ONLINE ISSN :3007-309X
PRINT ISSN :3007-3081

Journal of Medical & Health Sciences Review

COMPARATIVE ANALYSIS OF MOLECULAR AND CHEMILUMINESCENT ASSAYS FOR HBeAg AND ANTI-HBe ANTIBODIES QUANTIFICATION IN CHRONIC HEPATITIS B PATIENTS

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ARTICLE INFO

Keywords: Hepatitis B virus, HBeAg, Anti-HBeAb, CLIA, Real-time PCR, HBV DNA, Pakistan

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ABSTRACT

Hepatitis B virus (HBV) is a globally significant pathogen responsible for chronic liver infection, cirrhosis, and hepatocellular carcinoma. In Pakistan, where approximately 3% of the population is affected, HBV is considered endemic. Key risk factors include unsafe medical practices, needle reuse, and poor hygiene in healthcare and community settings. This study aimed to assess the correlation between HBV DNA quantification using real-time PCR and two chemiluminescence immunoassay (CLIA)-based serological markers—hepatitis B e antigen (HBeAg) and anti-hepatitis B e antibodies (Anti-HBeAb)—among patients with chronic hepatitis B in District Swabi, Pakistan. A total of 100 serum samples were collected from patients diagnosed with chronic hepatitis B at tertiary and rural healthcare centers. HBV DNA levels were measured using real-time PCR, while HBeAg and Anti-HBeAb levels were analyzed using CLIA. Most patients (89%) exhibited low HBeAg titers (1.6–5.0 IU/mL), suggesting minimal viral replication. Only a small proportion showed moderate (5–10 IU/mL; 2%), strong (20.1–30.0 IU/mL; 3%), or very strong (>30 IU/mL; 1%) reactivity. Anti-HBeAb was commonly detected, indicating potential viral seroconversion. The correlation between HBeAg and HBV DNA levels was found to be variable, with weak concordance in several cases. CLIA-based detection of HBeAg and Anti-HBeAb may serve as a cost-effective alternative to molecular assays in low-resource settings. However, due to their limited predictive value for viral load, they are best used in conjunction with molecular diagnostics for accurate disease staging and treatment monitoring.

INTRODUCTION

Hepatitis B virus (HBV) infection remains a persistent global health challenge, accounting for considerable morbidity and mortality worldwide(S. W. A. Shah et al., 2025). The World Health Organization estimates that over 296 million individuals are living with chronic HBV infection, with approximately 820,000 deaths each year due to cirrhosis, hepatocellular carcinoma (HCC), and liver failure related to chronic hepatitis B(Beard & Hill, 2024). HBV is a DNA virus primarily transmitted through perinatal exposure, unprotected sexual contact, unsafe injections, and exposure to infected blood or body fluids. In endemic regions such as Pakistan, the burden of HBV is particularly severe. Epidemiological data indicate that approximately 4% of the population are chronic carriers, and up to 38% of individuals show serological evidence of past exposure to HBV (Sievert et al., 2011). This high prevalence is compounded by inadequate vaccination coverage, unsafe therapeutic practices, re-use of syringes and razors, and limited access to diagnostics, especially in rural settings(Jones et al., 2011). Although the clinical spectrum of HBV ranges from inactive carriers to fulminant hepatitis, a significant portion of infected individuals remain asymptomatic during the immune tolerant phase, characterized by normal alanine aminotransferase (ALT) levels, high viral load (HBV DNA), and positive hepatitis B e antigen (HBeAg) status. HBeAg, a secreted product of the nucleocapsid gene, serves as a critical biomarker of active viral replication and infectivity, and is often associated with poorer prognosis and increased risk of vertical transmission(Invernizzi et al., 2016; Rizzo et al., 2022). From an immunological perspective, CHB is marked by T-cell exhaustion and impaired cytotoxic responses, especially in the presence of high antigenic loads. Seroconversion from HBeAg to anti-HBe antibodies is typically accompanied by a decline in HBV DNA levels, indicating partial immune control and clinical remission(de Almeida Pondé, 2021). As such,

quantitative analysis of HBeAg and anti-HBeAb offers meaningful insight into disease stage, therapeutic response, and viral clearance mechanisms(Kouame, 2021). Quantitative HBV DNA testing via real-time PCR is currently considered the gold standard for monitoring viral replication and guiding antiviral therapy. However, its application is limited in low-resource environments due to high cost, need for specialized equipment, and technical complexity. In contrast, chemiluminescence immunoassays (CLIA) offer a cost-effective, automated, and sensitive alternative for detecting viral antigens and antibodies(Pan et al., 2023; M. Shah et al., 2025). CLIA-based quantification of HBeAg and anti-HBeAb has shown promise in reflecting disease activity, especially in contexts where molecular diagnostics are inaccessible(Lempp et al., 2021). Despite the growing adoption of CLIA in diagnostic laboratories, few studies have rigorously evaluated its correlation with HBV DNA levels, particularly in high-burden countries like Pakistan(Waheed et al., 2019). A better understanding of this relationship could optimize resource allocation, reduce reliance on molecular testing, and improve patient stratification in remote or underserved regions(Al-Worafi, 2024).

This study investigated the correlation between molecular detection of HBV DNA and CLIA-based quantification of HBeAg and anti-HBeAb in patients with chronic hepatitis B. By comparing these diagnostic approaches, we seek to validate the clinical utility of CLIA as a viable surrogate marker for HBV monitoring in resource-limited healthcare settings.

