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Gender-Based Disparities in Hepatitis B Virus X Gene Detection in the Patients Undergoing Antiviral Treatments

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ABSTRACT

The Hepatitis B Virus (HBV) is a 3.2 kb long virus belonging to the Hepadnavirus family. It has a variety of clinical symptoms, with chronic hepatitis like liver cirrhosis and hepatocellular cancer, based on immunological interactions between the virus and the host. Compared to the other genes of HBV, the X gene is highly conserved in the virus's genomic characteristics. Many mutations in the X gene of HBV can lead to the severity and other disease complications. This study was designed to determine the HBV X gene's detection in local patients' serum samples. Out of 40 collected samples from HBV patients, 24 (12 from each male and female patient) were identified as chronically HBV samples through various diagnostic approaches such as ICT rapid test, ELISA, and Real-time PCR. The samples used for DNA extraction yielded an excellent concentration of DNA ranging from 2.4ng/µl to 9.8 ng/µl. The HBV X gene-specific primers set showed results at 55°C for Nested PCR. The results were confirmed with gel electrophoresis. A band size of 597bp compared with the 1kb and 50bp DNA ladder was observed. The PCR-amplified products were purified and sent for sequencing. The sequencing results have significantly helped to analyze the sequences of the X gene (Consensus Sequence of local isolates) using bioinformatics tools like nBLAST, BioEdit, Expasy, MEGA11, and Phylogenetic analysis. The study indicated that despite taking antiviral treatment, the detection of the HBV X gene in chronically infected male patients is more than in female patients. The statistical analysis determined a significant difference (p<0.05) between the detection of the HBV X gene in males and females. In the future, this study will contribute to designing more specific assays and combined targeted therapies for Chronic HBV infection caused explicitly by mutations in the HBV X gene.

Keywords: Hepatitis B Virus X Gene, HBx Gene Detection, HBV Local Isolates, Gender disparities, Chronic Hepatitis B Virus Infection.

1. INTRODUCTION

HBV infection remains the most significant and common public health burden. According to a WHO report, 257 million people worldwide are chronically infected with HBV, even though one-third has been exposed to it. They are more likely to get cirrhosis and hepatocellular cancer (Xia and Guo, 2020). The genome of HBV is around 3.2 kb. It contains loosened-up round DNA (rcDNA), which is partially double-stranded and comprised of an entire coding minus strand (−) and an incomplete noncoding plus strand (+) with a fixed 5' end and a variable-size 3′ end (Li et al., 2020). HBV genome comprises four open reading

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frames that overlap each other. It includes P, S, C, and X (Zhou et al., 2022). The X gene partially overlaps the polymerase gene at nt 1374-1621 and the pre-core/core gene at nt 1814-1838 (Benhenda et al., 2009). The X ORF encodes the X protein (HBxAg), which is believed to be involved in several procedures, including transcriptional activation, cell cycle control, signaling, and DNA repair (He et al., 2023). It is associated with the onset and progression of hepatocellular carcinoma (HCC), contributing to genomic instability, telomerase reactivation, epigenetic alterations, and cell death regulation.

The detection and clinical relevance of the HBx gene in local human isolates have pivotal implications for both research and clinical applications. Understanding the genetic and functional variations of HBx across populations can inform personalized treatment strategies and prognostic biomarker identification. The divergent hypotheses regarding HBx function whether it predominantly promotes viral replication or influences cellular signaling pathways and the potential link between specific HBx variants and HCC development necessitate resolution for guiding future research and therapies. Despite global awareness of HBV, molecular studies of the HBV X gene in Pakistan are lacking, prompting the need for this study's investigation.

The gender disparity in hepatocellular carcinoma (HCC) is particularly noticeable in cases related to hepatitis B virus (HBV). Previous studies have found that HBV is a sex-dimorphic virus. The androgen pathway boosts HBV transcription through the androgen response element (ARE) in viral enhancer I (EnhI). Conversely, the estrogen pathway hinders HBV transcription by preventing HNF4 α from binding to the corresponding site in EnhI (Morozov et al., 2023). The HBV X protein acts as a positive transcriptional coregulator to enhance androgen receptor-mediated gene expression, potentially contributing to the vulnerability of males to microbial infections and cancer development (Zhou et al., 2022). Thus, it is expected that sex hormones and immune responses, along with viral factors, contribute to sex/gender disparity in the outcome and progression of hepatitis B and C virus infections (Panasiuk et al., 2022).

