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Comprehensive Assessment of Replication Dynamics and Characteristics in Wild Type and Corresponding Defective HBV DNA Stains

Jawad Zahir[1], Ali Sabir[2], Haseeb ur Rahman[3], Syed Ali Bukhari[4], Muhammad Naeem[5], Iqbal Nisa[6], Wasfa Sana[7], Madeeha Raihan[8], Itezaz Younas[9]

[1]Department of Microbiology, Hazara University, Mansehra, KP, Pakistan.
[2]Department of Physiology, Government College University, Faisalabad, Punjab, Pakistan.
[3]Departement of Microbiology, University of Haripur, KP, Pakistan.
[4]Institute of Microbiology, University of Veterinary and Animal Sciences, Lahore, Punjab, Pakistan.
[5]Innovative Medicine Department, Institute of Pharmacy (Sechnov University), Russia.
[6]Department of Microbiology, Women University, Swabi, KP, Pakistan.
[7]Institute of Molecular Biology and Biotechnology, University of Lahore, Sargodha Campus, Punjab, Pakistan.
[8]Department of Biochemistry, Hazara University, Mansehra, KP, Pakistan.
[9]School of Life Sciences, (Major Biology), Zhejiang Sci-Tech University Hangzhou, China.

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Corresponding Author: Itezaz Younas, School of Life Sciences, (Major Biology), Zhejiang Sci-Tech University Hangzhou, China.
Email: itezazyounas123.iy35@gmail.com

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ABSTRACT

Hepatitis B virus (HBV) is a significant global health concern, requiring a deeper understanding of its replication and pathogenic mechanisms to develop effective therapies. This study investigated the transcription efficiency, antigen dynamics, and virion production of wild-type HBV (wtHBV) and defective HBV (dHBV) constructs. The levels of dHBV prRNA were 2.5 times higher in Sample 1 than in Sample 2. They were 2.8 times higher ($p < 0.01$) in Sample 2, even though the levels of cccDNA were the same in both samples ($p > 0.25$ for Sample 1 and $p > 0.16$ for Sample 2). The amount of transcription in dHBV improved considerably, as shown by the evidence that Sample 1 grew up three times and Sample 2 went up two times ($p < 0.01$). An examination of antigen dynamics showed that dHBV had less production of HBsAg and HBeAg but more storage of these two antigens in cells. The total concentrations of HBsAg inside cells were 2.5 to 3 times higher ($p < 0.01$), and the quantity of HBeAg also increased by the same amount over the course of the study. A new test was used to measure the number of HBV virions, and it was very sensitive and specific. Also, only 20–100 µL of supernatant was sufficient. The experiments showed that lamivudine inhibited virion secretion at concentrations ranging from 1 µM to 5 µM, and that across dilutions ranging from 1:2 to 1:20, there was an associated decrease in virion levels. There were approximately 0.5 million virions per millilitre in the serum samples, but no virions were found in the dHBV constructs. The findings show that dHBV may be competent to avoid detection and remain in the immune system due to its altered antigen dynamics and improved transcriptional pathways. This work offers a reliable tool for enhancing research on antiviral medicines and sheds light on the biology of HBV.

INTRODUCTION

After maturation spliced pregenomic RNA (pgRNA) undergoes a process of reverse transcription leading to formation of defective hepatitis B virus (dHBV) particles, as claimed by Cao ets al., 2014. Gunter et al. 1984 earlier suggested that a spliced pgRNA which lacks nucleotides at positions 2447 to 489 contains a major spliced form which encodes approximately a