Materials and Methods

Study Design and Setting

This cross-sectional study was conducted at the Bacha Khan Virology Laboratory, Swabi, Khyber Pakhtunkhwa, Pakistan, over a six-month period from September 2023 to February 2024.

Sample Collection

A total of 100 clinical blood samples were collected from patients diagnosed with chronic hepatitis B across various tertiary care hospitals and rural health centers in the District Swabi region. Blood samples were obtained using sterile phlebotomy procedures. All participants provided informed consent, and demographic and clinical data were documented using a standardized proforma.

Inclusion and Exclusion Criteria

Inclusion criteria: Individuals with confirmed chronic hepatitis B infection.

Exclusion criteria: Patients with non-HBV hepatitis (e.g., HCV, HAV, autoimmune hepatitis) or co-infections were excluded.

Sample Processing

Blood samples were centrifuged at 3000 rpm for 5 minutes to separate serum. Approximately 3 mL of serum was collected from each sample and stored in sterile Eppendorf tubes at –20°C until further analysis.

Molecular Confirmation of HBV Infection

Nucleic Acid Extraction

Viral DNA was extracted from 200 µL of serum using a Sansure magnetic bead-based automatic extraction system (NE48, Biotech China), following the manufacturer's protocol. Viral nucleic acids were purified through a process of physical and chemical lysis and binding to a solid-phase magnetic surface, ensuring removal of cellular contaminants.

Quantitative Real-Time PCR (qPCR)

Quantification of HBV DNA was carried out using the Sansure HBV DNA PCR Diagnostic Kit (Sansure Biotech, China) on a Bio-Rad CFX96™ Multiplex Real-Time PCR Thermal Cycler. The assay employed TaqMan-based fluorescent probes designed to target conserved regions of

the HBV genome. Fluorescence signals were detected using the FAM channel for HBV DNA quantification, and the HEX channel served to verify internal control amplification. To minimize false positives, the assay included uracil-N-glycosylase (UNG) enzyme for pre-PCR contamination control. ROX dye was used as a passive reference to normalize fluorescence fluctuations across wells.

PCR Cycling Protocol

Table 1: PCR Cycling Protocol

Step	Temperature	Duration	Cycle(S)
Enzyme Activation (Ung)	50 °C	2 minutes	1
Initial Denaturation	95 °C	2 minutes	1
Denaturation	95 °C	15 seconds	45
Annealing And Extension	58 °C	30 seconds	45
Final Hold	25 °C	10 seconds	1

Fluorescence signal intensities were recorded at the end of each extension phase. Amplification curves were automatically analyzed using Bio-Rad CFX Manager™ software to derive cycle threshold (Ct) values.

Detection of HBeAg and Anti-HBe Using Chemiluminescence Immunoassay (CLIA)

Detection and semi-quantification of HBeAg and anti-HBe antibodies were performed using a two-step chemiluminescence immunoassay (CLIA) according to the manufacturer's protocol. Briefly, microtiter wells were labeled for patient samples, calibrators, and quality control standards. A volume of 50 µL of each sample or calibrator was added to the designated wells, followed by 50 µL of enzyme-conjugated reagent. The contents were mixed manually for 10 seconds, then incubated at 37°C for 30 minutes under a covered plate to prevent evaporation. Following incubation, the wells were washed five times with 300 µL of wash buffer, allowing a 10-second soak during each cycle to ensure thorough removal of unbound materials. Subsequently, 100 µL of chemiluminescent substrate was added to each well and incubated for 5 minutes at room temperature in the dark to allow signal development. The luminescent signal was measured using a luminometer and recorded as Relative Light Units (RLU), which is proportional to the concentration of antigen or antibody in the sample.

Statistical Analysis

All statistical analyses were performed using SPSS version 16.0 (IBM Corp., USA). Descriptive statistics (meaning standard deviation, median, range, or percentages, as appropriate) were computed for demographic and clinical variables. The relationship between HBV DNA levels and HBeAg/anti-HBe levels was evaluated using Pearson's correlation coefficient. A two-tailed p-value of <0.05 was considered statistically significant.

RESULTS

HBeAg and HBeAb serological analyses were performed on 100 patient samples using CLIA and RT PCR for molecular detection, respectively. A high percentage of 89% of patients have low HBeAg (low from 1.500 1.6 to 5.00 IU/ml), and 11% have low reactivity (1.6 to 5.00 IU/ml), according to the association between HBeAg and HBeAb. Two patients exhibit moderate reactivity (5 to 10, 1.6 to 5.00 IU/ml), three patients exhibit strong reactivity (20.1 t0 30.0, 1.6 to 5.00 IU/ml), and one patient exhibits extremely strong reactivity (more than 30 IU/ml).

