigation found that RT-PCR has a 66.7% sensitivity, a 100% specificity, and a 100% positive predictive value. Both the diagnostic accuracy and the negative predictive value are 75%.

Strategies for development, adaptation, and prevention may benefit from more research on genotype distribution. The advancement of the illness and its consequences may be averted with early detection.

SUMMARY

- The research comprised 66 individuals who had a prospective history of hepatitis B virus infection.
- A meriscreen fast card test for HBsAg and an RT-PCR for HBV DNA were performed concurrently on the blood and serum samples taken from the patients.
- The majority of the patients, 25.7%, were between the ages of 21 and 30.
- Of the patients, 32 were men and 34 were females, making it 51.5% female.
- A very little medical history was present in 66.7% of individuals. Yet, 10 individuals, or 15.6%, had previously had blood transfusions.

- Twenty-seven individuals, or 40.9%, did not get a Hepatitis B vaccine, whereas twenty-six, or 39.4%, did. Of the remaining 13 patients, 19.7 percent were unaware of whether or not they had been vaccinated.
- Two instances (or 3.05 percent) were found to be positive on serology by meriscreen card test, according to analysis by HBsAg.
- Four instances (6.1%) were found to have HBV DNA after PCR analysis.
- Two patients (or 50%) are between the 31–40 age bracket, one(25%) is between the ages of 21 and 30, and one(25%) is between the ages of 51 and 60.
- When looking at the distribution of Hepatitis B positive patients by age group, there was no statistically significant difference ($P>0.05$).
- Hepatitis B positive individuals were more common in men than in females, however this difference was not statistically significant ($P>0.05$).
- Of the four patients who tested positive, two (or 50%) showed PCR concordance and two (or 50%) showed PCR discordance.
- Occult hepatitis, in which there is no HBsAg but HBV DNA in the blood, may explain the contradictory PCR findings shown in this investigation.
- The sensitivity of PCR for HBV DNA is 66.7%, specificity is 100%, positive predictive value is 100%, and accuracy is 75%.

REFERENCES

1. World Health Organization.2020.Hepatitis B. Fact sheet. World Health Organization, Geneva, Switzerland.
2. Sibnarayan Datta. An overview of molecular epidemiology of HBV in India; Virology Journal 2008, 5:156.
3. Alexandra Valsamakis. Molecular Testing in the Diagnosis and Management of Chronic Hepatitis B. Clinical Microbiology reviews. July 2007; 20(3):426-439.
4. Pankaj Puri. Tackling Hepatitis B disease burden in India. J Clin Exp Hepatol 2014; 4: 312-319.
5. Kurein T, et al. (2005). Hepatitis B Infection In Tamil Nadu, India. Indian J Med Res; 121: 670-675
6. Francis J M. Update on diagnosis, management and prevention of Hepatitis B Virus infection. Clinical microbiological reviews, April 1999; 12(2):351-366.
7. Margiue Patlak, Hepatitis B story. National academy of Sciences Febuary 2000:1-8
8. Christoph Seeger, Fabein Zoulim, William S Mason. Fields virology; 6thEdition 2013.; Philadelphia: Lippincott Williams
9. Wolfram H.Gerlich and Micheal Kann. (2005) Topley and Wilson .10th Edition.London:Hodder Arnold.