The study aims to detect the HBV X gene in chronically HBV infected male and female patients who are going under antiviral treatments. This analysis will involve PCR amplification and subsequent sequencing of the X gene. Through a comprehensive analysis of HBx sequences and detection in correlation with clinical outcomes, this study seeks to provide a more nuanced understanding of HBx's role in HBV infection. The research also holds significant potential for improved or early diagnosis of Hepatitis B viral infection and

surveillance in Pakistan. It may contribute to more accurate diagnosis, treatment, and prevention strategies for chronic HBV infection.

2. MATERIALS AND METHODS

2.1. Ethics Statement

The current research is approved by Ethical Research Committee (ERC) and endorsed by Institutional Review of Board (IRB) of Forman Christian College University (FCCU), Lahore Pakistan.

2.2. Sample Collection

90 samples (45 from each HBV infected male and female patients) were collected from different labs, including Liver Clinic, General Hospital, and Minhaj Lab Lahore, Pakistan during the month of October and November 2022. The samples with chronic hepatitis B that tested positive were used for this research. Centrifugation separated the plasma from the blood for 10 minutes at 4 °C at 5000 rpm. The isolated plasma was used for analysis.

2.3. Serological Tests

HBV infection was found using four different screening and diagnostic techniques for confirmation and side-by-side comparison. The four screening and diagnostic steps performed were Rapid ICT tests (CTK Biotech immune-chromatographic strips), ELISA (HBsAg ELISA, Equipar, Italy), and RT PCR (SYSTAAQ HBV Real-Time Polymerase chain reaction kit). Among 90 samples, a total 44 samples (22 each from male and female) were confirmed to have chronic HBV infection and used for the study.

2.4. Viral DNA Extraction

A favor-prep viral nucleic acid extraction kit was used. The HBV DNAs were isolated from 44 patients' serum samples that showed positive ICT and ELISA tests. Extracted DNA samples were transported and kept at -20 °C for further processing. DNA concentrations were measured using a Nanodrop spectrophotometer.

2.5. HBV X gene Amplification through Nested PCR

The outer sense and anti-sense primers were designed using a primer 3 tool. Outer and inner sense primers were utilized for PCR rounds 1 and 2. A 30µl of the PCR reaction mixture was prepared. The PCR reaction mixture consists of 15µl of fresh Thermo Scientific DreamTaq Green Master Mix (2X), 1µl forward primer, 1µl reverse primer, 3 µl nuclease-free H2O, and 10µl HBV extracted viral DNA sample. For 2nd round, 2µl from the previous 1st round PCR product was taken as a DNA template to make 20µl of the PCR reaction mixture. The PCR

condition for both rounds was: initial denaturation at 94 °C for 5 minutes, 30 cycles of denaturation step at 94 °C for 45sec, annealing at 55 °C, extension at 72 °C for 1 min, and the final extension at 72 °C for 10 minutes. The details of the primers are mentioned in table No. 1. The 2nd PCR product sizes of 597bp were visualized on 1.5% agarose gel to confirm HBV positive samples.

Table 1. Primer sequences details including primer names, sequences, annealing temperature, no. of nucleotides, and GC content.

Primer Names	***Primer Sequence***	***Annealing temperatures***	***No. of nucleotides***	***GC content***
Outer sense primer F1	5'-ATTGATTGGAAAGTMTGTM-3'	48.8	19	26%
Outer sense primer R1	5'-TCCACAGTAGCTCCAAATTCTTT-3'	58.1	23	39%
Inner sense primer F2	5'-CGCTTGTTTTGCTCGCAGC-3'	61.2	19	58%
Inner sense primer R2	5'-GGCACAGCTTGGAGGCTTG-3'	64.5	19	63%

Note: F1 = Forward Primer 1, R1 = Reverse Primer 1; F2 =Forward Primer 2, R2 = Reverse Primer 2.