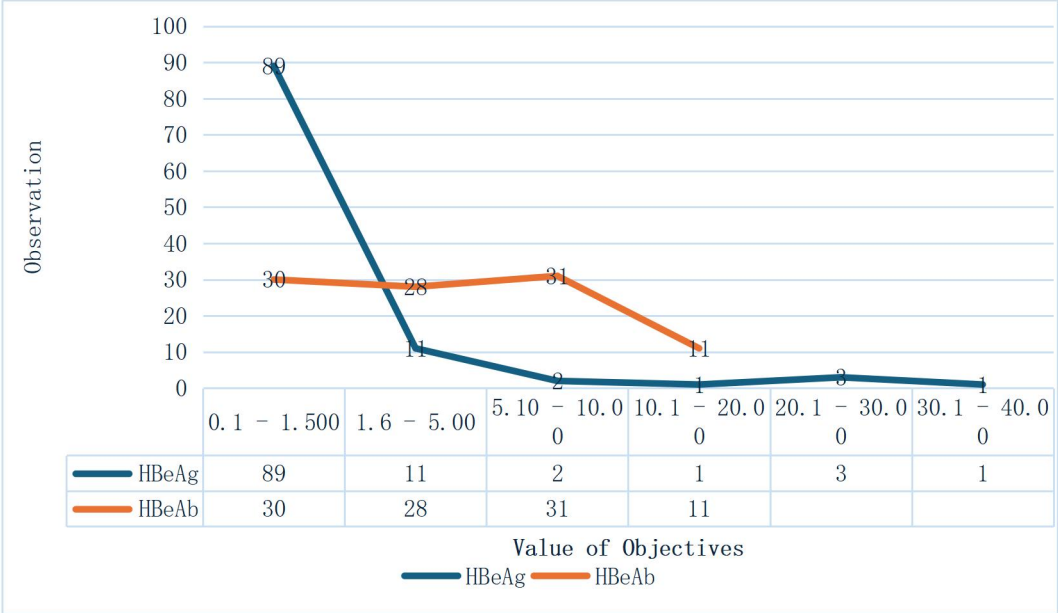


Figure 1: Line graph showing correlation between the Anti HBeAg and HBeAb reactivity.

Table 2: Column statistics of the age range of study participants who were reported positive for HBV

Number of values	100
Minimum	5.000
25% Percentile	29.00
Median	39.00
75% Percentile	54.00
Maximum	78.00
10% Percentile	23.00
90% Percentile	68.90
Mean	42.00
Std. Deviation	16.42
Std. Error of Mean	1.642
Lower 95% CI of mean	38.74
Upper 95% CI of mean	45.26
Coefficient of variation	39.1%
Geometric mean	38.59
Geometric SD factor	1.550
Sum	4200

Age range of participants

Figure 2: Arrange of the study participants who were reported positive for HBV

The age distribution of the study participants was analyzed, revealing a total of 100 observations. The age range varied widely, with the youngest participant being 5 years old and the oldest 78 years old. The descriptive statistics further delineate the age distribution, indicating that 25% of the participants fell below the age of 26, while the median age was 39 years. Additionally, 75% of the participants were under the age of 55. A deeper analysis was conducted, revealing that the 10th percentile of the age range was 19 years, while the 90th percentile was 70 years, demonstrating the breadth of age representation within the sample. The mean age of the participants was calculated to be 41.01 years, with a standard deviation of 19.94 years. The standard error of the mean was determined to be 1.061. Confidence intervals at the 95% level were computed, indicating a lower bound of 38.92 years and an upper bound of 43.10 years for the mean age. The coefficient of variation was determined to be 48.62%, suggesting moderate variability in age among the participants. The geometric mean age was found to be 35.52 years, with a geometric standard deviation factor of 1.791. In total, the sum of ages across all participants amounted to 14,477 years, providing a comprehensive overview of the age distribution within the studied population.

Table 3: Column statistics of the age range of male study participants who were reported positive for HBV

Number of values	60
Minimum	5.000
25% Percentile	27.25
Median	39.00
75% Percentile	48.75
Maximum	78.00
10% Percentile	22.00
90% Percentile	65.00
Mean	40.45
Std. Deviation	16.68
Std. Error of Mean	2.154
Lower 95% CI of mean	36.14
Upper 95% CI of mean	44.76
Coefficient of variation	41.25%
Geometric mean	36.74
Geometric SD factor	1.610
Sum	2427

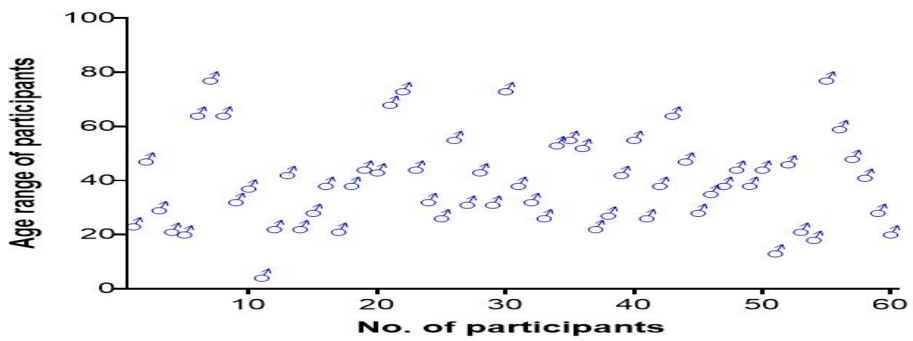


Figure 3: Age range of the male study participants who were reported positive for HBV

The male age distribution of the study participants was analyzed, revealing 60 in total of 100 observations. which ranging from a minimum of 5 years to a maximum of 78 years. The median value in years shows 39 (25%) 27.25, 75th percentile at 48.75. The 10th percentile is at 22.00 and the 90th percentile is at 65.00. The mean of the male participant values is 40.45, with a standard deviation of 16.68 and a standard error of the mean at 2.154. The lower 95% confidence interval of the mean is at 36.14 and the upper interval is at 44.76 respectively. The coefficient of variation is calculated to be 41.25%, while the geometric mean is 36.74 with a geometric standard deviation factor of 1.610. The sum of all values in the data set comprehensive is 2427.

