

Detection of Hepatitis B Virus DNA Positive among the Hepatitis B Virus Surface Antigen Positive Sample in a Tertiary Care Hospital of Mid-Western Nepal

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ABSTRACT

Introduction: Hepatitis B virus infection poses a serious risk to public health around the globe, especially in developing countries, and is leading cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. Hepatitis B virus DNA is used now more often to detect Hepatitis B virus infection in cases when serologic markers are insufficient, to detect viral replication and infectivity, and to assess prognosis and treatment. The study aimed to determine detection of hepatitis B DNA (HBV DNA) among Hepatitis B surface antigen (HBsAg) among positive samples in a tertiary care hospital of western Nepal.

Methods: A descriptive cross-sectional study was conducted at Department of Microbiology, Nepalgunj Medical College Teaching Hospital from January 2021 to July 2024 after receiving ethical approval from hospital Institutional Review Board. The serum samples of all patients were screened for HBsAg using Immuno-Chromatography Technique, and HBV DNA was further detected from HBsAg-positive sera by Real-time PCR test using Alto Star 1.5 HBV PCR Kit. This study included 53432 patients (inpatients and outpatients) attending hospital from January 2021 to July 2024. Point estimate at 99% Confidence Interval was calculated.

Results: Of 53,432 patients, 358 (0.67%) (0.58%–0.76% at 99% Confidence Interval) were confirmed as HBsAg positive. The mean age of patients who tested HBsAg-positive was 29.89 ± 17.06 years. The highest number of HBsAg-positive samples came from age 21-30, with 110 (30.72%) samples. Out of 358 HBsAg-positive samples, 272 (75.97%) yielded HBV DNA test positive.

Conclusions: The HBsAg positive samples is higher compare to study done in similar setting. High rate of HBsAg-positive/HBV DNA-negative results indicates that rapid diagnostic tests can yield a substantial number of results not indicative of active replication.

Keywords: DNA; HBsAg; polymerase chain reaction, prevalence.

INTRODUCTION

Hepatitis B (HB) is a significant global health problem with serious liver infection caused by Hepatitis B virus. and is the leading cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. The World Health Organization (WHO) estimates 1.5 million new cases of Hepatitis B yearly, and 296 million individuals worldwide are estimated to have a chronic infection.¹ Highest rates of HBV infection are seen in Sub-Saharan Africa and East Asia, where 5–10%

of general population is estimated to be infected; in contrast, the Indian Subcontinent has a 2–5% prevalence rate varies.^{2,3} Nepal's National Strategy for Viral Hepatitis B and C 2023-2030, published by the NCASC and WHO, cites an HBsAg seroprevalence of 0.9% in the general population.⁴ Enzyme linked immunosorbent assay (ELISA) and chemiluminescent microparticle immunoassays (CMIA) are two primary techniques used nowadays in clinical laboratories to detect HBV serological markers. Quantitative HBV DNA detection provides a clear distinction between the various

stages of infection by directly and flexibly monitoring HBV DNA replication in the serum.⁵ Detecting HBV DNA is a reliable marker of replication activity; higher HBV DNA titers have been associated with both an increased risk of hepatocellular carcinoma and a more rapid progression of the disease.⁶

The study aimed to determine detection of hepatitis B surface antigen among samples by using real-time PCR method and rapid diagnostic method in a tertiary care hospital of western Nepal.

METHODS

This descriptive cross-sectional study was conducted at the Department of Microbiology, Nepalgunj Medical College Teaching Hospital (NGMCTH), Kohalpur, Banke. The data were obtained from the laboratory register of the Clinical Microbiology Laboratory and Molecular Laboratory of NGMCTH. This study included 53432 patients (inpatients and outpatients) attending NGMCTH from January 2021 to July 2024. The institutional review committee of NGMCTH approved the study protocol [20/81-82 dated 27 September 2024].

The sample size was calculated using formula:

$$n = Z^2 \times p \times q / e^2$$

$$= 2.64^2 \times 0.1 \times 0.9 / 0.01^2$$

$$= 5972$$

Where,

n= required sample size

Z= 2.64 at 99% confidence interval

p= prevalence taken from previous studies

q= 1-p

e= margin of error, 1%

Taking non-response rate 10%, the calculate sample size is 6636. However, 53432 samples were taken for the study.