2.6. PCR Product Purification and DNA Sequencing

The GeneJET PCR Purification Kit (#K0701) was utilized to purify the amplified products. The purified HBV X gene DNA samples were sent to Macrogen Inc. for Sanger sequencing.

2.7. Mutational Analysis

Mutational analysis for the HBV X gene was done using bioinformatics tools like Molecular evolutionary genetic analysis (MEGA version 11) and Phylogenetic Tree.

2.8. NCBI Submission

The trimmed consensus sequence (made using the Bioedit tool) of HBV X Gene was then submitted to NCBI with BankIT numbers 2719198 and 2719361. GenBank has provided the accession number for our HBV X gene nucleotide sequences.

2.9. Statistical Analysis

A Chi-square test of independence was performed for association analysis of HBV X Gene detection with the study groups. The test was performed by using the IBM SPSS Statistics 20.

3. RESULTS

3.1. Confirmation of the Detection of HBV X Gene in Male and Female Patients

After 2nd round of PCR, the confirmed amplified PCR products were visualized on 1.5% agarose. 7µl of PCR amplified DNA samples were loaded with 5 µl of Ethridium Bromide,

which helped them visualize on the Gel Doc (Fisher Scientific, Gel Doc-It ® 310, Denmark). Both 1kb and 50bp ladders were used to compare the size of the X gene, as shown in figure 1. HBV X gene detection in HBV chronically infected male patients was more expressed than in female patients as also graphically represented in figure 2.