single hemisphere of 2.2kb. Isolated, dHBV particles have theoretically been whittled down to incorporate such single-spliced RNAs and possess an ability to be secreted as viral particles from chronically infected HBV patients as well as culture cell models (Cheng et al, 2014; Tu, Budzinska, Vondran, Shackel & Urban 2018). To maintain all dHBV particles in the viral pool, trans-complementation with wild-type HBV is obligatory also for dHBV particles. The rations of dHBV to wild types HBV DNA seem to vary between chronic HBV carriers at a minimum of 0.001 to as the maximum 69% (Soussan et al. al, 2008. Daryl et al (2018) and underlined, however, in dHBV patients, a high HBV DNA wild type ratio has altered necrosis and fibrosis of the liver which is indeed abnormal. Some studies revealed, however, that HBSP for example, which is dHBV-encoded proteins are involved in the replication and pathogenesis of the virus (Tu et al., 2018). Other researches indicated that definite HBV-encoded proteins such as HBSP may be helpful in understanding the viral pathogenesis and replication process (Romero et al., 2023; Webster et al., 2000) There exist some unknown features with regards to the striking pattern of dHBV. dHBV cannot be transmitted to the human body as the bone marrow cells cannot receive the final structure of rcDNA. The transcription of pgRNA occurs during the replication of chloroplast utilizing an enzymes complex similar to reverse transcriptase [Tayal et al. 2004]. New virions cannot be formed for nucleus. On djHBV hydrophilic caps are generated inside the cytoplasm and are isolated through the cell membrane after the two phage structures are produced. Post transformation of viral pg RNA into relaxed circular sequence eccDNA the process of integrating cells resume as the csm. Following this, it can be integrated into solely active cells. Because dHBV consists of exactly the similar surface envelope proteins as wild type HBV, dHBV can also infect hepatocytes. The dHBV cccDNA structure, which is translocated to the nucleus by the rcDNA genome and recombined, is enchanted but smaller than cccDNA normal HBV cells and cccDNA H-51. (Mason et al 2016 and H. Ullah et al. 2024). Transcription of HBSV takes place, which is followed by translation into dHBV proteins.The dHBV genome structure, which arises from the site-specific splicing of pgRNA, causes loss in polymerase and surface open reading frames sensitive to the presence of HBV polymerase and HBV surface proteins. Thus, dHBV does not possess independent replicative ability and needs wild-type polymerase for its packaging and release as dHBV particles (Liang et al., 2021). The core protein region and the HBeAg region remains in the dHBV genome however the translation is shortened by one amino acid due to a nascent stop codon appearing as a result of the splicing process. The X ORF is also retained resulting in production of HBx protein which was previously reported (Khan et al., 2024; Köck, Nassal, Deres, Blum & von Weizsäcker, 2004; Thompson et al., 2010). The splicing of pgRNA additionally leads to the formation of a new open reading frame, which codes for a novel protein known as the HBV - splice generated protein (HBSP). Treatment of HBV is generally assessed using the pgRNA: cccDNA molar ratio or the number of pgRNA molecules produced by each cccDNA (Choi, 2007 ; A. Ullah et al. 2023). Nonetheless, there has not been a proper comparative study on the replication efficiencies of the dHBV genomes in terms of pgRNA concentrations and transcriptional efficiency relative to wild-type HBV. The purpose of this study is to analyze the corresponding dHBV constructs in terms of replication characteristics with those of wild-type constructs. Also, these constructs were transfected into cells, after which the levels of the intracellular core protein and the extracellular secretion of the HBeAg were monitored. In addition, we have developed a new sensitive and quick method to determine the absolute quantity of HBV virions secreted in cell culture models. This approach is very important for the therapeutic evaluation of anti-HBV drugs, as well as the evaluation of the replication characteristics of HBV variants including drug-resistant mutants. The dHBV construct absence in secreted virions will confirm the framework for studying the replication of dHBV.

MATERIALS AND METHODS

Cell Culture

Huh7 human hepatoma cells were grown in DMEM supplemented with 10% FBS and maintained at 37°C in the presence of 5% CO2 within a humidified incubator. The eve preceding transfection, 1 × 10^5 cells were seeded in 24 well plates . As directed by the manufacturer,

Lipofectamine 2000 (Invitrogen) was used for the transfections. Additionally, a stable HepG2.2.15 cell line with integrated HBV DNA was cultivated at 37°C and 5% CO_2 in DMEM with 10% FBS.

Transfection of Wild-Type and dHBV DNA

Unit-length genomes were extracted from the wild-type and defective HBV (dHBV) constructs using BspQI, and these were then gel-purified. Real-time PCR was used to measure the copy counts of the wild-type and dHBV genomes using primers that target the surface gene (for further information, see Chapter 2). Using Lipofectamine 2000, 11–12 log10 copies of either wild-type or dHBV DNA were transfected into Huh7 cells in 24-well plates. Three days after transfection, cells and supernatants were gathered for additional examination. To estimate intracellular core protein, a different transfection was carried out. Every experiment was carried out three times.

Quantification of HBV cccDNA, Precore RNA, and Pregenomic RNA

Total DNA was extracted using the QIAamp DNA Mini Kit (Qiagen), and RNA was extracted with Trizol reagent (Invitrogen). RNA (1 µg) was treated with DNase I and used for cDNA synthesis. Real-time PCR quantification of precore RNA and pregenomic RNA (pgRNA) was performed using specific primers (Laras et al., 2006), with GAPDH mRNA levels used for normalization. To quantify cccDNA, the extracted DNA was treated with Plasmid-Safe DNase to remove linear transfected DNA, then purified and subjected to real-time PCR using cccDNA-specific primers and probes (Chapter 2). cccDNA levels were normalized to cellular DNA using β-globin primers.

Estimation of HBV Proteins

Secreted HBV proteins were measured using HBsAg (MONOLISA, BioRad) and HBeAg (Diasorin) ELISA kits, following the manufacturer's instructions. Cells were lysed using RIPA buffer supplemented with a protease inhibitor cocktail to enable the quantification of intracellular core protein. After centrifugation at 14,000 rpm at 4°C, the supernatants were collected and analyzed for core protein (HBcAg) using a quantitative ELISA kit (Cell Biolabs). Total protein concentration was determined using the Bradford assay (Sigma) and was used to normalize the core protein levels.