All the patients were requested by the clinician to perform HBsAg screening and HBV DNA detection upon positive findings with RDT as a confirmatory test and to monitor viral load. The appropriate amount of blood was collected using an aseptic technique. The serum samples of all patients were screened for HBsAg using the Immuno-Chromatography Technique (ICT). Commercially available ICT kit (HEPA CARD: J. Mitra & Co., Pt. Ltd, New Delhi, India) was used, and samples were processed according to the manufacturer's guidelines. The Kit that we used could detect 0.5ng/ml HBsAg.⁷

Viral DNA was extracted from serum samples using the Spin

Star™ Extraction Kit (ADT Biotech, Malaysia), following the manufacturer's instructions. The quality and quantity of the extracted DNA were confirmed functionally by the subsequent amplification of the Internal Control (IC) within the AltoStar® 1.5 HBV PCR Kit (Altona Diagnostics), which was successfully detected in all samples, verifying the absence of PCR inhibitors and the success of the extraction. Briefly, 200 µL lysis buffer and 50 µL proteinase K with 15 µL carrier RNA were added to a 1.5-ml tube. Then, 200 µL of serum was added to the top mix. The samples were incubated at 65 °C for 15 min, and then 280 µL of ethanol was added. Centrifuging was performed at 8000 rpm for 1 min. 500 µL wash buffer was added and centrifuged in the same manner as previously. Finally, 50 µL elution buffer was added and centrifuged. The final extraction was kept at 4 °C for real-time PCR.

The DNA extract was subjected to PCR using the Alto Star 1.5 HBV PCR Kit. for the proliferation of a 134-bp region of the genome of hepatitis B. Before the start, the patient serum sample and reaction components were brought to room temperature. For each patient sample, four microtubes (0.2 ml) were used for positive and negative control reactions for real-time PCR and positive and negative controls for extraction, respectively. Forty-five microliters of Master Mix and 2.5 µL internal controls were added to the tubes. Subsequently, 45-µl patient sample and control were added to them. The sequence of the forward primer was 5'-TAT GTT TCC CTC CTG CTG CT-3', and the sequence of the reverse primer was 5'-CCC CCA ACT CCC AAT TCTAT-3'. For undetectable HBV DNA levels, the assay's cut-off value was < 10 HBV DNA IU/ml (1 IU per millilitre = 5.68 copies per millilitre). Therefore, samples with HBV DNA levels ≥ 10 IU/ml (56.8 copies per ml) were deemed positive.^{9,10}

The data were entered into SPSS v. 25 software. Quantitative data was reported as percentages. Other demographic data, like age and sex, were also noted. The data were cleaned manually and analysed using SPSS v. 25. Values were presented as mean ± standard deviations (M±SD).

RESULTS

Of the 53432 patients, 358 (0.67%) (0.58%–0.76% at 99% Confidence Interval) were confirmed as HBsAg positive and were further assessed for HBV viral load and DNA testing. Among the 358 HBsAg seropositive blood samples, a total of 272 (75.98%) blood samples were HBV DNA positive.

The mean age of patients who tested HBsAg positive was 29.89 ± 17.055 years, with a minimum and maximum age of 1 and 69 years, respectively. We classified the age range into seven categories: <10, 11-20, 21-30, 31-40, 41-50, 51-60, and >60 years. Of the 358 samples, the highest number of HBsAg-positive samples came from age 21-30, with

110 (30.72%) samples, followed by those aged 51-60 with 55 (15.36%), and >60 years with 54 (15%), respectively (Table 1).

Table 1. Age and gender wise distribution of HBsAg-positive patients (n= 358).

Age group in year	Gender		Total
	Male	Female	
≤10	2	0	2 (0.55)
11-20	17	6	23 (6.42)
21-30	59	51	110 (30.72)
31-40	40	23	43 (0.55)
41-50	26	25	51 (12)
51-60	39	16	55 (15.36)
>60	41	13	54 (15.08)
Total	224	134	358 (100)

Among 358 HBsAg-positive samples, 272 (75.9%) yielded HBV DNA test positive. The maximum number of HBV DNA-positive patients was among males: 178 (79.46%), followed by females: 94 (70.1%). The male-to-female ratio for HBV DNA positivity was 1.89:1 (Table 2).

Table 2. The gender wise distribution of HBV DNA positivity and negativity.

Gender	HBV DNA (Positive)	HBV DNA (Negative)
Male	178 (79.46)	46 (20.54)
Female	94 (70.1)	40 (29.85)
Total	272 (75.9)	46 (24.1)

The mean age of patients who tested HBV DNA positive was 29.47 ± 16.51 years. Of the 358 samples, 272 (76%) tested positive for HBV DNA. The highest number of HBV DNA positive samples came from age 21-30, with 78 (28.67%) samples, followed by those aged >60 with 45 (16.54%), 51-60 years with 44 (16.18%), 31-40 years with 42 (15.44%), and 11-20 years with 21(7.72%), respectively (Table 3).

