

DOI: [doi.org/10.55627/mmc.001.01.0022](https://doi.org/10.55627/mmc.001.01.0022)**Research Article****Analysis of the HBV Small S Gene Partial Sequences and its Implications for Detection, Prevention and Treatment in Pakistani Patients**Ambreen Masroor Khokhar<sup>1</sup>, Saeed Khan<sup>1\*</sup>, Maria Zahid<sup>1</sup>, Amanullah Lail<sup>2</sup>, Bilal Ahmed Khan<sup>1</sup>, Asif Iqbal Khan<sup>1</sup>, Samina Baig<sup>3</sup>, Shumaila NM Hanif<sup>4</sup>, Hizb Ullah Khan<sup>5</sup>, Asif Ullah Khan<sup>5</sup>, Amjad Khan<sup>6</sup><sup>1</sup>Provincial Public Health Laboratory, Dow University of Health Sciences, Ojha Campus Karachi, Pakistan.<sup>2</sup>Department of Pediatrics Dow International Medical College, Dow University of Health Sciences, Karachi, Pakistan.<sup>3</sup>Microbiology Lab, Dow University of Health Sciences Karachi, Pakistan.<sup>4</sup>University of Pikeville, Kentucky College of Osteopathic Medicine Kentucky, USA.<sup>5</sup>Department of Biochemistry, Abdul Wali Khan University Mardan, Pakistan.<sup>6</sup>Ineos Oxford Institute for Antimicrobial Research, University of Oxford, 12 Mansfield Road, Oxford, OX1 3TA, UK.\*Correspondence: [saeed.khan@duhs.edu.pk](mailto:saeed.khan@duhs.edu.pk)© The Author(s) 2021. This article is licensed under a Creative Commons Attribution 4.0 International License. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>.**Abstract**

Hepatitis B virus (HBV) causes significant morbidity and mortality throughout the world, especially in developing countries. In Pakistan, the HBV infection rate is one of the highest in the world and about one third of infected population is co-infected with hepatitis C virus (HCV) and hepatitis D virus (HDV). In the present study, we isolated HBV from 49 HBV mono-infected and 25 HBV/HCV co-infected Pakistani patients and classified them based on the partial sequences of S gene. We further investigated mutations in these sequences that might result in the failure of hepatitis B surface antigen (HBsAg) detection, as well as vaccination and treatment failure. The D and D1 were identified as the most prevalent HBV genotype and sub-genotype respectively in Pakistani samples. The same genotype/sub-genotype pattern was observed for the HBV/HCV co-infected patients. We identified several mutations in small S gene, which are previously reported to have roles in HBV diagnosis and treatment. Especially, the sT127P mutant, previously known to be implicated in vaccine escape, was prevalent with 98 and 96% frequencies in HBV mono-infected and HBV/HCV co-infected patients respectively. The findings of current study have implications with respect to prevention, diagnosis, and treatment of HBV infections in the Pakistani population.

**Keywords:** Hepatitis B virus, Pakistan, small S gene, mutations, vaccine escape, drug resistance**Introduction**

Hepatitis B virus (HBV) infection is a serious health issue contributing to significant morbidity and mortality worldwide, particularly in developing countries. It is a leading cause of acute as well as chronic hepatic infection, hepatocellular carcinoma, and liver cirrhosis. More than 2 billion people around the world have been infected by HBV, (Tan et al. 2021; Razavi-Shearer et al. 2018; Schweitzer et al. 2015) around 257-291 million of them chronically (Lim et al. 2020). In Pakistan, the infection rate of the HBV is increasing continually, with approximately 3-5% general population has been infected, according to a national

survey (Ali et al. 2011).

The HBV is classified into eight genotypes (i.e., A-H) (Norder et al. 2004), based on >8% variation over the entire genome (Arauz-Ruiz et al. 2002). These variations are partially due to host/virus interaction and partially due to their parallel evolution in distinct geographic regions (Kay and Zoulim 2007). HBV genotypes have been further divided into subtypes based on serological classification. Genotype A is subdivided into (A1-A4) which are predominant in Northwest Europe, North America, and Africa. Genotype B and C are mostly found in Asia and further

subdivide into several sub-genotypes (B1-B6 and C1-C5, respectively). Genotype D is sub-divided into (D1-D10) and distributed worldwide. The genotype E, G, and H, have no sub-genotypes, while genotype F is divided into (F1-F4) sub-genotypes (Kramvis, Kew, and François 2005). Genetic variation among the HBV genotypes and sub-genotypes plays an important role in the severity of the disease, its complications, and resistance to treatment, and vaccination response (Chu and Lok 2002). In Pakistan, the most common genotype of HBV was found to be D which accounts for more than 70% HBV genotypes prevalent in Pakistan (Mahmood et al. 2016).

The HBV genome is comprised of the four different genes, S (surface) including (Pre-S1, Pre-S2, small S), C (core), P (polymerase), and X gene which is involved in hepatocellular carcinoma. The small S protein, known as HBsAg, is one of the most important proteins because of its vital role in the viral binding to the host cell receptor and subsequent entry into the cell (Ganem and Prince 2004). Several mutations have been reported in the S gene. Most of the mutations are observed in the small S region, while some have also been found in the Pre-S1 and Pre-S2 regions. These mutations play important roles to in the failure of HBV vaccination, HBsAg detection, and immune escape (Coppola et al. 2015; Purdy 2007).

The HBV coinfection with hepatitis C virus (HCV) is another important healthcare problem, contributing significantly to the burden of chronic liver disease (i.e., active hepatitis, cirrhosis, and hepatocellular carcinoma)(Konstantinou and Deutsch 2015). Globally, the HCV/HBV co-infection account for a substantial fraction of chronic liver disease patients (Liu and Hou 2006). Both viruses transmit vertically as well as horizontally (Wasley et al. 2010). The presence of both viruses increases the progression rate of liver disease and leads to a higher risk of the tumor than the presence of each of these viruses alone (Kurtz 1999).

The objectives of the present study include identification of HBV genotypes and sub-genotypes isolated from Pakistani patients, based on the partial sequences of S gene. We also aimed to detect mutations in these regions that might result in the failure of HBV vaccination, HBsAg detection, and immune escape. Furthermore, we compared the genetic pattern at this locus between the HBV mono-infected and HBV/HCV co-infected cases.

## Methods and Material

### Human Samples

This prospective study was approved by the Institutional Review Board & Ethics Committee of Dow University of Health Sciences, Karachi. Participating individuals were briefed about the project and informed consent was obtained from all the participants. All the experiments were performed in accordance with relevant guidelines and regulations including 'Declaration of Helsinki'. The study cohort included 49-mono-infection of HBV and 25 of HBV/HCV co-infected patients. Yellow cap tubes were used for blood collection, 200µl serum was separated and stored at -70 °C until further analysis.

### DNA Extraction and Amplification

Total DNA was extracted from the patient serum using QIAamp DNA Mini Kit (Qiagen Germany), and quantified by 1% agarose gel electrophoresis, and spectrophotometric analysis. S-gene amplification was done by using the nested PCR technique. Sense primer, 5'-GTGGTGGACTTCTCTCAATTTTC-3' and antisense primer 5'-CGGTAWAAAGGGACTCAMGAT-3' were used first. The second round of PCR was performed with the sense primer 5'-CAAGGTATGTTGCCCGTTTG-3', and antisense primer 5'-AAAGCCCTGCGAACCACTGA-3'.