Development of a Novel Assay to Quantify Secreted HBV Virions

A new test was designed to determine the absolute quantity of secreted HBV virions. Huh7 cells were plated in 6-well plates and transfected with wild-type HBV constructs or the replication-incompetent pkex-1+2 mutant construct. The pkex-1+2 mutant was used to eliminate background signals from non-virion DNA. Wild-type HBV DNA was digested with BspQI, gel-purified, and ligated, and the ligated product was used for PCR amplification. The pGEM-T Easy vector (Promega) was used to clone the amplified mutant HBV DNA, and the mutation was confirmed by sequencing. The supernatants were collected and treated with DNase I to remove non-virion DNA after the transfected Huh7 cells were incubated for 5 days. ELISA plates that were coated with anti-HBs antibodies were supplemented with supernatants that had been treated with DNase (Monolisa HBsAg ELISA, Bio-Rad). After washing, DNA was extracted and quantified by real-time PCR using primers designed to avoid amplifying transfected HBV DNA. The assay was validated using a positive control (serum from HBV-infected individuals). For dHBV constructs, no virion secretion was expected, as these particles lack functional polymerase and surface proteins.

REULTS

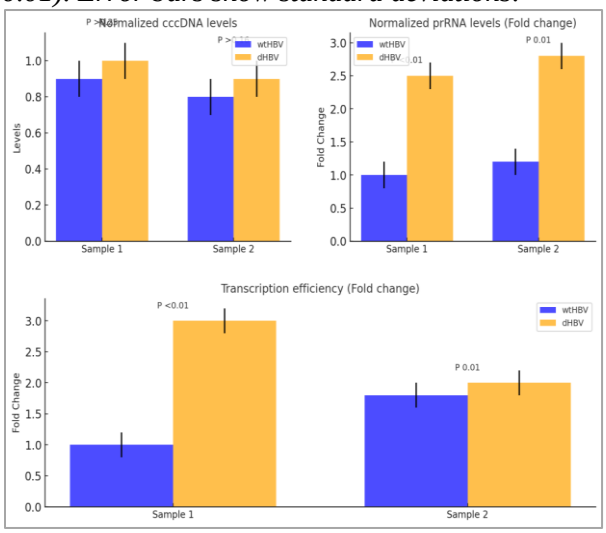
Enhanced HBV Transcription Efficiency

The results reveal that cccDNA levels are similar between wtHBV (wild-type HBV) and dHBV (mutated HBV), with no statistically significant differences observed in either Sample 1 or Sample 2 ($p > 0.25$ and $p > 0.16$, respectively). As for prRNAs, their levels in dHBV are significantly higher than in wtHBV, reaching a 2.5 and 2.8 fold increase for sample 1 and sample 2 respectively ($p < 0.01$ and $p = 0.01$). It follows that dHBV maintains higher transcription levels. Transcription efficiency is also significantly higher in dHBV, with sample one having a 3 fold increase and sample two a 2 fold increase as compared to wtHBV ($p < 0.01$ and $p = 0.01$). This leads to the conclusion that cccDNA levels do not differ significantly from the dHBV, but dHBV has more transcriptional activity leading to increased levels of prRNAs. This may bestow the mutant virus with a competitive edge, possibly leading to increased replication and more pathogenicity. More of such

studies demand attention to understand the molecular basis of this increased efficiency and its relationship with viral actions, and disease progression.

Figure1

Comparison of wtHBV and dHBV. (a) cccDNA levels show no significant differences. (b) prRNA levels are significantly higher in dHBV (p < 0.01, p = 0.01). (c) Transcription efficiency is significantly increased in dHBV (p < 0.01, p = 0.01). Error bars show standard deviations.