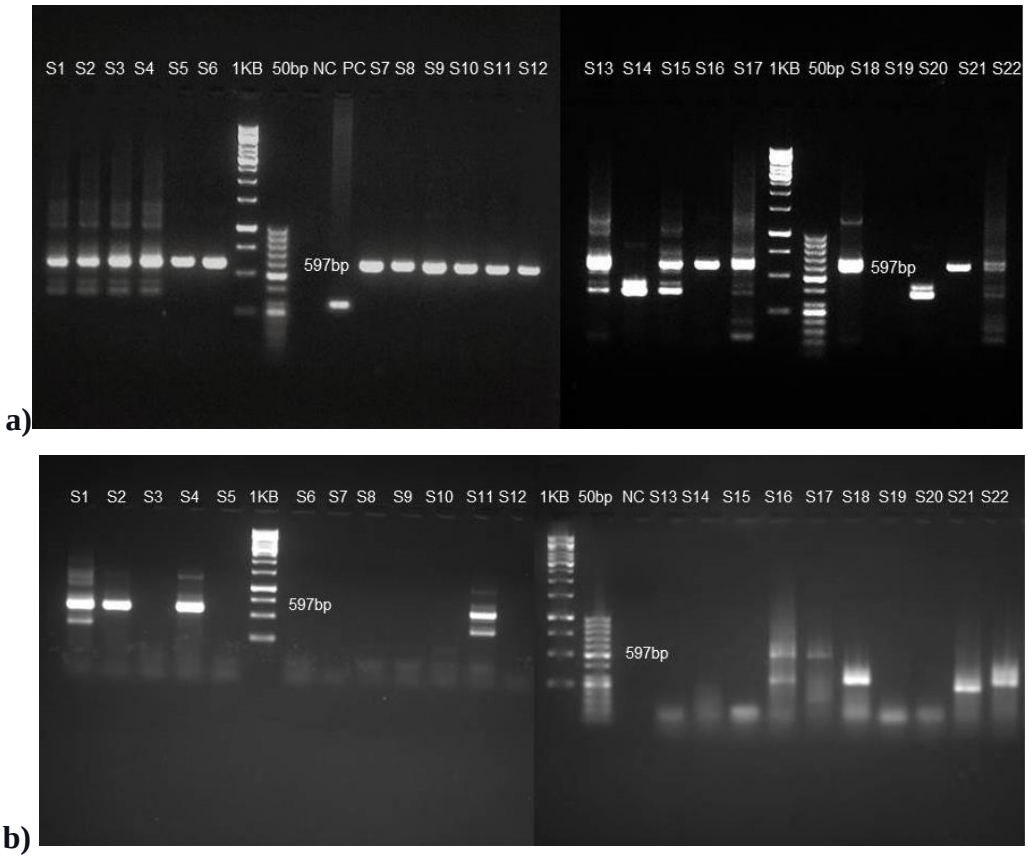

Figure 1. Confirmation of HBV X Gene Detection through Gel Electrophoresis showing band size of 597bp: a) Detection of HBV X Gene in chronically HBV Infected Male Patients; b) Detection of HBV X Gene in chronically HBV Infected Female Patients. Abbreviations: Sample 1 (S1) to Sample 22 (S22), Negative Control (NC), Positive Control (PC).

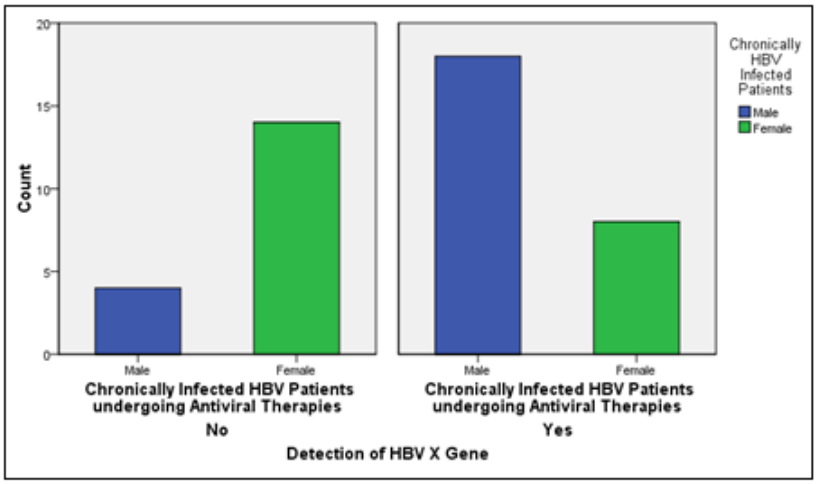

Figure 2. Histograms showing detection frequency of HBV X gene in chronically infected HBV male and female patients undergoing antiviral treatments

3.2. Mutational Analysis

3.2.1. MEGA 11

The Consensus sequence of HBV X gene was prepared using Bioedit tool. It is then processed for further mutational analysis using MEGA 11. A total of 20 Sequences were selected from Blast alignment results with respective consensus sequence of HBV X gene. Multiple Sequence Alignment was done using a Clustal W feature in MEGA 11 Software. Highly conserved regions were observed, which depicted that the X gene region is highly conserved in HBV local isolates.

3.2.2. Phylogenetic Tree

Neighbor likelihood method was used to construct Phylogenetic tree to see the relationship between the consensus sequence of X gene of Pakistani HBV isolates and other GenBank isolates. The scale of 0.001 shows about 0.001% of genetic variation/changes in the consensus region of the X gene with different HBV alignments as shown in figure 3.