### HBV DNA Sequence Analysis

The PCR products were purified and submitted for commercial DNA sequence services, Macrogen Korea. The quality of the sequences was checked by using the staden package and Finch TV. Trimming of sequences was performed by the Lasergene package v.7.1 (DNASTAR Inc UA) to yield high quality sequences draft.

### Structure Analysis

The reference genomes (A-H) of (HBV were downloaded from the NCBI reference genotype tool (Rožanov et al. 2004). High quality trimmed sequence data was compared with the reference genomes. The DnaSP v6.0 package (Rozas et al. 2017) was used to calculate the sequence composition of parsimony informative sites and haplotypes. The Arlequin v3.5 software (Excoffier, Laval, and Schneider 2007) was used to find out the population genetic statistics, like Fst analysis (pairwise fixation index), analysis of molecular variance (AMOVA), and Nei's distance (D<sub>A</sub>)

to estimate the nucleotide differences among the individual sequences.

### Mutation Analysis of S gene sequences

The analyses of mutations that might be responsible for drug resistance, HBsAg detection, and vaccine escape were performed by the online tools, i.e. HIV-Grade: HBV-Drug Resistant Interpretation (DRI) (Neumann-Fraune et al. 2014) and Gen2Pheno HBV (Beggel et al. 2012a). The two tools differ in their approach to estimate whether a particular mutation is implicated in the detection, therapy, or vaccine escape.

## Results

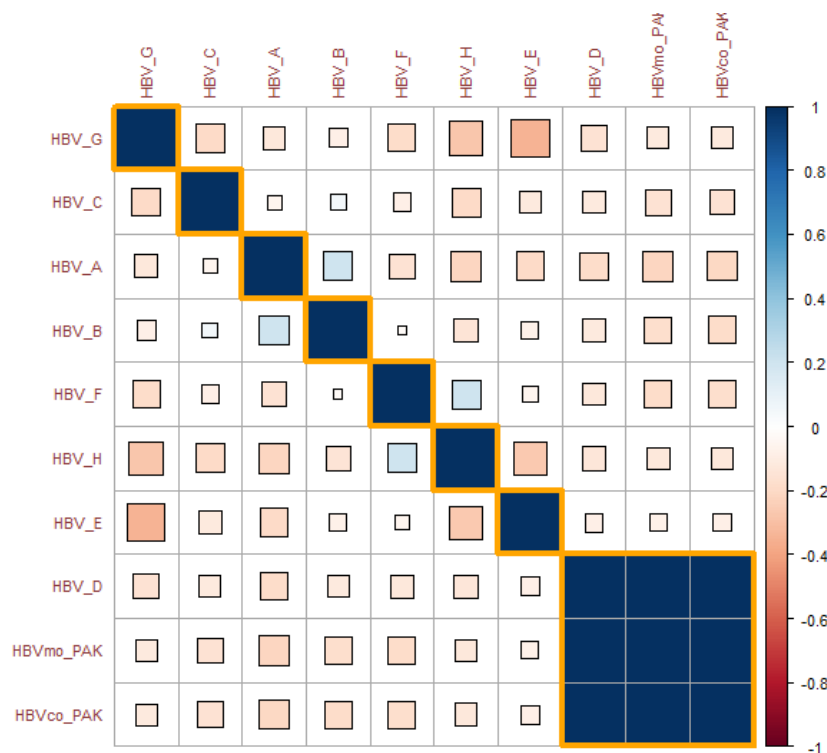
### Distance Correlation Matrix

A low genetic distance was observed between the sample sequences generated in present study against the reference genotype D through pairwise Fixation correlation index (Fst) (Figure 1). Fst, the most widely used statistical method for studying population

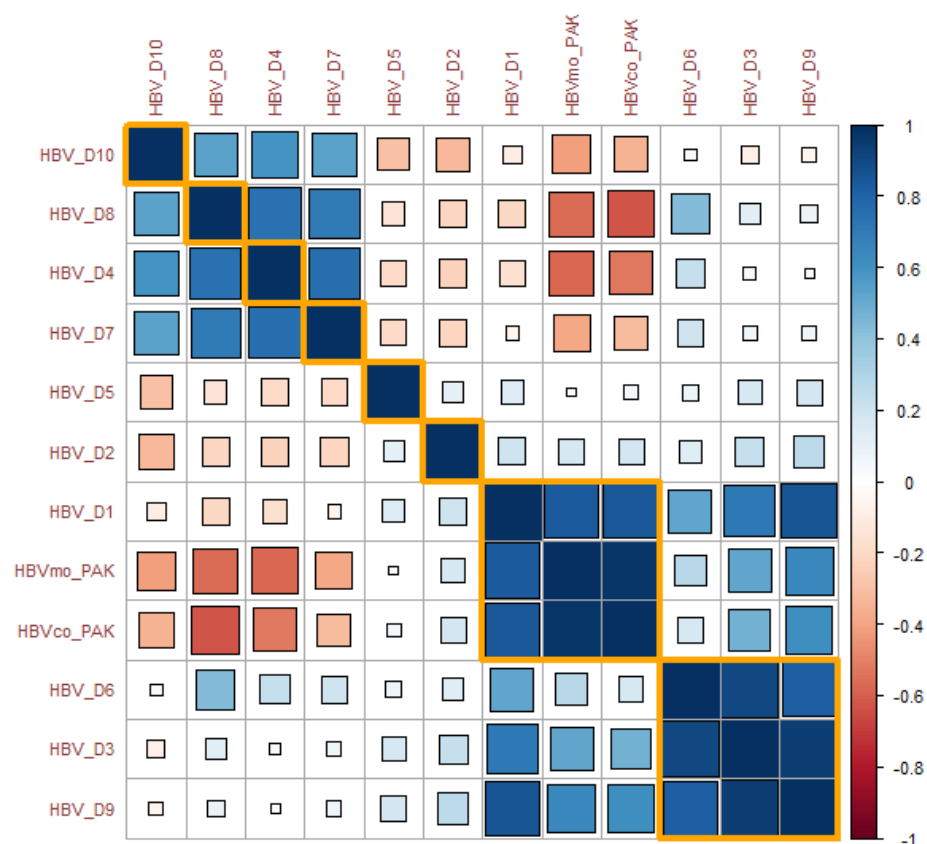
genetics, was employed to ascertain genetic structure of the sequences obtained

from HBV mono-infected and HBV/HCV co-infected patients. A high correlation was observed between the sequences and the reference genotype D using Pearson construction matrix by R-package. Sequences from HBV mono-infected and HBV/HCV co-infected patients shared >98% similarity and clustered together with the genotypes D. However, other reference genotypes (i.e., A, B, C, E, F, G, H) showed less than <2% similarity with our sequences (Figure 1).