Table 4: Column statistics of the female age range of study participants who were reported positive for HBV

Number of values	40
Minimum	22.00
25% Percentile	29.50
Median	42.00
75% Percentile	59.00
Maximum	74.00
10% Percentile	24.30
90% Percentile	69.00
Mean	44.33
Std. Deviation	15.95
Std. Error of Mean	2.522
Lower 95% CI of mean	39.22
Upper 95% CI of mean	49.43
Coefficient of variation	35.98%
Geometric mean	41.54
Geometric SD factor	1.445
Sum	1773

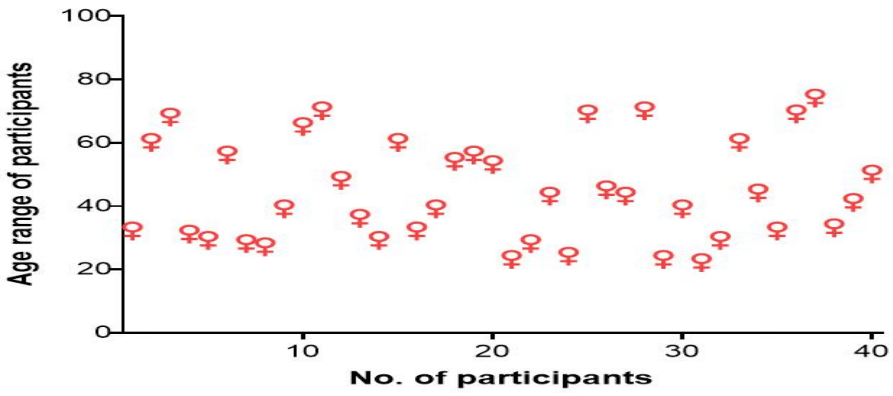


Figure 4: Age range of the female study participants who were reported positive for HBV

The observation of 100 HBV patient show female age range positivity is 40 in number which statistically analysis includes the minimum age value (22.00), the 25th percentile (29.50), the median (42.00), the 75th percentile (59.00), and the maximum value (74.00). Additionally, it shows the 10th percentile (24.30) and the 90th percentile (69.00), the mean (44.33), standard deviation is (15.95), standard error of the mean is less than (2.522), lower and upper 95% confidence intervals of the mean (39.22 and 49.43) respectively, coefficient of variation (35.98%), geometric mean (41.54), geometric standard deviation factor (1.445), and the sum of the values (1773). These values are used to describe the distribution and central tendency in study population.

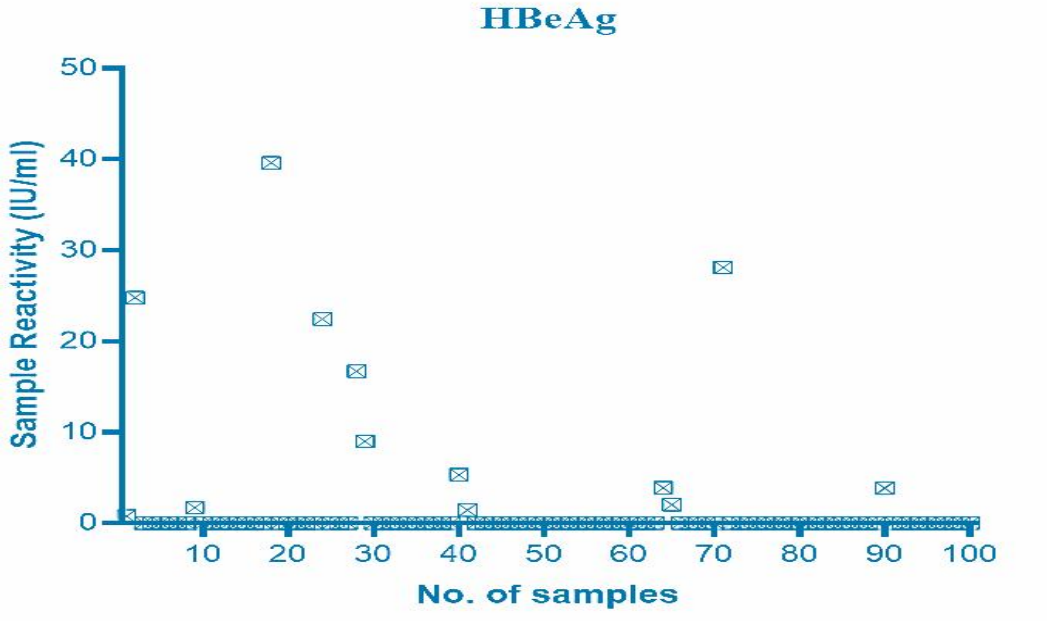


Figure 5: HBV antigen reactivity (IU/ml) and immunity in individuals with chronic hepatitis B as studied during current.