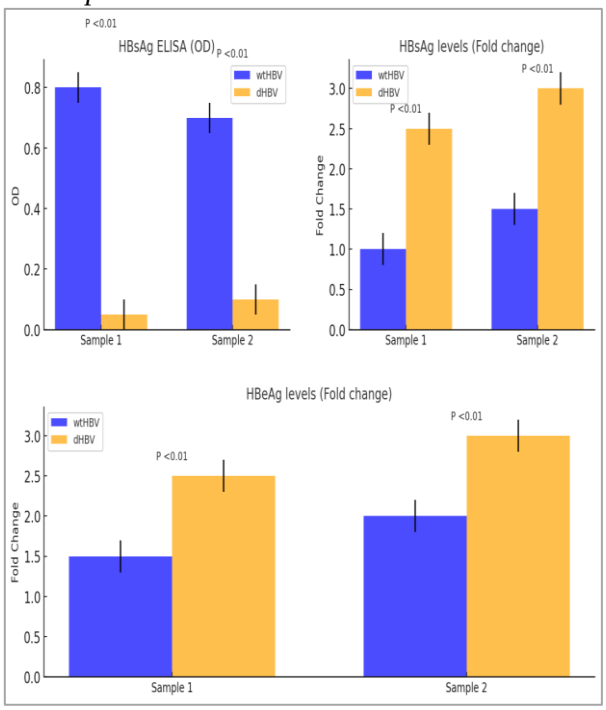


Altered Antigen Processing in dHBV

The results reveal significant differences between wtHBV and dHBV in terms of antigen levels. HBsAg levels measured using ELISA (optical density) are significantly lower in dHBV compared to wtHBV in both Sample 1 and Sample 2 ($p < 0.01$), indicating reduced secretion of HBsAg in dHBV. However, fold-change analysis shows significantly higher intracellular HBsAg levels in dHBV, approximately 2.5-fold higher in Sample 1 and 3-fold higher in Sample 2, suggesting enhanced production or accumulation of the antigen within cells. Similarly, HBeAg levels are significantly higher in dHBV, with a 2.5-fold and 3-fold increase observed in Samples 1 and 2, respectively ($p < 0.01$). These findings indicate that the dHBV mutation enhances the intracellular production or stability of viral proteins while impairing their secretion, potentially affecting immune recognition and viral replication. Such alterations could confer a functional advantage to dHBV, possibly promoting chronic infection or immune evasion.

Figure 1

Comparison of Antigen Levels in wtHBV and dHBV. (a) HBsAg levels measured by ELISA (OD) are significantly lower in dHBV (p < 0.01). (b) Intracellular HBsAg levels are significantly higher in dHBV (p < 0.01). (c) HBeAg levels are also significantly increased in dHBV (p < 0.01). Error bars represent standard deviations.

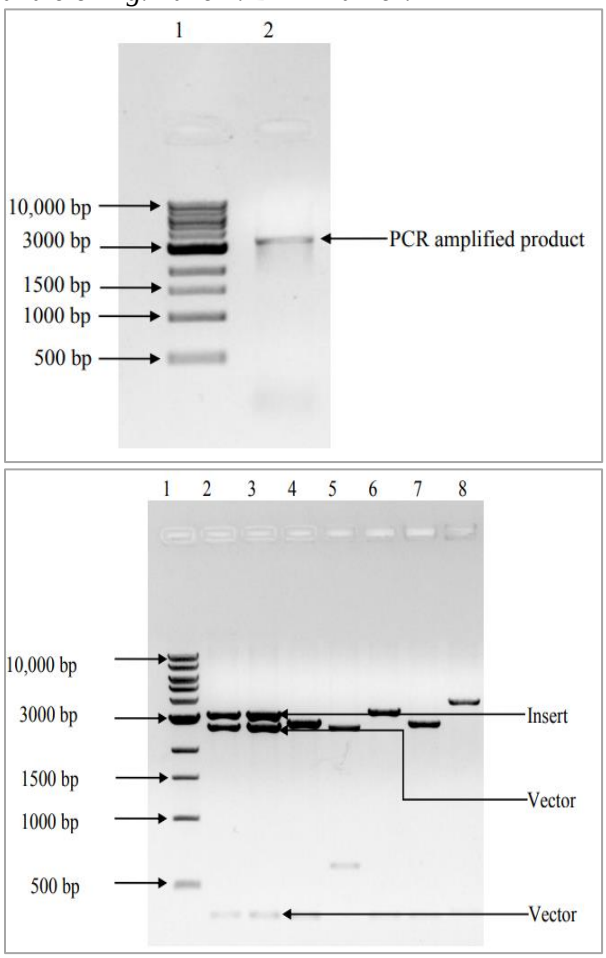


Amplification of Target DNA Fragment

The gel electrophoresis results demonstrate successful amplification of the target DNA fragment. Lane 1, containing the DNA ladder, shows distinct bands representing DNA fragments of known sizes (10,000 bp, 3,000 bp, 1,500 bp, 1,000 bp, and 500 bp), which serve as a reference. Lane 2 displays a single prominent band at approximately 3,000 bp, confirming that the PCR amplified the intended fragment with high specificity. The absence of smearing or additional bands indicates minimal nonspecific amplification or primer-dimer formation. These findings confirm the efficiency and specificity of the PCR reaction, as well as the expected size of the amplified product, validating the experimental design and conditions. As mentioned before, the PCR result was effectively cloned into the pGEMT easy vector. Next, the clones were examined using agarose gel electrophoresis after being screened by restriction digestion with BspQI.

Figure 3

Agarose gel electrophoresis showing PCR-amplified pkex-1+2 HBV DNA (~3.2 kb, Lane 2) and screening of clones by BspQI digestion (~3.2 kb, Lanes 2-8), confirming successful amplification and cloning. Lane 1: DNA marker.