Likewise, we found a high correlation of >90% between the sub-genotype D1 and our sequences from HBV mono-infected as well as HBV/HCV co-infected patients. Low genetic difference was also observed between our samples and reference sub-genotypes D3, and D9, showing more than >60% similarity. Other sub-genotypes (D2, D4, D5, D6, D7, D8, D10) displayed significant distinction and exhibited <10% similarity with HBV mono-infected and HBV co-infected sample sequences (Figure 2).



**Figure 1. Correlation plot between various HBV genotypes and our samples based on the pairwise Fst values.** The Fst values for each genotype were calculated using the Arelquin software, and used the Pearson construction matrix for 'correplot' using R package. The plot shows hierarchical clustering and correlation constructed between the genotypes. The minimum genetic distinction value and high correlations are depicted by the dark brackets and large sizes.



**Figure 2. Correlation plot between various HBV Sub-genotypes and our samples based on the pairwise Fst values.** The Fst values for each genotype were calculated using the Arelquin software, and Pearson construction matrix for ‘correplot’ using R package. The plot shows hierarchical clustering and correlation between the sub-genotypes. The minimum genetic distinction value and high correlations are depicted by the dark brackets and large sizes.

AMOVA was performed to elucidate the genetic structure to ascertain groups with the lowest variation. The AMOVA significances were interpreted with 99000 permutations. The low number of pairwise nucleotide difference (Nei’s:  $D_A$ ) was found between the genotype D and HBV mono-infected, HBV/HCV co-infected sequences congruent to abovementioned pairwise analyses (Figure 3). The low genetic distance showed that Pakistani samples (both mono-infected and co-infected) belong to the HBV genotype D. Likewise, low mean pairwise population genetic differences ( $\pi_{xy}$ ) were observed between HBV mon-infected, HBV/HCV co-infected sequences and genotype D. Greater genetic differences ( $\pi_{xx}$ ) were found within the HBV mono-infected samples as compared the HBV/HCV co-infected group (Figure 3).

Moreover, the HBV mono-infected and HBV/HCV co-infected sequences generated in current study displayed lowest genetic differentiation (i.e.,  $D_A$ , and  $\pi_{xy}$ ) to HBV sub-genotype, D1 (Figure 4). The genetic distance between our samples and the sub-genotype D3 was also found to be low, though it was higher than for D1 sub-genotypes. The sub-genotype D9 showed high similarity with our sequenced samples of HBV patients, although the genetic distance ( $D_A$ ) was higher than for sub-genotypes D1 and D3. Our samples showed high genetic distinction when compared to the other sub-genotypes (D2, D4, D5, D6, D7, D8, D10) (Figure 4).

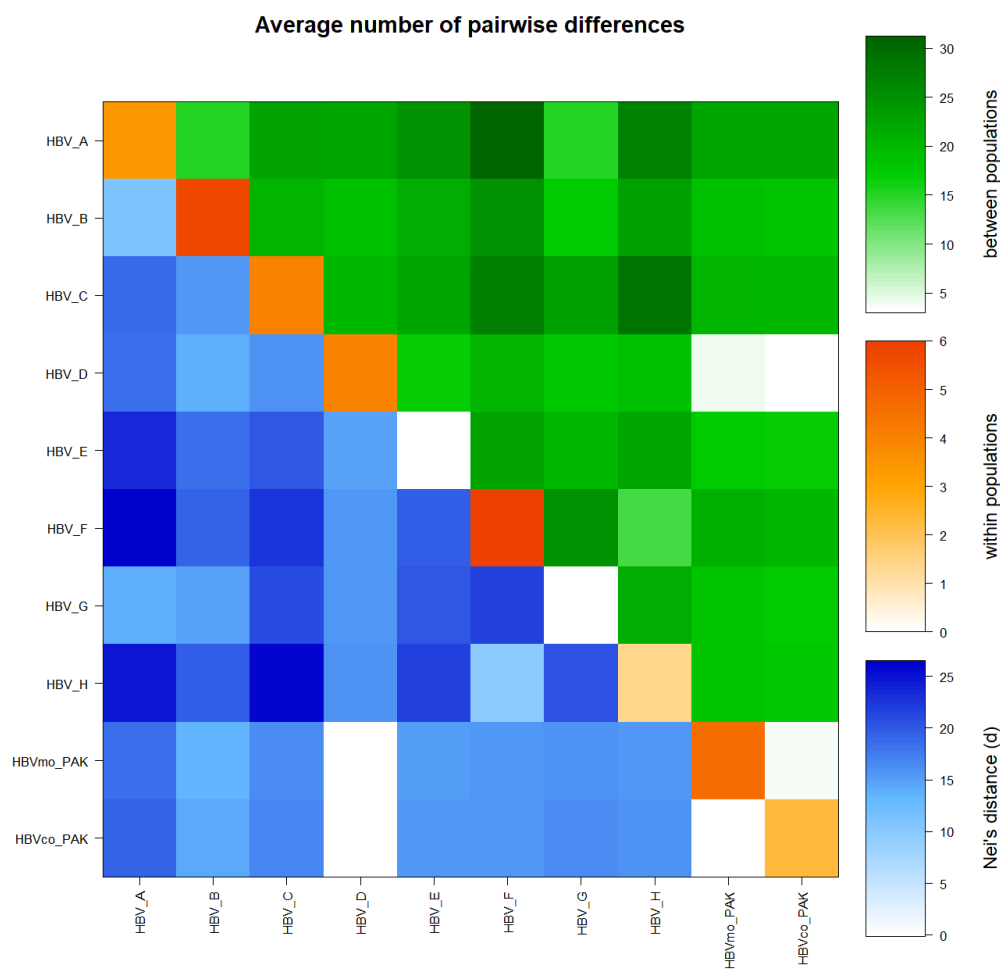
### Mutational Analysis

A total of 24 samples (96%) showed mutations in HBV/HCV co-infected group as determined with the