Table 5: Descriptive Analysis of HBe Ag data

Number of values	100
Minimum	0.01000
25% Percentile	0.01000
Median	0.01000
75% Percentile	0.01000
Maximum	39.61
10% Percentile	0.01000
90% Percentile	1.978
Mean	1.605

Std. Deviation	6.069
Std. Error of Mean	0.6069
Lower 95% CI of mean	0.4007
Upper 95% CI of mean	2.809
Coefficient of variation	378.1%
Geometric mean	0.02315
Geometric SD factor	9.290
Sum	160.5

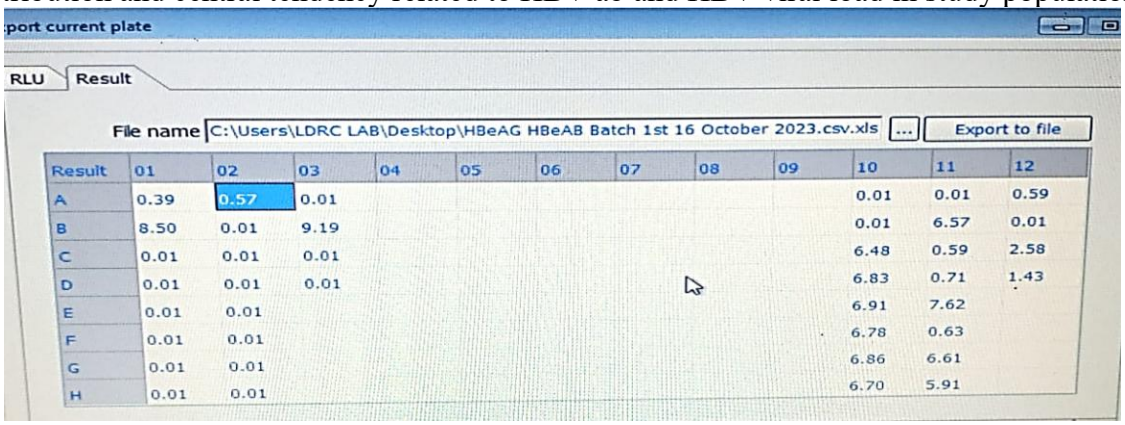
The above table shows the HBV antigen reactivity in 100 observed chronic hepatitis patients. It includes the minimum value (0.01000), the 25th percentile (0.01000), the median (0.01000), the 75th percentile (0.01000), and the maximum value (39.61) respectively. Additionally, it shows the 10th percentile (0.01000), the 90th percentile (1.978), the mean (1.605), standard deviation (6.069), standard error of the mean (0.6069), lower and upper 95% confidence intervals of the mean (0.4007 and 2.809), coefficient of variation (378.1%), geometric mean (0.02315), geometric standard deviation factor (9.290), and the sum of the values (160.5). These values help describe the distribution and central tendency related to HBV Ag and HBV viral load in study population.

Table 6: Descriptive Analysis of HBe-Ab data

Number of values	100
Minimum	0.001000
25% Percentile	0.6800
Median	3.050
75% Percentile	6.600
Maximum	17.94
10% Percentile	0.02680
90% Percentile	7.558
Mean	3.868
Std. Deviation	3.497
Std. Error of Mean	0.3497
Lower 95% CI of mean	3.174
Upper 95% CI of mean	4.562
Coefficient of variation	90.41%
Geometric mean	1.460
Geometric SD factor	8.112
Sum	386.8

The above table shows the HBV antibodies reactivity in 100 observed chronic hepatitis patients. It views the minimum value (0.001000), the 25th percentile (0.6800), the median (3.050), the

75th percentile (6.600), and the maximum value (17.94). Additionally, it shows the 10th percentile (0.02680), the 90th percentile (7.558), the mean (3.868), standard deviation (3.497), standard error of the mean (0.3497), lower and upper 95% confidence intervals of the mean (3.174 and 4.562), coefficient of variation (90.41%), geometric mean (1.460), geometric standard deviation factor (8.112), and the sum of the values (386.8). These useful values describe the distribution and central tendency related to HBV ab and HBV viral load in study population.



Result	01	02	03	04	05	06	07	08	09	10	11	12
A	0.39	0.57	0.01							0.01	0.01	0.59
B	8.50	0.01	9.19							0.01	6.57	0.01
C	0.01	0.01	0.01							6.48	0.59	2.58
D	0.01	0.01	0.01							6.83	0.71	1.43
E	0.01	0.01								6.91	7.62	
F	0.01	0.01								6.78	0.63	
G	0.01	0.01								6.86	6.61	
H	0.01	0.01								6.70	5.91	

Figure 6: Showing the results of HBe Ag and HBe Ab (chemiluminescence) immune assay
The above figure defines CLIA result of HBe Ag and Hbe Ab shows maximum 9.19 IU/ml and 6.91 IU/ml respectively. While the low value of Hbe Ab is 0.01 and same as 0.01 Hbe Ag.