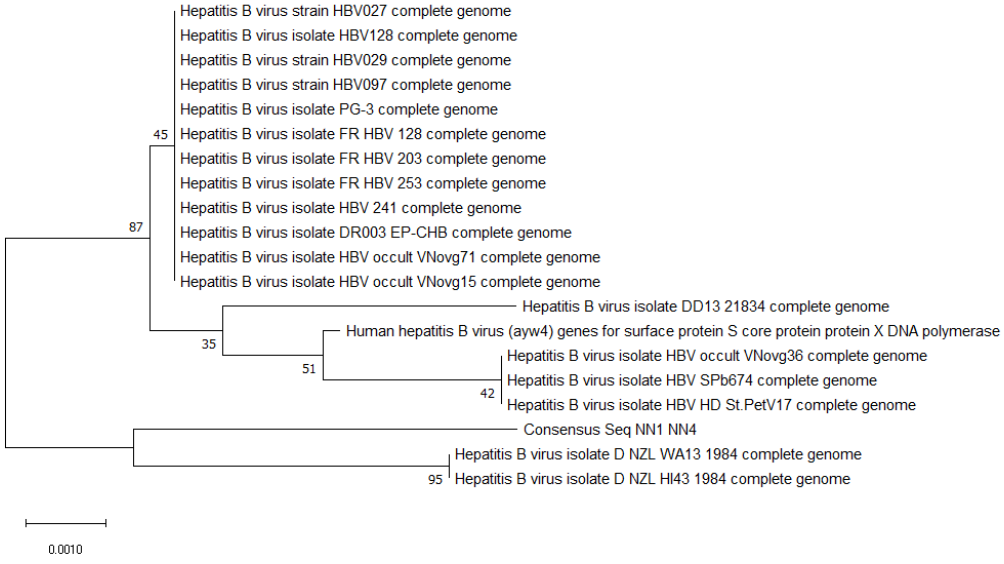

Figure 3. Phylogenetic tree of HBV X gene consensus sequence showing 0.001% genetic variation.

3.3. GenBank Accession Numbers for HBV X Gene Nucleotide Sequence

GenBank have provided the Accession Number for our HBV X gene consensus sequence; OR232197 and OR232198**.**

3.4. Statistical Analysis

Cross-tabulation was performed to investigate further the relationship association between the detection of HBV X Gene in male and female patients. The HBV X Gene detection percentages in male and females were 69.2% and 30.8% respectively as shown in table 2.

Table 2. Percentages of detection of HBV X Gene in Chronically HBV infected Male and Female patients undergoing antiviral therapies.

*Gender of chronically HBV Infected Patients * Detection of HBV X Gene Crosstabulation*					
			Detection of HBV X Gene		*Total*
			No	*Yes*	
Chronically HBV Infected Patients	Male	Count	4	18	22
		% within chronically HBV Infected Patients	18.2%	81.8%	100.0%
		% within Detection of HBV X Gene	22.2%	69.2%	50.0%
	Female	Count	14	8	22
		% within chronically HBV Infected Patients	63.6%	36.4%	100.0%
		% within Detection of HBV X Gene	77.8%	30.8%	50.0%
Total		Count	18	26	44
		% within Gender of chronically HBV Infected Patients	40.9%	59.1%	100.0%
		% within Detection of HBV X Gene	100.0%	100.0%	100.0%

These patterns align with the chi-square test results, supporting the observed association more. The test of independence revealed that the detection of the HBV X gene in male patients undergoing antiviral therapies is found to be significantly different from the detection of the HBV X Gene in females undergoing antiviral therapies at (p<0.05) as the p-value (0.002) is less than 0.05 as shown in table 3.

Table 3. Chi Square Test for HBV X Gene Detection in Male and Female.

Chi-square Test			
	Value	**df**	***p-value***
Pearson Chi-Square	9.402[a]	1	.002
Continuity Correction[b]	7.615	1	.006
Likelihood Ratio	9.831	1	.002
Fisher's Exact Test			
Linear-by-Linear Association	9.188	1	.002
N of Valid Cases	44		

Note: df = degree of freedom; N = Number.

4. DISCUSSION

HBx protein is a transcriptional coactivator. It plays a vital role in carcinogenesis because it modulates essential tumor suppressor genes and apoptotic regulators and interferes with DNA repair pathways (Khan et al., 2022). The HBV X gene is the smallest yet functional gene among other HBV genes. By facilitating HBV replication and integrating into the host genome, the HBx gene and the protein it encodes are heavily involved in developing chronic hepatitis, cirrhosis, and liver cancer (Conners, 2023). Pakistan has a long history of hepatitis B virus infection. The incidence of HBV in Pakistan is alarming due to a lack of understanding about HBV prevention and treatment. HBV carriers in Pakistan number between 7-9 million, with a carrier prevalence of 3-5% and an annual infection rate constantly rising. Describing the genotypes and sub-genotypes of HBV infections is necessary to comprehend their epidemiology, transmission, pathogenicity potential, and clinical outcome. In Pakistani HBV isolates, patients with HBV positive disease frequently have mutations in the Enh-II region of the X gene linked to liver cirrhosis (LC) and hepatocellular carcinoma (HCC). The mutations identified in chronic hepatitis (CH) patients are concerning since many people may develop LC or HCC due to these mutations (Brown et al., 2022).