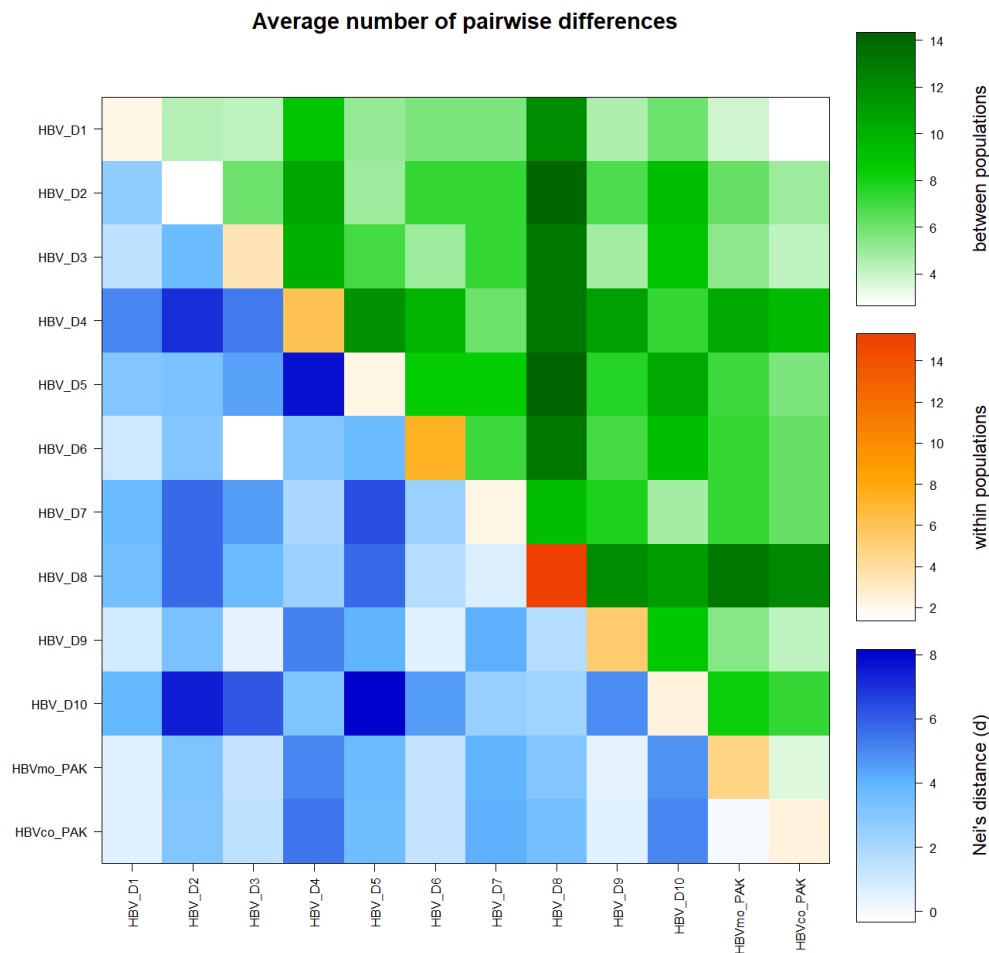
G2P tool, while mutations were found in all 49 samples in the HBV mono-infected group (Table 1). The most frequent mutation identified by G2P tool was T127P which was detected in 96% co-infected samples and 97.95% in mono-infected samples. The second most frequent mutation identified by the G2P tool in the mono-infected group was Y134F which was not detected in any samples of the co-infected group. Both I82S and P120S were the most common mutations in the co-infected group after T127P with a frequency of 8% each. P120S was absent in the mono-infected group

while I82S was found at 4.08%.

Likewise, the DRI identified mutations in 22 mono-infected HBV samples (45%), while mutations were found only in 7 out of the 25 sequences in HBV/HCV co-infected group (Table 2). E164G was the most frequent mutation observed in the mono-infected group at 6.12% which was not detected in the co-infected group. The most frequent mutations in the co-infected group were I82S (8%) and P120S (8%). While P120S was not detected in the mono-infected group, I82S was observed in the mono-infected group at 4.08%.



**Figure 3. Mean Pairwise differences for HBV genotypes.** The graph compares genetic structures of various HBV reference genotypes with our HBV mono-infected and HBV/HCV co-infected samples. It shows the genetic difference based on the three different statistical method, between samples- $\pi_{xy}$  (green above diagonal); within-samples- $\pi_{xx}$  (orange diagonal); and the net number of nucleotide difference among samples- $D_A$  (blue below diagonal).



**Figure 4. Mean Pairwise differences for HBV sub-genotypes.** The graph compares genetic structures of various HBV reference sub-genotypes with our HBV mono-infected and HBV/HCV co-infected samples. It shows the genetic difference based on the three different statistical method, between samples- $\pi_{xy}$  (green above diagonal); within-samples- $\pi_{xx}$  (orange diagonal); and the net number of nucleotide difference among samples- $D_A$  (blue below diagonal).

### Discussion

This study was conducted to determine the sequence-based genotypes/sub-genotypes and mutations in the small S gene of HBV mono-infected and HBV/HCV co-infected patients from Pakistan. The results show that the most common genotype and sub-genotype in Pakistani patients were D and D1 respectively (Figures 1 & 2). Previous studies from Pakistan also reported a high prevalence of genotype D in most of regions of Pakistan (Baig et al. 2007; Alam et al. 2007; Noorali et al. 2008), except in the Punjabi population where genotype C is mostly prevalent (M, S, and S 2004). About 1.6% infected patients with HBV in Pakistan show multiple

genotypes (Zeng et al. 2004). The genotype D causes a more severe disease (Kao, Liu, and Chen 2002) and is less responsive to the interferon treatment as compared to other genotype such as A and B (Sablon and Shapiro 2004; Erhardt et al. 2000). Previous reports of HBV genotype prevalence shows that B and C are the predominant genotypes in South Asia outside Pakistan and Afghanistan. For example, the predominant genotype in Afghanistan is D (Amini-Bavil-Olyaei et al. 2006), while India shows a mixed genotypes pattern with mostly A, C, and D genotypes (Thakur et al. 2002; Gandhe, Chadha, and Arankalle 2003; Kumar et al. 2005). In fact, seven genotypes (A-G) of the HBV were found in Asia (Toan et al. 2006).

**Table 1. Mutations and their frequencies in HBV Small S protein as predicted by Geno2Peno online tool.**

(ND-not detected).

Mutations	Mono-infected		Co-infected	
	(n)	%	(n)	%
S114T	1	2.04%	ND	-
R122K	1	2.04%	ND	-
T131N	1	2.04%	ND	-
T127P	48	97.95%	24	96%
Y134F	3	6.12%	ND	-
S143T	2	4.08%	ND	-
G159A	1	2.04%	ND	-
F161Y	1	2.04%	ND	-
A168V	1	2.04%	ND	-
P70T	1	2.04%	ND	-
C76L	1	2.04%	ND	-
V184A	2	4.08%	ND	-
P70R	1	2.04%	ND	-
W172C	2	4.08%	ND	-
G71A	1	2.04%	ND	-
W74C	1	2.04%	ND	-
M75R	1	2.04%	ND	-
L77Q	1	2.04%	ND	-
R78P	1	2.04%	ND	-
I82C	1	2.04%	ND	-
L88M	1	2.04%	ND	-
I92S	1	2.04%	ND	-
D144N	2	4.08%	ND	-
I82S	2	4.08%	2	8%
Y134R	1	2.04%	ND	-
A166D	1	2.04%	ND	-
E164G	3	6.12%	ND	-
L186P	2	4.08%	ND	-
T189I	2	4.08%	1	4%
I110L	1	2.04%	ND	-
C76Y	1	2.04%	ND	-
R79P	2	4.08%	ND	-
T140I	1	2.04%	ND	-
G71V	1	2.04%	ND	-
A166T	1	2.04%	ND	-
Y100C	1	2.04%	ND	-
L175S	1	2.04%	ND	-
Q101R	1	2.04%	ND	-
T131P	1	2.04%	ND	-
F83C	1	2.04%	ND	-
L88Q	1	2.04%	ND	-
Q101H	ND	-	1	4%
P120S	ND	-	2	8%
Y134N	ND	-	1	4%
G119I	ND	-	1	4%