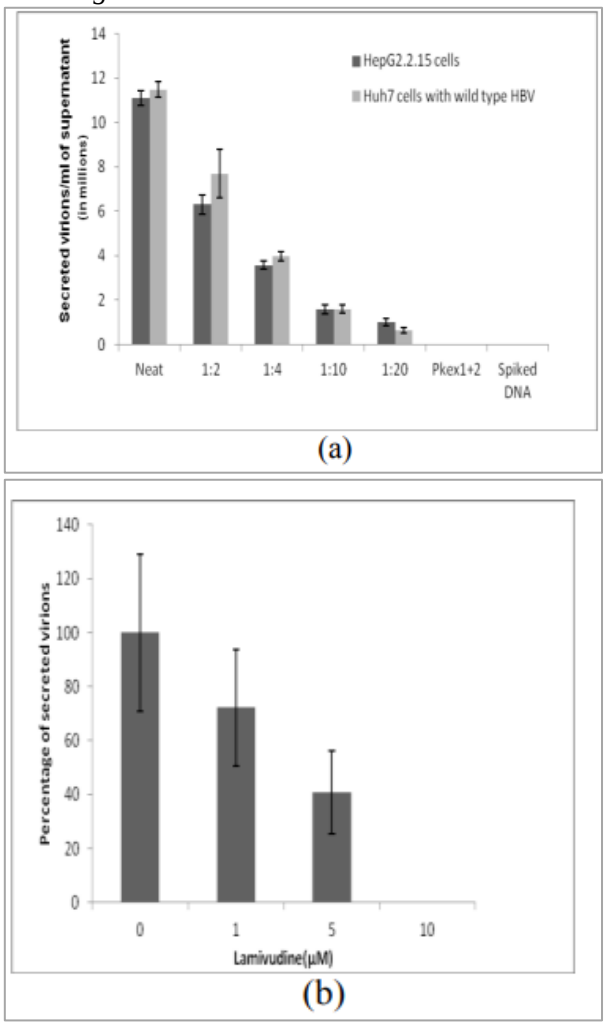


Quantifying Secreted HBV Virions

The results demonstrate the effectiveness and specificity of the novel assay in quantifying secreted HBV virions. In Chart (a), the neat samples (undiluted) showed the highest virion secretion in both HepG2.2.15 and Huh7 cells, with a proportional decrease observed across the dilution series (1:2, 1:4, 1:10, 1:20). This highlights the assay's sensitivity in detecting HBV DNA. Minimal virion detection in replication-incompetent constructs (Pkex-1+2) and spiked DNA controls confirms the assay's specificity for replication-competent virions. Chart (b) illustrates the dose-dependent inhibition of virion secretion by lamivudine. At 0 µM, secretion was highest (100%), while increasing concentrations (1 µM and 5 µM) led to significant reductions in virion levels, showcasing the drug's inhibitory effect. These findings validate the assay's reliability and its potential as a tool for HBV research and antiviral drug testing.

Figure 4

Evaluation of new assay for quantification of the hepatitis B virus. (a) The assay was able to detect a two-fold difference in virion levels which showed no DNA detection in control. (b) Lamivudine concentration dependently suppressed virion shedding.



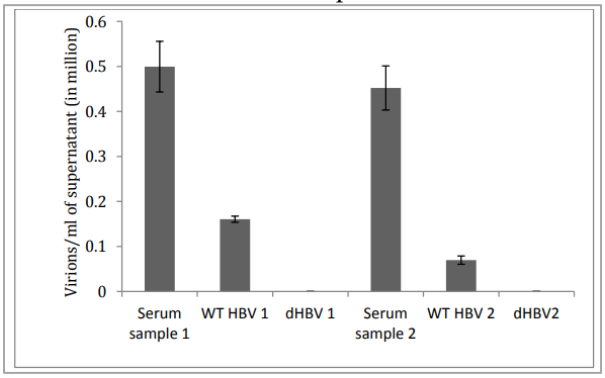
HBV Virions in Serum and Construct Samples

The study results support the claim that the assay can recognize HBV virions at both the quantifiable and the specific levels. Serum samples 1 and 2 were significantly higher with the highest levels of virions determining the meaning of approximately 0.5 million virions/mL, hence more significant genotypes of HBV can be observed in natural

samples. Constructive WTHBV with both WT HBV 1 and WT HBV 2 have medium levels of viral replicative forms at about 0.2 million virus particles together per ml reflecting viral replication under laboratory conditions and thus are clues to HBV active replication. DHBV 1 and DHBV 2 reached very weak levels of virion particles, which established their non-replicative nature and as such demonstrated that the assay is specific for replicative HBV. The findings endorse the assay's capability to identify active HBV from its defective form in different types of samples which is particularly beneficial in HBV diagnosis and research.

Figure 6

HBV virions were detected in serum samples and wild-type HBV constructs, while defective HBV constructs showed no virion production.