This study analyzes a collection of chronically infected HBV patients we acquired from a Pakistani medical facility. This study uses molecular analysis of HBV X gene detection in male and female genders undergoing antiviral therapies. The samples used in this study only represent some of the population of Lahore, Pakistan, because they were only drawn from the referred labs within a brief time. The primary objective of this study was to separate the entire or a portion of the X gene from the HBV genome. The study also focused on some previously employed strategies to determine which viral detection method is more suitable, efficient, and affordable to adopt, given the diagnostics setting in Pakistan. The amplicons of the X gene were sent to Macrogen to be sequenced. The entire sequenced DNA and the Mutational Analysis were analyzed using Bioinformatics tools like MEGA 11 software and Phylogenetic Tree.

Numerous conformance tests were conducted to obtain reliable results. The first diagnostic approach was an ICT rapid test kit. It was seen that ICT rapid test kits were easier to use and did not require any prior training, but the sensitivity of the kit needed to be higher. It means there were chances of false positives in the results since the viral load should be increased for more accurate results. Acute or chronic HBV infection is indicated by the

presence of HBsAg, with the possible exception of a brief time after receiving a dose of the HepB vaccination. Recovery from HBV infection is marked by the emergence of anti-HBs following a reduction in HBsAg (Ding et al., 2023). The kit is easy to use and gives quick results within 15-30 minutes, but since it is preferred for patients with a high viral load, this detection method is unsuitable. The second approach used to confirm the positive samples of the ICT rapid test kit was ELISA. This approach proved more sensitive and specific than the ICT rapid test, emitting detectable signals within 30 minutes. The chronic HBV-infected samples tested positive through ELISA, and the detection level was less than or equal to 10-15 ng of Hbs Ag /ml when measured at 50nm optical density. However, the ELISA HBsAg kit proved technical and took longer to execute. The third approach to the detection of HBV was real-time PCR. It is the most advanced of all approaches since it allows quantification and qualification of the virus. This method is commonly used to characterize genes or DNA fragments of interest. The only disadvantage of this method is the increased possibility of false positives since the contamination rate is high, and the procedure requires utmost precision to perform in a contamination-free environment. Nested PCR was performed at the very end, a reliable yet technical method. The primary purpose of this approach was to amplify a complete or partial fragment of the X gene region of HBV. This approach is more time-consuming than real-time PCR as it is a qualitative technique. Although the RT-PCR method is recognized as a precise and reliable approach suitable for screening for HBV, it should be suggested for making a final and accurate diagnosis of patients suspected of having the virus. During the research journey, we encountered an unusual scenario that can be relatable to the previous findings. Some female samples were proved to be HBV positive by RT PCR, but when they were run on a gel, no detection of the HBV X gene was found. When the data/sample's history was retrieved, it was known that antiviral therapies significantly reduced viral load by the time they reached the recovery phase.

In the case of male patients who were undergoing antiviral treatments, it was observed that no significant recovery was shown. The applied statistical analysis of Chi square test has shown a p value of <0.05 with indicates increase detection of HBV X gene in males as compared to females undergoing antiviral therapies. This analysis might demonstrate the sexual dimorphism in chronic hepatitis B virus infection. The HBV X gene detection in males can be because of the androgen receptor, as it has been shown to promote HBV-induced hepatocarcinogenesis through modulation of HBV RNA transcription. Detection levels of genes related to the estrogen response pathway differ between male and female QD-HCCs,