The genotype identification in our analysis was accomplished based on the small S gene because it is a more stable and conserved region as compared to the other Pre-S genes (Lindh et al. 1998). The S gene is also an important part of the coding region, helping in the formation of the HBV virions (Chotiyaputta and Lok 2009; Hsu and Yeh 2011). Our analysis of the small S gene sequences using pairwise genetic comparisons shows that the genotype D had low genetic difference among the investigated sequences. Likewise, the sub-genotype D1 exhibited the minimum genetic difference against the HBV mono-infected and HBV/HCV co-infected sequences obtained in the Pakistani individuals while the genotype D3 and D9 showed moderate genetic differences (Figures 3 & 4). The S gene is responsible for HBsAg that includes a major hydrophilic region (MHR). Mutations in the MHR are associated with the vaccine escape. This happens due to the mutations in the MHR region leading to conformational changes in HBsAg, reducing its affinity towards antibody to HBsAg and resulting in the immune escape (Tian et al. 2007; Cooreman, Leroux-Roels, and Paulij 2001). The genotype identification in our analysis was accomplished based on the small S gene because it is a more stable and conserved region as compared to the other Pre-S genes (Lindh et al. 1998). The S gene is also an important part of the coding region, helping in the formation of the HBV virions (Chotiyaputta and Lok 2009; Hsu and Yeh 2011). Our analysis of the small S gene sequences using pairwise genetic comparisons shows that the genotype D had low genetic difference among the investigated sequences. Likewise, the sub-genotype D1 exhibited the minimum genetic difference against the HBV mono-infected and HBV/HCV co-infected sequences obtained in the Pakistani individuals while the genotype D3 and D9 showed moderate genetic differences (Figures 3 & 4). The S gene is responsible for HBsAg that includes a major hydrophilic region (MHR). Mutations in the MHR are associated with the vaccine escape. This happens due to the mutations in the MHR region leading to conformational changes in HBsAg, reducing its affinity towards antibody to HBsAg and resulting in the immune escape (Tian et al. 2007; Cooreman, Leroux-Roels, and Paulij 2001).

The G2P and DRI are the two most frequently employed tools to study drug-resistance, detection and vaccine escape mutations in the HBV genome (Neumann-Fraune et al. 2014; Beggel et al. 2012b). Analysis by G2P exhibited a prevalence of the HBsAg mutations in HBV/HCV samples at 96%, while DRI showed mutations in 14.3% samples. In HBV mono-infected samples, the G2P showed that 45% samples have mutations while DRI detected mutations in all samples. These results may have implications for patients who have a false-negative diagnosis of HBV due to these mutations. Such cases may contribute to the development of chronic carriers (Foy et al. 2012).

Several HBsAg mutations (Table 1 & 2), observed in this study, have previously been shown to have a significant role in vaccine, and immune escape (i.e., sD144A, sY100C, sP70T, sY134N, sI110L, sQ129H, sY134F, sS143T, sV184A, sT189I, sT127P) (Sayan et al. 2010; Verheyen et al. 2012; Pal et al. 2013; Coppola et al. 2015; Hosseini et al. 2019). The frequency of some of the mutations such as sT127P was very high both in HBV mono-infected (48 out of 49 or 98%) and HBV/HCV co-infected samples (24 out of 25 or 96%). While each of the mutations, sQ129H, sD144A, sP120S, and sY100C has been found in <3% samples, these mutations are reported to have clinical significance in vaccine escape (Nguai et al. 1997; Luongo et al. 2015; Ireland et al. 2000; Lee et al. 2001; Lazarevic 2014). Several mutations detected in our study are not previously reported for any role in vaccine escape and require further investigation to elucidate their role(s) in this phenomenon, if any.

Around, 3-5% Pakistani population is infected with the chronic carrier HBV infection that incurs substantial treatment costs (Ali et al. 2011; Abdullah et al. 2019). The commonly used drugs for the treatment of HBV infection in Pakistan are Adefovir, Tenofovir, Entecavir, Telbivudine, and Lamivudine (Abbas et al. 2010). In our analysis, a single mutation (A181S) which is previously associated with drug-resistance to Adefovir in the HBV mono-infection individuals, was observed in 4% samples. No other mutation found in our analysis is previously linked with resistance to the actions of Tenofovir, Entecavir, Telbivudine, and Lamivudine. However, due to the limited sequenced sample size, these results may not be good enough for

**Table 2. Mutations and their frequencies in HBV Small S protein as predicted by HBV-drug resistance interpretation (DRI) online tool. (ND-not detected).**

Mutations	Mono-infected		Co-infected	
	(n)	%	(n)	%
P70T	1	2.04%	ND	-
C76L	1	2.04%	ND	-
F83C	2	4.08%	ND	-
Q101R	1	2.04%	ND	-
T131P	1	2.04%	ND	-
V184A	2	4.08%	ND	-
I82C	1	2.04%	ND	-
L88M	1	2.04%	ND	-
A166T	1	2.04%	ND	-
Y100C	1	2.04%	ND	-
T68S	1	2.04%	ND	-
P70R	1	2.04%	ND	-
G71A	1	2.04%	ND	-
W74C	1	2.04%	ND	-
M75R	1	2.04%	ND	-
L77Q	1	2.04%	ND	-
R78P	1	2.04%	ND	-
R79P	2	4.08%	ND	-
L175S	1	2.04%	ND	-
G71V	1	2.04%	ND	-
P127T	1	2.04%	1	4%
Y134R	1	2.04%	ND	-
A166D	1	2.04%	ND	-
S143T	1	2.04%	ND	-
D144N	1	2.04%	ND	-
E164G	3	6.12%	ND	-
L186P	1	2.04%	ND	-
I92S	1	2.04%	ND	-
T189I	1	2.04%	1	4%
C76Y	1	2.04%	ND	-
Y134F	1	2.04%	ND	-
W172C	2	4.08%	ND	-
L199V	1	2.04%	ND	-
Y135S	1	2.04%	ND	-
D144A	1	2.04%	ND	-
T140I	1	2.04%	ND	-
I82S	2	4.08%	2	8%
I110L	1	2.04%	ND	-
L88Q	1	2.04%	ND	-
Q101H	ND	-	1	4%
P120S	ND	-	2	8%
Y134N	ND	-	1	4%
G119I	ND	-	1	4%
Q129H	ND	-	2	8%

clinical decision-making. Additional studies with large sample size and proper patient follow up with respect to treatment response and efficacy are necessary before generalizing these results.

We conclude that the HBV genotype and sub-genotype most prevalent in Pakistani patients with HBV mono-infection as well as HBV/HCV co-infection, are D and D1, respectively. Some of the mutations found in the small S gene during our analysis have roles in detection, treatment, and vaccine escape. However, further studies are necessary to reproduce and authenticate these findings.

### Conflict of interest

The authors declare that they have no competing interests.

### Funding

The authors thank Dow University of Health Sciences Karachi for the financial support in carrying out this research project.

### Ethics approval

Yes. The study was approved by the Institutional Review Board & Ethics Committee of Dow University of Health Sciences, Karachi.

### Consent forms

Yes. Consent forms were obtained from the participating patients.