Table 3. The age distribution of HBV DNA positivity and negativity.

Age group in year	Total No. of samples	HBV DNA Positive (n%)	HBV DNA Negative (n%)
≤ 10	2	2 (0.73)	0
11-20	23	21 (7.72)	2 (2.32)

21-30	110	78 (28.67)	32 (37.21)
31-40	63	42 (15.44)	21(24.41)
41-50	51	40 (14.70)	11(12.79)
51-60	55	44 (16.18)	11(12.79)
>60	54	45 (16.54)	9 (10.46)
Total	358	272 (100)	86 (100)

Among 272 samples that tested positive for HBV DNA, the highest positive rate (95.22%) was found in patients of the Medicine department, followed by Gynaecology (2.57%) and Orthopaedics (0.73%) departments (Table 4).

Table 4. The clinical speciality-wise distribution of HBV DNA positivity and negativity.

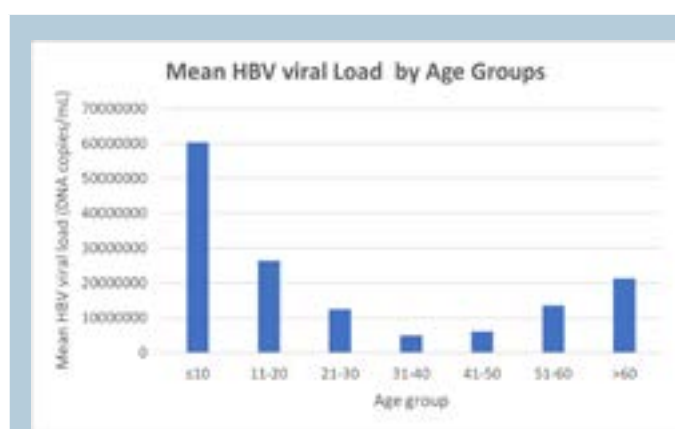
Department	Total No. of samples	HBV DNA Positive n (%)	HBV DNA Negative n (%)
Medicine	340	259 (95.22)	81 (94.18)
Gynaecology and Obstetrics	10	7 (2.57)	3 (3.49)
Surgery	4	4 (1.47)	0 (0)
Orthopaedics	4	2 (0.73)	2 (2.32)
Total	358	272 (100)	86 (100)

The observed mean viral load value, determined through statistical analysis, is $13500031.02 \pm 36672207.52$ copies/ml with a 95% Confidence Interval (CI) (lower mean: 9122345.4; upper mean: 17877716.6). The mean viral load found for the age group 21-30 was $12535404.8 \pm 35291479.5$ DNA copies/ml and $21366913.74 \pm 48870950.32$ DNA copies/ml for age group >60 years. The median value at 75% Percentile is calculated as 284134.2 DNA copies/ml. The median HBV viral loads for males and females were 35392 and 3135126 DNA copies/ml, respectively (Table 5).

Table 5.The age-wise distribution of the HBV viral load.

Age group in year	n	Mean $\pm$ SD	Median	Lower 95% CI	Upper 95% CI
≤ 10	2	60390811.6 $\pm$ 1579123.9	60390811.6	46202946.5	74578676.7
11-20	21	26476123.3 $\pm$ 42297458.2	189000	7222551.1	45729695.4
21-30	78	12535404.8 $\pm$ 35291479.5	31528	4578405.6	20492403.9
31-40	42	5009071.2 $\pm$ 18328748.8	31455	-702568.96	10720711.4
41-50	40	6132512.7 $\pm$ 29410209	23240	-3273328.5	15538353.8
51-60	44	13642591.7 $\pm$ 38634070.6	17402	1896760.7	25388422.8
>60	45	21366913.7 $\pm$ 48870950.3	105224	6684454.8	36049372.6
Total	272	13500031 $\pm$ 36672207.5	35126	9122345.4	17877716.6

The mean viral load by age group is shown in Figure 1.