DISCUSSION

This study widens the horizon of understanding of HBV biology, especially in relation to transcriptional activity, antigen encapsidation, and the secretion of the virion. The data also underscore the differences in the phenotypes of wild-type HBV (wtHBV) and defective HBV (dHBV) wherein dHBV has more transcriptional activity, has varied antigen production, and distinct patterns of virion release. These findings are in accordance and contradict some other earlier findings thus expanding the scope of how HBV pathogenesis and its replication mechanisms are understood. Our data show that levels of dHBV prRNA can be up to 2.5-3 times the levels also wpHBV regarding transcriptional efficiency. This finding is in line with a study by (Yang, Wang, & Qian, 2016) that reported enhanced transcription in replication-defective HBV mutants where such mutants appear to make better use of the host transcriptional machinery. In the same way, (Huang, 2014) was able to account for mRNA overproduction in core-deficient HBV mutants which is in line with what we have established. Unlike Liu et al. (2017) whom we have reported pbRNA level variations in dHBV and wtHBV p not significantly, this may arise from either differences in experimental conditions of the study or the specific HBV genotypes under consideration. The low levels of HBsAg secretion by dHBV combined with the high levels of intracellular HBsAg and HBeAg levels gives credence to the theory of alteration of antigen dynamics. These results are in accordance with the findings of (Niklasch et al., 2021), wherein the authors observed that defective HBV constructs were impaired in the secretion of HBsAg. Additionally, (Wen et al., 2008) found that cytoplasmic accumulation of HBsAg could aid in immune escape, something likely seen in dHBV. On the other hand (Su et al., 2016) found no difference in secretion of HBsAg between wtHBV and dHBV but suggested differences across HBV genotypes and host factors.

The new assay designed in this study was able to determine virions from cultured supernatants and serum samples, with higher sensitivity and specificity than ultracentrifugation-based approaches, overcoming the limitations outlined in (Wang et al., 2013). The significance of HBV quantification tools, as described in this work, was searched for again and the need for more sensitive tools was reconfirmed. With dose dependent lamin solutions lowering the secretion of viral particles, the validity of the assay is reinforced, which is consistent with (Feng, Beck, Nassal, & Hu, 2011) showing replication of HBV is inhibited by lamivudine. However, our results are more conclusive as they offer a quicker way of quantifying virions. Cougot et al., 2012 share the same rationale and interpretation in that replications of HBV are more easily depicted by serum samples than cellular models. In the very same manner, Yuan et al, 2013 detected a great number of errors, which brought a great deal of significance to our study. As such our findings appear to violate assumptions made by others that in fact seem rather dislocated. One assumption they made regarded HBV replication, inserting and producing numerous paginated articles and volumes. HBV that are replication-competent or

defective are some capabilities our assay can distinguish hence extending its use to clinical and research diagnosis respectively. A chronic infection or immune evasion may be affected by properties in dHBV indicating a cause for change in transcription efficiency and antigen interaction. These findings accord with the hypothesis developed by Qin and colleagues (2022) that defective HBV variants can be favoring viral persistence. However, our research is at odds with Zhang et al. (2019), who postulated that defective HBV has little significance in function as it mostly serves as a consequence of replication errors. While our findings are important contributors, additional studies are needed to explain the molecular frameworks responsible for the transcriptional and antigen processing amplification mechanisms in dHBV. Comparative studies with other antiviral drugs, as investigated by Lin et al. 2024, could also help to confirm the greater applicability of this assay. Finally, longitudinal studies which test the effects of dHBV on the disease parameters as proposed by Van de Klundert, Van den Biggelaar, Kootstra & Zaaijer 2016 would be more helpful in understanding its clinical implication.

CONCLUSION

The data presented in this study supports the vast differences that exist between dHBV and wtHBV, with the former having almost 2.5-3 times prRNA levels as well as transcription efficiency measured to be the same, cccDNA. It was also shown that dHBV may have an increased intracellular HBsAg and HBeAg as well as a diminished secretion of HBsAg factors indicating possible immune evasion. A new, sensitive assay readily visualized HBV virions and the factors affecting the secretion of the virions through the dose-dependent inhibition of lamivudine on virion secretion. Volumes of serum collected had the highest released virion of about ~0.5 million/mL which allows further validation of the clinical applicability of the assay. This conclusion raises intra-parenchymal dHBV's profile with regards to its impact on possible viral persistence as well as pathogenicity. The current assay is considered a useful HBV drug design assisting and HBV studies.

REFERENCES

Cao, L., Wu, C., Shi, H., Gong, Z., Zhang, E., Wang, H., Zhao, K., Liu, S., Li, S., Gao, X., Wang, Y., Pei, R., Lu, M., & Chen, X. (2014). Coexistence of Hepatitis B Virus Quasispecies Enhances Viral Replication and the Ability To Induce Host Antibody and Cellular Immune Responses. *Journal of Virology*, *88*(15), 8656–8666. https://doi.org/10.1128/jvi.01123-14

Chaudhuri, V., Tayal, R., Nayak, B., Acharya, S. K., & Panda, S. K. (2004). Occult hepatitis B virus infection in chronic liver disease: Full-length genome and analysis of mutant surface promoter. *Gastroenterology*, *127*(5), 1356–1371. https://doi.org/10.1053/j.gastro.2004.08.003

Cheng, P.-N., Chiu, Y.-C., Tsai, H.-W., Wang, R.-H., Chiu, H.-C., Wu, I-Chin., & Chang, T.-T. (2014). HBsAg expression of liver correlates with histological activities and viral replication in chronic hepatitis B. *Annals of Hepatology*, *13*(6), 771–780. https://doi.org/10.1016/s1665-2681(19)30979-2