### Authors contribution

AMK and SK conceptualized the study, collected samples and wrote the final manuscript, MZ, AL, BAK, AIK, SB, and SNMH helped in analysis and writing the first draft, HUK, and AUK did the experimental analysis and helped in initial manuscript writing, SK, and AK supervised the whole project and wrote the final manuscript.

### Acknowledgements

Authors acknowledge the support of the Dow University of Health Sciences Karachi for creating work friendly environment for conducting such scholarly activities.

## References

- Abbas, Zaigham, Wasim Jafri, Saeed Hamid, and Pakistan Society for the Study of Liver Diseases. 2010. "Management of Hepatitis B: Pakistan Society for the Study of Liver Diseases (PSSLD) Practice Guidelines." *Journal of the College of Physicians and Surgeons--Pakistan: JCPSP* 20 (3): 198–201. <https://doi.org/03.2010/JCPSP.198201>.
- Abdullah, Sikander, Sarmad Zahoor, Muhammad Ahmad Rao, Syed Maaz Abdullah, Sadia Asif, Abdul Wajid, and Abdul Rehman Zia Zaidi. 2019. "Seroprevalence of Hepatitis B Virus in Blood Donors at a Large Teaching Hospital of Pakistan: A Potential Health Policy Concern." *Journal of Applied Hematology* 10 (1): 29.
- Alam, Muhammad Masroor, Sohail Zahoor Zaidi, Salman Akbar Malik, Shahzad Shaukat, Asif Naeem, Salmaan Sharif, Mehar Angez, and Javed Aslam Butt. 2007. "Molecular Epidemiology of Hepatitis B Virus Genotypes in Pakistan." *BMC Infectious Diseases* 7 (October): 115. <https://doi.org/10.1186/1471-2334-7-115>.
- Ali, Muhammad, Muhammad Idrees, Liaqat Ali, Abrar Hussain, Irshad Ur Rehman, Sana Saleem, Samia Afzal, and Sadia Butt. 2011. "Hepatitis B Virus in Pakistan: A Systematic Review of Prevalence, Risk Factors, Awareness Status and Genotypes." *Virology Journal* 8 (March): 102. <https://doi.org/10.1186/1743-422X-8-102>.
- Amini-Bavil-Olyaei, Samad, Seyed-Moayed Alavian, Ahmad Adeli, Ramin Sarrami-Forooshani, Farzaneh Sabahi, Elham Sabouri, Hamid-Reza Tavangar, Mohammad Azizi, and Fereidoun Mahboudi. 2006. "Hepatitis B Virus Genotyping, Core Promoter, and Precore/Core Mutations among Afghan Patients Infected with Hepatitis B: A Preliminary Report." *Journal of Medical Virology* 78 (3): 358–64. <https://doi.org/10.1002/jmv.20547>.
- Arauz-Ruiz, Patricia, Helene Norder, Betty H. Robertson, and Lars O. Magnius. 2002. "Genotype H: A New Amerindian Genotype of Hepatitis B Virus Revealed in Central America." *The Journal of General Virology* 83 (Pt 8): 2059–73. <https://doi.org/10.1099/0022->

- 1317-83-8-2059.
- Baig, Saeeda, Anwar Ali Siddiqui, Waqaruddin Ahmed, Huma Qureshi, and Ambreen Arif. 2007. "The Association of Complex Liver Disorders with HBV Genotypes Prevalent in Pakistan." *Virology Journal* 4 (November): 128. <https://doi.org/10.1186/1743-422X-4-128>.
- Beggel, Bastian, Maria Neumann-Fraune, Matthias Döring, Glenn Lawyer, Rolf Kaiser, Jens Verheyen, and Thomas Lengauer. 2012a. "Genotyping Hepatitis B Virus Dual Infections Using Population-Based Sequence Data." *The Journal of General Virology* 93 (Pt 9): 1899–1907. <https://doi.org/10.1099/vir.0.043042-0>.
- . 2012b. "Genotyping Hepatitis B Virus Dual Infections Using Population-Based Sequence Data." *The Journal of General Virology* 93 (Pt 9): 1899–1907. <https://doi.org/10.1099/vir.0.043042-0>.
- Chotiyaputta, Watcharasak, and Anna S. F. Lok. 2009. "Hepatitis B Virus Variants." *Nature Reviews. Gastroenterology & Hepatology* 6 (8): 453–62. <https://doi.org/10.1038/nrgastro.2009.107>.
- Chu, Chi-Jen, and Anna S. F. Lok. 2002. "Clinical Significance of Hepatitis B Virus Genotypes." *Hepatology (Baltimore, Md.)* 35 (5): 1274–76. <https://doi.org/10.1053/jhep.2002.33161>.
- Cooreman, Michael P., Geert Leroux-Roels, and Wilma P. Paulij. 2001. "Vaccine- and Hepatitis B Immune Globulin-Induced Escape Mutations of Hepatitis B Virus Surface Antigen." *Journal of Biomedical Science* 8 (3): 237–47. <https://doi.org/10.1007/BF02256597>.
- Coppola, Nicola, Lorenzo Onorato, Carmine Minichini, Giovanni Di Caprio, Mario Starace, Caterina Sagnelli, and Evangelista Sagnelli. 2015. "Clinical Significance of Hepatitis B Surface Antigen Mutants." *World Journal of Hepatology* 7 (27): 2729–39. <https://doi.org/10.4254/wjh.v7.i27.2729>.
- Erhardt, A., U. Reineke, D. Blondin, W. H. Gerlich, O. Adams, T. Heintges, C. Niederau, and D. Häussinger. 2000. "Mutations of the Core Promoter and Response to Interferon Treatment in Chronic Replicative Hepatitis B." *Hepatology (Baltimore, Md.)* 31 (3): 716–25. <https://doi.org/10.1002/hep.510310323>.
- Excoffier, Laurent, Guillaume Laval, and Stefan Schneider. 2007. "Arlequin (Version 3.0): An Integrated Software Package for Population Genetics Data Analysis." *Evolutionary Bioinformatics Online* 1 (February): 47–50.
- Foy, Matthew C., Chloe L. Thio, Hyon S. Hwang, Melissa Saulynas, James P. Hamilton, Derek M. Fine, and Mohamed G. Atta. 2012. "False-Negative Hepatitis B Virus (HBV) Surface Antigen in a Vaccinated Dialysis Patient with a High Level of HBV DNA in the United States." *Clinical and Vaccine Immunology : CVI* 19 (5): 820–22. <https://doi.org/10.1128/CVI.05696-11>.
- Gandhe, Swati S., Mandeep S. Chadha, and Vidya A. Arankalle. 2003. "Hepatitis B Virus Genotypes and Serotypes in Western India: Lack of Clinical Significance." *Journal of Medical Virology* 69 (3): 324–30. <https://doi.org/10.1002/jmv.10292>.
- Ganem, Don, and Alfred M. Prince. 2004. "Hepatitis B Virus Infection--Natural History and Clinical Consequences." *The New England Journal of Medicine* 350 (11): 1118–29. <https://doi.org/10.1056/NEJMra031087>.
- Hosseini, Seyed Y., Neda Sanaei, Mohamad-Reza Fattahi, Seyed A. Malek-Hosseini, and Jamal Sarvari. 2019. "Association of HBsAg Mutation Patterns with Hepatitis B Infection Outcome: Asymptomatic Carriers versus HCC/Cirrhotic Patients." *Annals of Hepatology* 18 (4): 640–45. <https://doi.org/10.1016/j.aohep.2018.12.006>.
- Hsu, Chao-Wei, and Chau-Ting Yeh. 2011. "Emergence of Hepatitis B Virus S Gene Mutants in Patients Experiencing Hepatitis B Surface Antigen Seroconversion after Peginterferon Therapy." *Hepatology (Baltimore, Md.)* 54 (1): 101–8. <https://doi.org/10.1002/hep.24363>.
- Ireland, Jacqueline H., Barbara O'Donnell, Ashraf A. Basuni, Joy D. Kean, Lesley A. Wallace, George K. Lau, and William F. Carman. 2000. "Reactivity of 13 in Vitroexpressed Hepatitis B Surface Antigen Variants in 7 Commercial Diagnostic Assays." *Hepatology* 31 (5): 1176–82. <https://doi.org/10.1053/he.2000.6407>.
- Kao, Jia-Horng, Chun-Jen Liu, and Ding-Shinn Chen. 2002. "Hepatitis B Viral Genotypes and Lamivudine Resistance." *Journal of Hepatology* 36 (2): 303–4. [https://doi.org/10.1016/s0168-8278\(01\)00246-x](https://doi.org/10.1016/s0168-8278(01)00246-x).