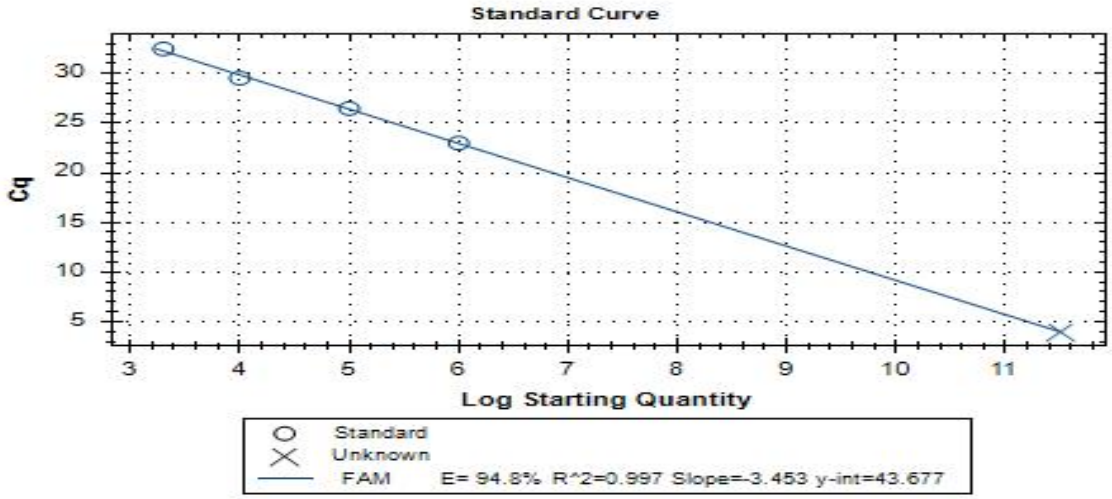


Figure 7: Showing the standard graph of HBV RT PCR Calibrators

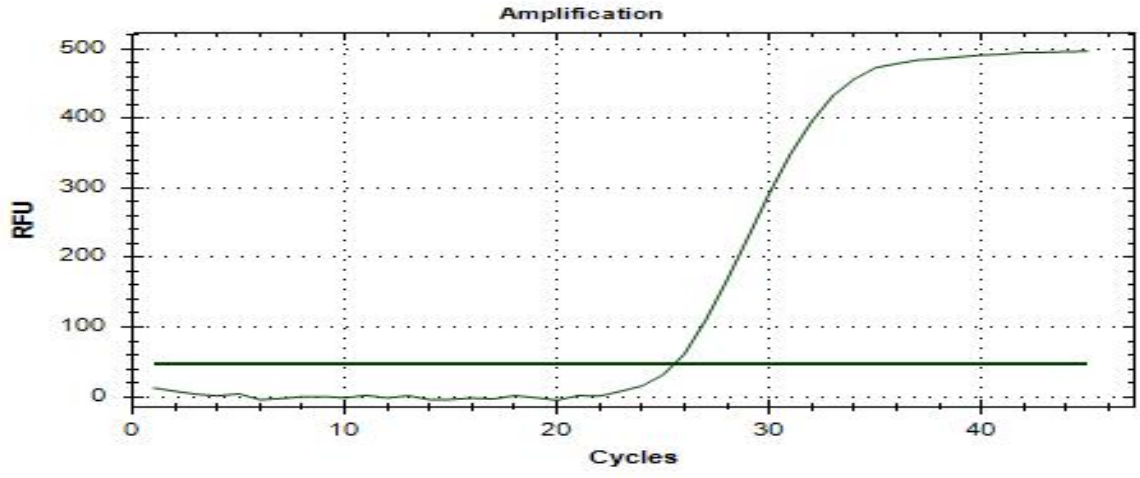


Figure 8: Showing the Internal Control graph Cq value is 26.1 of HBV RT PCR

Above standard graph of HBV real time PCR reaction shows the efficiency of PCR reaction which up to 94.8 % with all high to low calibrator Cq value.

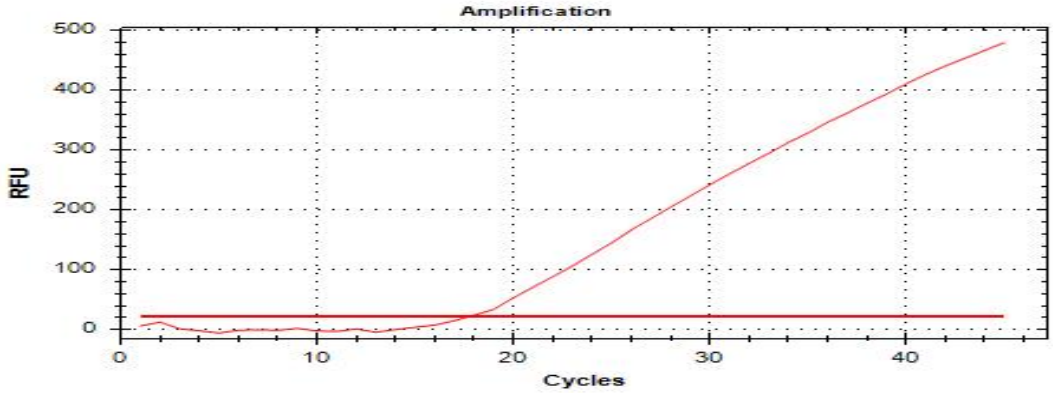


Figure 9: Showing the high viral load graph of HBV RT PCR Graph showing the maximum viral load of patient with early Cq value 19.1, shows precise sensitivity of HBV quantitative PCR. Viral load of linearity reaches up to 2.7 million as per reaction of manufacture.