**Figure 1.** Prevalence of HBV viral DNA among different age group

DISCUSSION

Hepatitis B virus (HBV) infection poses a serious risk to public health around the globe, especially in developing countries.¹¹ The first detectable antigen during an acute HBV infection is HBsAg, which is used as a screening test. It is detectable in the sera 2–8 weeks before the onset of symptoms and disappears in 2–6 months.¹² Before the detection of HBV DNA by molecular diagnostic procedures, HBeAg was considered the most significant indicator of viraemia. However, it

has been recognized that assessing HBeAg and anti-HBe as viraemia indicators is not a valid approach since the discovery of HBeAg negative mutants.¹³

The present study was conducted among patients attending a tertiary care hospital at Kohalpur, Banke, to detect HBsAg and further HBV DNA in HBsAg positive patients. During the study period, a total of 358 samples were tested positive for HBsAg out of 53432 samples tested. In this study, the prevalence of HBsAg among patients is 0.67 %, slightly lower than the prevalence rate in Nepal (0.9%).¹⁴ This confirms the declining trends of HBV infection worldwide, and in Asian and African countries in particular. This could be a consequence of the integration of hepatitis B vaccination in developing and underdeveloped countries, where higher levels of immunization are observed among young people.¹⁵ In this study, out of 358 HBsAg positive patients, 224 were male (62.5%) and 134 were female (37.4 %), constituting male: female ratio as 1.67:1. This discrepancy in gender distribution within the research population would be explained by the fact that men are more exposed to risk factors. This could be due to a higher prevalence of HBV infection in males as they are more exposed to the risk factors. In Nepal, females are mostly involved in household chores due to cultural preferences, whereas men are better educated and work outside the home or in the agricultural field. Furthermore, men are more likely to indulge in activities that transmit the infection,

such as visiting barbers and having several sexual partners, and also a common practice of nose/ear piercing in certain communities of Nepal, which increases their risk of contracting the disease. This increased HBV infection in males has been observed in several other studies as well.^{16,17}

In the present study, HBV DNA was detected in 75.9% of HBsAg positive specimens. Several other studies also have reported HBV DNA positivity in HBsAg positive patients, including Kùlah et al. 18 53%, and Bati N et al.¹² 71%. According to studies conducted on patients with HBsAg, 29 % of Bati N et al.¹⁰ reported HBV DNA negativity. Similar to previous studies, we have discovered that 24.02 % of HBsAg positive patients in our study had HBV DNA negative.

In the present study, males were the majority of 272 (75.98 %) serum samples that yielded positive HBV DNA test results, with 178 patients (65.4%). It also indicated a significant relationship between the existence of HBV DNA and gender. There was no significant association between the existence of HBV DNA and age of the patient. The present study showed that HBV DNA is more prevalent in the 21-30 age group. In contrast, the study by Uddin M et al. (2014)¹⁹ from Bangladesh has reported the highest prevalence of HBV DNA in the age group 36-50 years.

In the present study, mean viral load value determined was $13500031.02 \pm 36672207.52$ copies/ml with a 95% Confidence Interval (CI) (lower mean: 9122345.4; upper mean: 17877716.6). The mean viral load found for the age group 21-30 was $12535404.8 \pm 35291479.5$ DNA copies/ml and $21366913.74 \pm 48870950.32$ DNA copies/ml for age group >60 years. Median value at 75% percentile is calculated as 284134.2 DNA copies/ml.

In Nepal, the prevalence of HBV infection varies among populations. According to the National Strategy for Viral Hepatitis Prevention and Control (2023–2030),²⁰ the estimated HBsAg seroprevalence in the general population is approximately 0.9%. However, higher rates are observed in certain high-risk groups, such as people who inject drugs (PWID), where prevalence can reach 5–7%, and among incarcerated individuals and migrants.²¹

HBsAg is still important as a serologic marker for the de-

tection of hepatitis B virus infection. Furthermore, HBV DNA detection is the most important molecular test in determining viral replication and infectivity, and also in the evaluation of antiviral response and the prognosis. The major limitation of the study was that we have used the RDT kit for screening the HBsAg positive cases and HBV DNA PCR for confirmation of the HBV infections using the test with different sensitivity and specificity, which made it difficult to evaluate the true prevalence of HBV DNA among patients and compare the prevalence between HBsAg positivity and HBV DNA positivity. The prevalence of HBV DNA could have been estimated if all patients had tested both for HBsAg and HBV DNA in the same study population. Other limitation of this study is that HBV DNA PCR was not performed on RDT-negative samples. Therefore, we cannot calculate the absolute sensitivity and specificity of the RDT or rule out occult HBV infection in the seronegative population. Future prospective studies should include PCR testing on a subset of negative samples to address this important question.

CONCLUSIONS

The overall prevalence rate of HBsAg observed was slightly lower than the prevalence rate in Nepal. HBV DNA positivity is high in the age group of 21–30 years, and males are more infected than females. Our study highlights a critical limitation of serological assays in confirming active viremia. The integration of real-time PCR is therefore imperative in the clinical algorithm for HBV diagnosis to confirm active infection, determine treatment eligibility, and prevent the misclassification of patients.

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Conflict of Interest: None.

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