- Kay, Alan, and Fabien Zoulim. 2007. "Hepatitis B Virus Genetic Variability and Evolution." *Virus Research* 127 (2): 164–76. <https://doi.org/10.1016/j.virusres.2007.02.021>.
- Konstantinou, Dimitris, and Melanie Deutsch. 2015. "The Spectrum of HBV/HCV Coinfection: Epidemiology, Clinical Characteristics, Viral Interactions and Management." *Annals of Gastroenterology: Quarterly Publication of the Hellenic Society of Gastroenterology* 28 (2): 221–28.
- Kramvis, Anna, Michael Kew, and Guido François. 2005. "Hepatitis B Virus Genotypes." *Vaccine* 23 (19): 2409–23. <https://doi.org/10.1016/j.vaccine.2004.10.045>.
- Kumar, Ashish, Sirish I. Kumar, Reeta Pandey, Sita Naik, and Rakesh Aggarwal. 2005. "Hepatitis B Virus Genotype A Is More Often Associated with Severe Liver Disease in Northern India than Is Genotype D." *Indian Journal of Gastroenterology: Official Journal of the Indian Society of Gastroenterology* 24 (1): 19–22.
- Kurtz, Robert C. 1999. "Hepatocellular Carcinoma and Coinfection with Hepatitis B and C." *Cancer* 86 (5): 741–43. [https://doi.org/10.1002/\(SICI\)1097-0142\(19990901\)86:5<741::AID-CNCR3>3.0.CO;2-M](https://doi.org/10.1002/(SICI)1097-0142(19990901)86:5<741::AID-CNCR3>3.0.CO;2-M).
- Lazarevic, Ivana. 2014. "Clinical Implications of Hepatitis B Virus Mutations: Recent Advances." *World Journal of Gastroenterology: WJG* 20 (24): 7653–64. <https://doi.org/10.3748/wjg.v20.i24.7653>.
- Lee, K. M., Y. S. Kim, Y. Y. Ko, B. M. Yoo, K. J. Lee, J. H. Kim, K. B. Hahm, and S. W. Cho. 2001. "Emergence of Vaccine-Induced Escape Mutant of Hepatitis B Virus with Multiple Surface Gene Mutations in a Korean Child." *Journal of Korean Medical Science* 16 (3): 359–62. <https://doi.org/10.3346/jkms.2001.16.3.359>.
- Lim, Joseph K., Mindie H. Nguyen, W. Ray Kim, Robert Gish, Ponni Perumalswami, and Ira M. Jacobson. 2020. "Prevalence of Chronic Hepatitis B Virus Infection in the United States." *Official Journal of the American College of Gastroenterology | ACG* 115 (9): 1429–38. <https://doi.org/10.14309/ajg.0000000000000651>.
- Lindh, M., J. E. Gonzalez, G. Norkrans, and P. Horal. 1998. "Genotyping of Hepatitis B Virus by Restriction Pattern Analysis of a Pre-S Amplicon." *Journal of Virological Methods* 72 (2): 163–74. [https://doi.org/10.1016/s0166-0934\(98\)00026-3](https://doi.org/10.1016/s0166-0934(98)00026-3).
- Liu, Zhihua, and Jinlin Hou. 2006. "Hepatitis B Virus (HBV) and Hepatitis C Virus (HCV) Dual Infection." *International Journal of Medical Sciences* 3 (2): 57–62. <https://doi.org/10.7150/ijms.3.57>.
- Luongo, Monica, Rosina Critelli, Antonella Grottola, Stefano Gitto, Veronica Bernabucci, Mirco Bevini, Chiara Vecchi, Giuliano Montagnani, and Erica Villa. 2015. "Acute Hepatitis B Caused by a Vaccine-Escape HBV Strain in Vaccinated Subject: Sequence Analysis and Therapeutic Strategy." *Journal of Clinical Virology: The Official Publication of the Pan American Society for Clinical Virology* 62 (January): 89–91. <https://doi.org/10.1016/j.jcv.2014.11.029>.
- M, Idrees, Khan S, and Riazuddin S. 2004. "Common Genotypes of Hepatitis B Virus." *Journal of the College of Physicians and Surgeons--Pakistan: JCPSP* 14 (6): 344–47. <https://doi.org/10.2004/jcpsp.344347>.
- Mahmood, Majid, Muhammad Asim Anwar, Azra Khanum, Nasib Zaman, and Abida Raza. 2016. "Distribution and Clinical Significance of Hepatitis B Virus Genotypes in Pakistan." *BMC Gastroenterology* 16 (1): 104. <https://doi.org/10.1186/s12876-016-0513-5>.
- Neumann-Fraune, Maria, Bastian Beggel, Rolf Kaiser, and Martin Obermeier. 2014. "Hepatitis B Virus Drug Resistance Tools: One Sequence, Two Predictions." *Intervirology* 57 (3–4): 232–36. <https://doi.org/10.1159/000361076>.
- Ngui, S. L., S. O'Connell, R. P. Eglin, J. Heptonstall, and C. G. Teo. 1997. "Low Detection Rate and Maternal Provenance of Hepatitis B Virus S Gene Mutants in Cases of Failed Postnatal Immunoprophylaxis in England and Wales." *The Journal of Infectious Diseases* 176 (5): 1360–65. <https://doi.org/10.1086/514133>.
- Noorali, Samina, Shazia Tabassum Hakim, David McLean, Shahana U. Kazmi, and Omar Bagasra. 2008. "Prevalence of Hepatitis B Virus Genotype D in Females in Karachi, Pakistan."