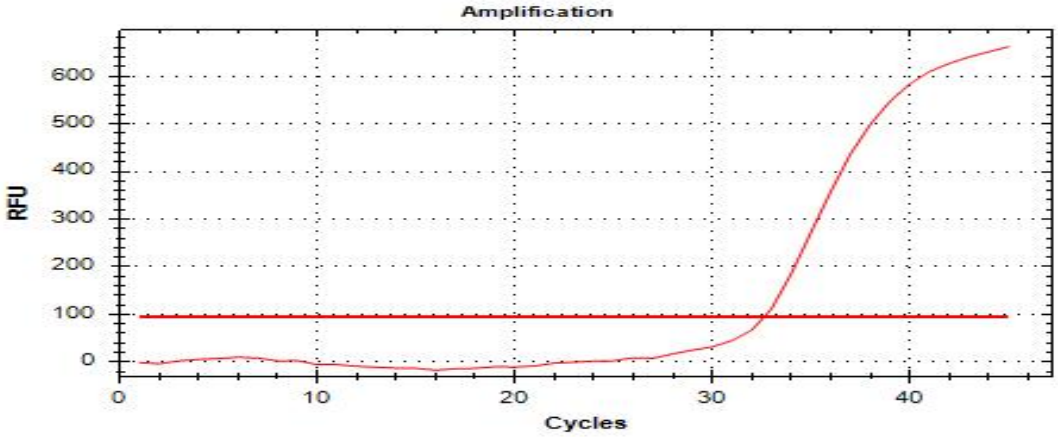


Figure 10: Showing the moderate viral load graph of HBV RT PCR

An illustrated graph showing the medium viral load of patients of applying the highly sensitive amplification kit which Cq value is 32.0.

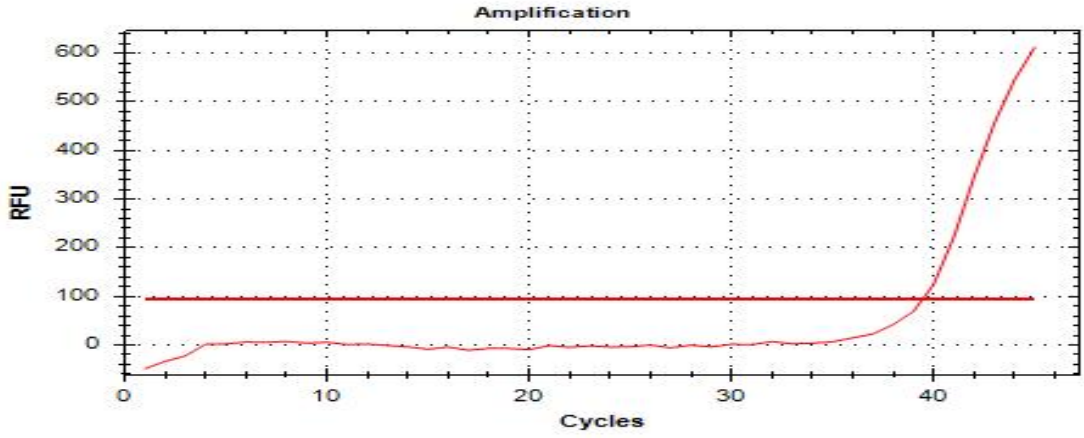


Figure 11: Showing the low viral load graph of HBV RT PCR
Sigmoidal curve of the above graph with very late Cq value 39.8 showing the very low viral load detection which are further co-related with Hbe Ag and Hbe Ab.

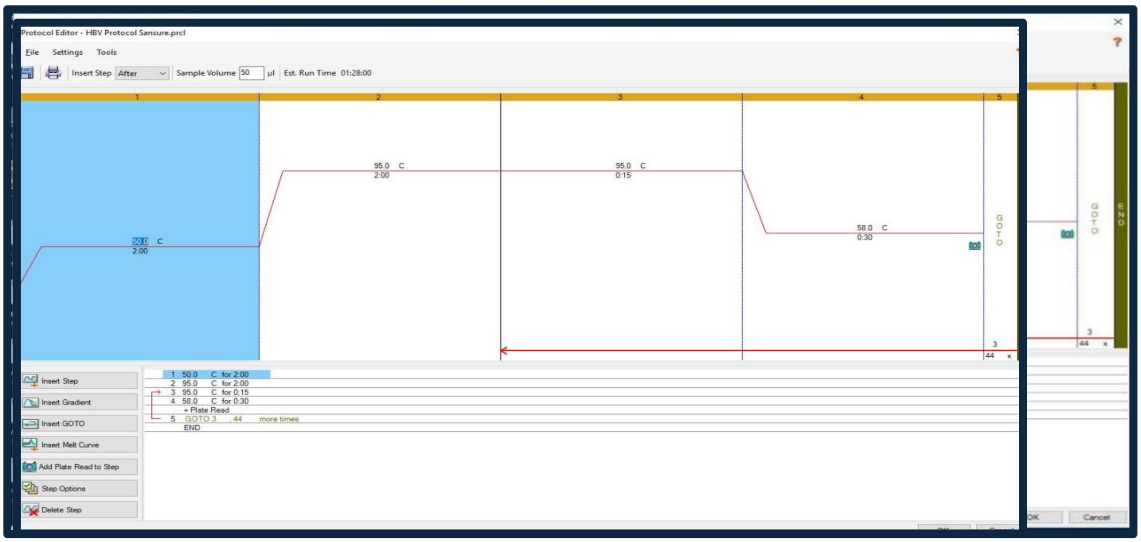


Figure 12: showing the whole stepwise procedure/protocol of SanSure kit performing the thrice steps of PCR with specified time, temperature and cycles
In contrast, the **mean Anti-HBe level** was **3.868 IU/mL**, with a narrower standard deviation of 3.497 and a CV of 90.4%. The antibody values ranged from **0.001 to 17.94 IU/ml.**