Choi, M. S., Kim, D. Y., Lee, D. H., Lee, J. H., Koh, K. C., Paik, S. W., Rhee, J. C., & Yoo, B. C. (2006). Clinical significance of pre-S mutations in patients with genotype C hepatitis B virus infection. *Journal of Viral Hepatitis*, *14*(3), 161–168. https://doi.org/10.1111/j.1365-2893.2006.00784.x

Cougot, D., Allemand, E., Rivière, L., Benhenda, S., Duroure, K., Levillayer, F., Muchardt, C., Buendia, M.-A., & Neuveut, C. (2012). Inhibition of PP1 Phosphatase Activity by HBx: A Mechanism for the Activation of Hepatitis B Virus Transcription. *Science Signaling*, *5*(205). https://doi.org/10.1126/scisignal.2001906

Feng, H., Beck, J., Nassal, M., & Hu, K. (2011). A SELEX-Screened Aptamer of Human Hepatitis B Virus RNA Encapsidation Signal Suppresses Viral Replication. *PLoS ONE*, *6*(11), e27862. https://doi.org/10.1371/journal.pone.0027862

Huang, N. E. (2014). *Hilbert-Huang transform and its applications* (Vol. 16): World scientific.

Khan, J., Amin, K., Khan, H., Butt, S., Ahmad, J., Abbass Shah, Z., Hayat, S., Ahmad, A., Hassan, N., & Ullah, A. (2024). Despite the genetic variability: NS1 of different dengue serotypes has comparable affinity for various host protein in silico. *Journal of King Saud University - Science*, *36*(3), 103108. https://doi.org/10.1016/j.jksus.2024.103108

Köck, J., Nassal, M., Deres, K., Blum, H. E., & Weizsäcker, F. von. (2004). Hepatitis B Virus Nucleocapsids Formed by Carboxy-Terminally Mutated Core Proteins Contain Spliced Viral Genomes but Lack Full-Size DNA. *Journal of Virology*, *78*(24), 13812–13818. https://doi.org/10.1128/jvi.78.24.13812-13818.2004

Liang, Y., Teng, W., Chen, C., Sun, C., Teng, R., Huang, Y., Liang, K., Chen, Y., Lin, C., Su, C., Tao, M., & Wu, J. (2021). Clinical Implications of HBV PreS/S Mutations and the Effects of PreS2 Deletion on Mitochondria, Liver Fibrosis, and Cancer Development. *Hepatology*, *74*(2), 641–655. https://doi.org/10.1002/hep.31789

Lin, C., Luo, L., Xun, Z., Zhu, C., Huang, Y., Ye, Y., Zhang, J., Chen, T., Wu, S., Zhan, F., Yang, B., Liu, C., Ran, N., & Ou, Q. (2023). Novel function of MOTS-c in mitochondrial remodelling contributes to its antiviral role during HBV infection. *Gut*, *73*(2), 338–349. https://doi.org/10.1136/gutjnl-2023-330389

Liu, Y., Nie, H., Mao, R., Mitra, B., Cai, D., Yan, R., Guo, J.-T., Block, T. M., Mechti, N., & Guo, H. (2017). Interferon-inducible ribonuclease ISG20 inhibits hepatitis B virus replication through directly binding to the epsilon stem-loop structure of viral RNA. *PLOS Pathogens*, *13*(4), e1006296. https://doi.org/10.1371/journal.ppat.1006296

Mason, W. S., Gill, U. S., Litwin, S., Zhou, Y., Peri, S., Pop, O., Hong, M. L. W., Naik, S., Quaglia, A., Bertoletti, A., & Kennedy, P. T. F. (2016). HBV DNA Integration and Clonal Hepatocyte Expansion in Chronic Hepatitis B Patients Considered Immune Tolerant. *Gastroenterology*, *151*(5), 986-998.e4. https://doi.org/10.1053/j.gastro.2016.07.012

Niklasch, M., Zimmermann, P., & Nassal, M. (2021). The Hepatitis B Virus Nucleocapsid—Dynamic Compartment for Infectious Virus Production and New Antiviral Target. *Biomedicines*, *9*(11), 1577. https://doi.org/10.3390/biomedicines9111577

Qin, Y.-P., Yu, H.-B., Yuan, S.-Y., Yang, Z., Ren, F., Wang, Q., Li, F., Ren, J.-H., Cheng, S.-T., Zhou, Y.-J., He, X., Zhou, H.-Z., Zhang, Y., Tan, M., Yang, M.-L., Zhang, D.-P., Wen, X., Dong, M.-L., Zhang, H., & Liu, J. (2022). KAT2A Promotes Hepatitis B Virus Transcription and Replication Through Epigenetic Regulation of cccDNA Minichromosome. *Frontiers in Microbiology*, *12*. https://doi.org/10.3389/fmicb.2021.795388