- Journal of Infection in Developing Countries 2 (5): 373–78. <https://doi.org/10.3855/jidc.200>.
- Norder, Helene, Anne-Marie Couroucé, Pierre Coursaget, José M. Echevarria, Shou-Dong Lee, Isa K. Mushahwar, Betty H. Robertson, Stephen Locarnini, and Lars O. Magnius. 2004. "Genetic Diversity of Hepatitis B Virus Strains Derived Worldwide: Genotypes, Subgenotypes, and HBsAg Subtypes." *Intervirology* 47 (6): 289–309. <https://doi.org/10.1159/000080872>.
- Pal, Ananya, Rajesh Panigrahi, Avik Biswas, Sibnarayan Datta, Neelakshi Sarkar, Subhashish Kamal Guha, Bibhuti Saha, Arup Banerjee, Sekhar Chakrabarti, and Runu Chakravarty. 2013. "Influence of HIV-Associated Degree of Immune Suppression on Molecular Heterogeneity of Hepatitis B Virus among HIV Co-Infected Patients." *Virology* 436 (1): 134–42. <https://doi.org/10.1016/j.virol.2012.11.003>.
- Purdy, Michael A. 2007. "Hepatitis B Virus S Gene Escape Mutants." *Asian Journal of Transfusion Science* 1 (2): 62–70. <https://doi.org/10.4103/0973-6247.33445>.
- Razavi-Shearer, Devin, Ivane Gamkrelidze, Mindie H. Nguyen, Ding-Shinn Chen, Pierre Van Damme, Zaigham Abbas, Maheeba Abdulla, et al. 2018. "Global Prevalence, Treatment, and Prevention of Hepatitis B Virus Infection in 2016: A Modelling Study." *The Lancet Gastroenterology & Hepatology* 3 (6): 383–403. [https://doi.org/10.1016/S2468-1253\(18\)30056-6](https://doi.org/10.1016/S2468-1253(18)30056-6).
- Rozanov, Mikhail, Uwe Plikat, Colombe Chappey, Andrey Kochergin, and Tatiana Tatusova. 2004. "A Web-Based Genotyping Resource for Viral Sequences." *Nucleic Acids Research* 32 (Web Server issue): W654–659. <https://doi.org/10.1093/nar/gkh419>.
- Rozas, Julio, Albert Ferrer-Mata, Juan Carlos Sánchez-DelBarrio, Sara Guirao-Rico, Pablo Librado, Sebastián E. Ramos-Onsins, and Alejandro Sánchez-Gracia. 2017. "DnaSP 6: DNA Sequence Polymorphism Analysis of Large Data Sets." *Molecular Biology and Evolution* 34 (12): 3299–3302. <https://doi.org/10.1093/molbev/msx248>.
- Sablon, Erwin, and Fred Shapiro. 2004. "Hepatitis B and C Genotyping: Methodologies and Implications for Patient Management." *Journal of Gastroenterology and Hepatology* 19 (s7): S329–37. <https://doi.org/10.1111/j.1440-1746.2004.03645.x>.
- Sayan, M., O. Sentürk, S. Ç Akhan, S. Hülagü, and M. B. Cekmen. 2010. "Monitoring of Hepatitis B Virus Surface Antigen Escape Mutations and Concomitantly Nucleos(t)ide Analog Resistance Mutations in Turkish Patients with Chronic Hepatitis B." *International Journal of Infectious Diseases: IJID: Official Publication of the International Society for Infectious Diseases* 14 Suppl 3 (September): e136–141. <https://doi.org/10.1016/j.ijid.2009.11.039>.
- Schweitzer, Aparna, Johannes Horn, Rafael T. Mikolajczyk, Gérard Krause, and Jördis J. Ott. 2015. "Estimations of Worldwide Prevalence of Chronic Hepatitis B Virus Infection: A Systematic Review of Data Published between 1965 and 2013." *Lancet (London, England)* 386 (10003): 1546–55. [https://doi.org/10.1016/S0140-6736\(15\)61412-X](https://doi.org/10.1016/S0140-6736(15)61412-X).
- Tan, Mingjuan, Ajeet S. Bhadoria, Fuqiang Cui, Alex Tan, Judith Van Holten, Philippa Easterbrook, Nathan Ford, et al. 2021. "Estimating the Proportion of People with Chronic Hepatitis B Virus Infection Eligible for Hepatitis B Antiviral Treatment Worldwide: A Systematic Review and Meta-Analysis." *The Lancet Gastroenterology & Hepatology* 6 (2): 106–19. [https://doi.org/10.1016/S2468-1253\(20\)30307-1](https://doi.org/10.1016/S2468-1253(20)30307-1).
- Thakur, Varsha, Rajkumar Chandra Guptan, Syed Naqui Kazim, Veena Malhotra, and Shiv Kumar Sarin. 2002. "Profile, Spectrum and Significance of HBV Genotypes in Chronic Liver Disease Patients in the Indian Subcontinent." *Journal of Gastroenterology and Hepatology* 17 (2): 165–70. <https://doi.org/10.1046/j.1440-1746.2002.02605.x>.
- Tian, Yongjun, Yang Xu, Zhenhua Zhang, Zhongji Meng, Li Qin, Mengji Lu, and Dongliang Yang. 2007. "The Amino Acid Residues at Positions 120 to 123 Are Crucial for the Antigenicity of Hepatitis B Surface Antigen." *Journal of Clinical Microbiology* 45 (9): 2971–78. <https://doi.org/10.1128/JCM.00508-07>.

- Toan, Nguyen L., Le H. Song, Peter G. Kremsner, Dinh N. Duy, Vu Q. Binh, Bernd Koeberlein, Stefan Kaiser, Reinhard Kandolf, Joseph Torresi, and C.-Thomas Bock. 2006. "Impact of the Hepatitis B Virus Genotype and Genotype Mixtures on the Course of Liver Disease in Vietnam." *Hepatology* (Baltimore, Md.) 43 (6): 1375–84. <https://doi.org/10.1002/hep.21188>.
- Verheyen, Jens, Maria Neumann-Fraune, Thomas Berg, Rolf Kaiser, and Martin Obermeier. 2012. "The Detection of HBsAg Mutants Expressed in Vitro Using Two Different Quantitative HBsAg Assays." *Journal of Clinical Virology: The Official Publication of the Pan American Society for Clinical Virology* 54 (3): 279–81. <https://doi.org/10.1016/j.jcv.2012.04.010>.
- Wasley, Annemarie, Deanna Kruszon-Moran, Wendi Kuhnert, Edgar P. Simard, Lyn Finelli, Geraldine McQuillan, and Beth Bell. 2010. "The Prevalence of Hepatitis B Virus Infection in the United States in the Era of Vaccination." *The Journal of Infectious Diseases* 202 (2): 192–201. <https://doi.org/10.1086/653622>.
- Zeng, Guo-Bing, Shu-Juan Wen, Zhan-Hui Wang, Li Yan, Jian Sun, and Jin-Lin Hou. 2004. "A Novel Hepatitis B Virus Genotyping System by Using Restriction Fragment Length Polymorphism Patterns of S Gene Amplicons." *World Journal of Gastroenterology: WJG* 10 (21): 3132–36. <https://doi.org/10.3748/wjg.v10.i21.3132>.