Discussion

This study investigated the correlation between molecular (HBV DNA quantification via real-time PCR) and serological (HBeAg and Anti-HBe via CLIA) markers in patients with chronic hepatitis B (CHB). Our results demonstrate that a significant majority (89%) of patients exhibited low HBeAg titers and correspondingly low viral loads, indicative of the inactive carrier state or immune control phase. This finding aligns with existing literature, which suggests that seroconversion from HBeAg to Anti-HBe is often accompanied by a decline in viral replication and improved clinical outcomes (Nyairo, 2018; Patel, 2024). Moreover, a high proportion of patients were Anti-HBe positive, supporting the notion that many had undergone seroconversion and were likely in a more immunologically controlled phase of infection. These observations reinforce the clinical value of Anti-HBe as a marker of reduced infectivity and viral replication. However, the study also identified a subgroup of patients with elevated HBeAg levels—including three patients with strong reactivity (20.1–30.0 IU/mL), one with very strong reactivity (>30 IU/mL), and two with moderate levels (5–10 IU/mL)—who exhibited high viral loads, consistent with active viral replication. This emphasizes the role of HBeAg quantification as a surrogate indicator of ongoing infection and disease activity, particularly in patients who may not yet have undergone seroconversion(Yu, 2024). Notably, these cases reaffirm the utility of HBeAg as a predictive marker for both treatment responsiveness and clinical progression. The observed

variation in the correlation between HBeAg and HBV DNA highlights the heterogeneous nature of CHB. While HBeAg is a valuable biomarker, it cannot wholly substitute molecular quantification in all clinical scenarios. Studies have shown that some HBeAg-negative patients may still carry significant viral loads, especially in the context of precore or basal core promoter mutations, leading to HBeAg-negative chronic hepatitis B(Mak et al., 2021). In our sample, 83% of patients had either non-detectable or low HBV DNA levels (<10 IU/mL), while 10% showed moderate viral load, 3% strong positivity, and 4% very strong positivity. These patterns support a moderate correlation between HBeAg titers and viral load, though the relationship is not linear across all clinical phases. The immunological window a period during which Anti-HBe appears only after the decline of HBeAg was also reflected in our findings. In certain cases, Anti-HBe antibodies were detectable only after a lag period, consistent with the dynamics of humoral immune response and delayed seroconversion. This underscores the importance of considering time-dependent serological evolution when interpreting these markers(Akabane et al., 2025; Mobahi, 2024). Importantly, this study validates the role of chemiluminescence immunoassays (CLIA) as a reliable, cost-effective diagnostic approach. In settings where real-time PCR is not economically feasible or logistically practical, CLIA offers a rapid, scalable alternative for HBV screening and monitoring. Its integration into national hepatitis control programs could enhance early detection, particularly in high-burden countries like Pakistan where access to molecular diagnostics remains limited(El-Shall et al., 2022). In contrast to nucleoside analogue-based studies, such as the one conducted between 2007 and 2010 that followed 48 HBeAg-positive patients after treatment cessation, our study is cross-sectional, highlighting the need for longitudinal monitoring. In that cohort, virologic relapses (HBV DNA > 10^4 copies/mL) and HBeAg reappearance were observed in a proportion of patients' post-treatment(Song et al., 2012). Future prospective studies in Pakistani cohorts are warranted to explore serological and virological relapse dynamics, especially following antiviral therapy. Lastly, the age distribution in our cohort revealed that the majority of HBV-infected individuals were middle-aged adults (35–55 years). This demographic profile mirrors national trends, where horizontal transmission and unsafe medical practices significantly contribute to HBV spread in adulthood(Gašpert, 2025; Khan et al., 2000).

Conclusion

This study demonstrates a significant correlation between HBV DNA levels and chemiluminescent measurements of HBeAg and Anti-HBe antibodies in patients with chronic hepatitis B. The findings validate the utility of CLIA as a sensitive and accessible method for evaluating viral activity and immune response, particularly where RT-PCR facilities are unavailable.

- 89% of patients had low HBeAg, often corresponding with low viral loads.
- Anti-HBe antibodies were more prominent in patients with lower HBeAg titers, suggesting effective immune control.
- High HBeAg levels were strongly associated with elevated viral loads, supporting the role of HBeAg as a surrogate marker.

Together, these results support the use of CLIA as a viable tool in clinical and epidemiological HBV monitoring strategies.

Future Perspectives

- Larger population-based studies across diverse regions are recommended to evaluate the association of HBeAg and Anti-HBe with HBV genotypes.

- Comprehensive serological panels including HBsAg, Anti-HBs, Anti-HBc, and liver function tests should be incorporated to enhance disease staging and monitoring.
- Further studies should investigate the longitudinal predictive value of HBeAg/Anti-HBe dynamics for therapy response and seroconversion rates.

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