suggesting a potential role of estrogen in HBV detection disparities. HCC driver genes: There are reported differences in mutation frequencies of HCC driver genes between male and female QD-HCCs, including TP53, TERT promoter, and ADGRB1. Other factors: Additional factors, such as hormonal differences and genetic variations, may contribute to the gender disparities in HBV expression in HCC (Chiu et al., 2007; Li et al., 2019). Several studies also show that the female sex has been linked to a better response to antiviral therapies like PEG-IFN-alpha. However, this effect is not universal. In a trial of more than 2000 HBV patients beginning treatment with entecavir, a gender advantage was also seen for nucleotide analogue therapy: a quicker virologic response was seen in treatment-naive females than in males. Male sex has been linked to failure to suppress HBV viremia, and females have a greater functional cure rate in HIV/HBV co-infected patients initiating antiviral medication (Yeh et al., 2023). Estrogen can repress HBV gene transcription by up-regulating estrogen receptors, potentially explaining the lower viral load and reduced liver cancer risk in HBV-infected women compared to men (Zhang et al., 2008). It is suggested that prophylactic antiviral medication should be given to patients with chronic HBV infection who receive anticancer immunotherapy to lower the risk of HBV reactivation and resulting hepatitis. Past HBV infection patients are at lower risk of HBV reactivation than patients with chronic HBV infection. In people with chronic and prior HBV infections, HBV reactivation rates were 1.0% and 0%, respectively. In HBV chronic infection, antiviral prophylaxis dramatically lowers the risk of HBV reactivation and related hepatitis (Kim et al., 2016). For the treatment of chronic HBV infection, a novel capsid assembly modulator (CAM) medication called GS-SBA-1 is being developed to improve viral suppression when combined with nucleoside analogues (NAs) (Xu et al., 2023). HBV integrations in liver diseases like HCC exhibit different sequences at junctions. It includes some virus-host chimera DNA (vh-DNA), which may be a distinguishing marker for individual HCC (Xu et al., 2022). The detection level of the X gene may be lower in patients who were sub-clinical or already had an infection that had resolved, which may be the likely explanation for why nested PCR for the X gene region is negative for positive RT-PCR of some samples.

The current study has also clarified the diagnostics discrepancies that will serve as a foundation for future investigation of additional potential cases. Complete amplification of the X gene was impossible, as this region overlaps with the pre-core region. However, we amplified the 597bp domain of the X gene. The confirmed PCR products were purified using a PCR product purification kit and were sent to Macrogen for sequencing of the HBV X

gene. A phylogenetic tree was also constructed using the obtained aligned sequences to understand the sample's evolutionary relationships and genetic variations. Identifying the X gene in HBV isolates and its sequence mutational analysis helped to characterize the X gene molecularly. It provided valuable insights into the genetic diversity of HBV strains leading to the chronicity of HBV infection. Established data to identify natural mutants of the HBx gene in Chinese patients with chronic liver disorders determined that the HBx mutant highly impacts cellular processes like apoptosis and cell proliferation (Ruggieri et al., 2018). Another study demonstrates that HBV X mutations can affect the HBx open reading frame and the overlapping genes. As a result, it can potentially progress to clinical severity, resulting in chronic hepatitis B virus (HBV) infection. The mutation in the X gene can significantly change host gene expression, particularly in the case of HCC, and can affect virus replication (Wang et al., 2012).

The limitation of the study regarding the clinical representation of the people from which the sample was obtained limits the clinical application of the viral sub-genotype. Longitudinal investigations of sub- genotype-specific infection in individuals are required to bridge this knowledge gap. However, in the future, this study could help explore the promoting factors that contribute to leading chronic hepatitis B virus infection. Based on this, significant targeted treatments can also be discovered.

5. CONCLUSION

To conclude, this study provides a significant overview of the methods used to diagnose HBV. However, it also investigates the detection of the HBV X gene in male and female patients. The study suggests that the interplay of androgen and estrogen signalling and genetic variations in HCC driver genes may influence the HBV X gene detection in these patients. Further research is needed to understand the mechanisms underlying these gender disparities fully.

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7. CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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