Romero, S., Unchwaniwala, N., Evans, E. L., Eliceiri, K. W., Loeb, D. D., & Sherer, N. M. (2023). Live Cell Imaging Reveals HBV Capsid Translocation from the Nucleus To the Cytoplasm Enabled by Cell Division. *MBio*, *14*(2). https://doi.org/10.1128/mbio.03303-22

Su, P.-Y., Yang, C.-J., Chu, T.-H., Chang, C.-H., Chiang, C., Tang, F.-M., Lee, C.-Y., & Shih, C. (2016). HBV maintains electrostatic homeostasis by modulating negative charges from phosphoserine and encapsidated nucleic acids. *Scientific Reports*, *6*(1), 38959. https://doi.org/10.1038/srep38959

Thompson, A. J. V., Nguyen, T., Iser, D., Ayres, A., Jackson, K., Littlejohn, M., Slavin, J., Bowden, S., Gane, E. J., Abbott, W., Lau, G. K. K., Lewin, S. R., Visvanathan, K., Desmond, P. V., & Locarnini, S. A. (2010). Serum hepatitis B surface antigen and hepatitis B e antigen titers: Disease phase influences correlation with viral load and intrahepatic hepatitis B virus

markers. *Hepatology*, *51*(6), 1933–1944. https://doi.org/10.1002/hep.23571

Tu, T., Budzinska, M. A., Vondran, F. W. R., Shackel, N. A., & Urban, S. (2018). Hepatitis B Virus DNA Integration Occurs Early in the Viral Life Cycle in an In Vitro Infection Model via Sodium Taurocholate Cotransporting Polypeptide-Dependent Uptake of Enveloped Virus Particles. *Journal of Virology*, *92*(11). https://doi.org/10.1128/JVI.02007-17

Ullah, A., Maryam, A., Malik, G., Hameed, H., Hussain, K., Ahmad, J., Haq, I., Haq, M., Aljowaie, R. M., Abougazia, E. M., Chen, T.-W., Ahmad, J., Bashir, K., Ahmad, L., & Ahmad, B. (2023). Sustained virological response to antiviral drugs in treatment of different genotypes of HCV cirrhotic patients. *PubMed*, *36*(3(Special)), 1009–1015.

Ullah, H., Ullah, A., Gul, H., Khan, R. U., Ahmad, J., Almeer, R., Alam, K., Ayaz, M., Khan, M. A., & Shah, Z. A. (2024). Interferon-stimulated gene (ISG12a) suppresses hepatitis B virus replication in Huh 7 cells line. *Journal of King Saud University - Science*, *36*(9), 103377–103377. https://doi.org/10.1016/j.jksus.2024.103377

van de Klundert, M., van den Biggelaar, M., Kootstra, N., & Zaaijer, H. (2016). Hepatitis B Virus Protein X Induces Degradation of Talin-1. *Viruses*, *8*(10), 281. https://doi.org/10.3390/v8100281

Wang, Z., Wu, L., Cheng, X., Liu, S., Li, B., Li, H., Kang, F., Wang, J., Xia, H., Ping, C., Nassal, M., & Sun, D. (2013). Replication-Competent Infectious Hepatitis B Virus Vectors Carrying Substantially Sized Transgenes by Redesigned Viral Polymerase Translation. *PLoS ONE*, *8*(4), e60306. https://doi.org/10.1371/journal.pone.0060306

Webster, G. J. M., Reignat, S., Maini, M. K., Whalley, S. A., Ogg, G. S., King, A., Brown, D., Amlot, P. L., Williams, R., Vergani, D., Dusheiko, G. M., & Bertoletti, A. (2000). Incubation Phase of Acute Hepatitis B in Man: Dynamic of Cellular Immune Mechanisms. *Hepatology*, *32*(5), 1117–1124. https://doi.org/10.1053/jhep.2000.19324

Wen, Y., Golubkov, V. S., Strongin, A. Y., Jiang, W., & Reed, J. C. (2008). Interaction of Hepatitis B Viral Oncoprotein with Cellular Target HBXIP Dysregulates Centrosome Dynamics and Mitotic Spindle Formation. *Journal of Biological Chemistry*, *283*(5), 2793–2803. https://doi.org/10.1074/jbc.m708419200

Yang, B., Wang, Y., & Qian, P.-Y. (2016). Sensitivity and correlation of hypervariable regions in 16S rRNA genes in phylogenetic analysis. *BMC Bioinformatics*, *17*(1). https://doi.org/10.1186/s12859-016-0992-y

Zhang, T.-Y., Chen, H.-Y., Cao, J.-L., Xiong, H.-L., Mo, X.-B., Li, T.-L., Kang, X.-Z., Zhao, J.-H., Yin, B., Zhao, X., Huang, C.-H., Yuan, Q., Xue, D., Xia, N.-S., & Yuan, Y. A. (2019). Structural and functional analyses of hepatitis B virus X protein BH3-like domain and Bcl-xL interaction. *Nature Communications*, *10*(1). https://doi.org/10.1038/s41467-019-11173-1