

ANTIVIRAL RESPONSE OF DRUGS USED AGAINST HBV PATIENTS OF KHYBER PAKHTUNKHWA, PAKISTAN

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(Received, 7<sup>th</sup> January 2023, Revised 24<sup>th</sup> October 2023, Published 25<sup>th</sup> October 2023)

**Abstract** Hepatitis B virus is an ample cause of end-stage liver diseases and hepatocellular carcinoma. Effective treatment in high-risk countries such as Pakistan can help delay or prevent these consequences. The existing study aims to evaluate the response rates of antiviral drugs tenofovir and entecavir (6-48 months) based on different clinical parameters. Sera collected from HBV patients (43) subjected to DNA extraction, followed by real-time PCR detection. Furthermore, ICT was performed to detect HBs-Ag and ELISA for HBe-Ag. Response rate after 6 months of tenofovir treatments showed 100% normal creatinine and ultrasound and ALT (50%) and while in the case of entecavir, each ALT and ultrasound normalization (66.7%), showed creatinine (100%). The fatty liver was reported 50% and 33.3% after tenofovir and entecavir treatment, respectively. The response after 12 months of treatment with tenofovir showed normalization of ALT and ultrasound (84.6%), bilirubin and creatinine normalization (92.3%), and fatty liver (15.4%). Whereas bilirubin and creatinine levels showed (100%) normal, ALT and ultrasound normalization (80%) with 20% of patients having congenital left lobe of the liver after entecavir. Patients profiles after 24 months of tenofovir treatment showed normal ALT and ultrasound (85.7%), bilirubin (100%), and renal impairment observed in patients (14.3%). The 24 months entecavir treatment showed significant improvement in various clinical parameters normalization with 100% such as ALT, bilirubin, and creatinine in all patients. The efficacy of entecavir showed a significant response as compared to Tenofovir. Furthermore, nucleoside/nucleotide analogues enhanced its efficacy with longer treatment duration.

[Citation: Hassan N., Amin, F.U., Bashir K., Irshad M., Jamil S., Munawar N., Haqqani S., Shabir, H., Khan, M.J. (2023). Antiviral response of drugs used against HBV patients of Khyber Pakhtunkhwa, Pakistan. Bull. Biol. All. Sci. Res. 8: 49. doi: <https://doi.org/10.54112/bbasr.v2023i1.49>]

**Keywords:** Hepatitis B virus, Entecavir, Tenofovir, Bilirubin, Creatinine, ALT

## Introduction

Hepatitis B virus (HBV) is a small, enveloped, partially double stranded DNA virus that is strain of the Hepadnaviridae family (Ott et al., 2012). The genome has circular DNA that consists of 3.2 kilo base pair, encoding proteins. HBeAg is secreted dimer protein (role in replication of virus) and HBcAg (core antigen or capsid protein for capsid formation). HBs-Ag is a surface antigen consisting of large, intermediate, and small surface envelope glycoproteins, polymerase (reverse transcriptase action) and HBx is small antigen, for transcription regulation to initiate infection (Seeger and Mason, 2015). Hepatitis B viral infection is a comprehensive liver problematic with significant disease and fatality and affects over 350 million populations worldwide.

Almost 5% of the world's people are infected with HBV (Manzoor et al., 2011).

Chronic HBV infection has been categorized into five phases according to the level of the presences of HBe and HBs antigen, level of HBV DNA, ALT (alanine aminotransferase), and incidence or absence of liver inflammation (Cooke et al., 2010; Cornberg et al., 2017; Liaw and Chu, 2009; Yang et al., 2016). The CHB patients are currently treated with two main primary then with nucleoside/nucleotide analogue (NA) or interferon- $\alpha$  (Terrault et al., 2016). Currently, there are five approved (NA), telbivudine (ADV), adefovirdipivoxil (ADV), entecavir (ETV), lamivudine (LAM) and tenofovir disoproxil fumarate (TDF) for HB treatment. These can be divided as HBV shows high resistance to LAM, ADV and TBV and less resistance to ETV, TDF, TAF (Lok et al.,

2017; Pei et al., 2017). The treatment with a potent and low-frequency resistance (i.e., ETV, TDF) is expected to have long-standing antiviral drug efficacy associated to unnoticeable HBV DNA level (Terrault et al., 2018). These NA are effective in HBV infected patients to suppress viral loads, HBe-Ag seroconversion, getting alanine aminotransferase (ALT) normalization, and improving liver fibrosis (But et al., 2010). It is reported that the threat of cirrhosis and hepatocellular carcinoma (HCC) can be decreased by persistent and effective suppression of HBV DNA. Therefore, long-term treatment (NA therapies) for chronic hepatitis B is used to accomplish complete virology suppression, with loss hepatitis B surface antigen (HBs-Ag) (Lai and Yuen, 2008).

Conventional interferon- $\alpha$  is the initial therapy approved for the cure of chronic HBV. This therapy mainly helps effort through immunomodulation and decline viral load. The main advantages are increased seroconversion rates to antibody (anti-HBe) against HBeAg, finite duration, the absence of resistance, and preventing packing of genomic viral RNA into the core protein (Clercq et al., 2010; Wu et al., 2019). Interferon has severe adverse effects, including diseases such as influenza, anorexia, thyroid dysfunction, hepatitis burst, autoimmune disease, and neuropsychiatric defects like depression, irritability, and even suicidal trend (Wang and Chen, 2014). Interferon is augmented by another compound, polyethylene glycol (PEG), called Pegylated-interferon, licensed in 2005 and preferred to use finite duration over conventional interferon (Chang and Suh, 2008). The current aimed to explore the efficiency of different antiviral drugs used to treat HBV patients.

## Materials and Methods

### Study design

This experimental study was conducted in tertiary care hospital labs in Peshawar from January 2019 to December 2020. Blood samples were collected from all tested individuals through an authorized technician. Samples were centrifuged at 3000 rpm for 2 minutes to isolate the plasma and stored at  $-20^{\circ}\text{C}$ . HBV patients were included in this study with oral nucleoside/nucleotide analogue. Blood samples from HB patients were analyzed every 6 months or when necessary for following assessment according to HBV DNA level, ALT, bilirubin, creatinine, and liver disease.

### Screening Tests

HBsAg was detected by ICT (Immunochromatographic test), and ELISA tested HBeAg. The samples were screened primarily by using ICT strip to separate the positive sample of HBsAg, and the Bios kit was used to confirm the positive sample of HBe-Ag.

### Real time PCR

DNA was extracted from 200ml of a sample using DNA extraction kit (GF-1 kit). The extracted DNA

was subjected to PCR for the detection of HBV. The Promotor<sup>TM</sup> HBV Hepatitis virus quantitative test kit was used for HBV DNA detection by real-time time-PCR. It contained all the reagents and enzymes for the specific amplification of the HBV genome.

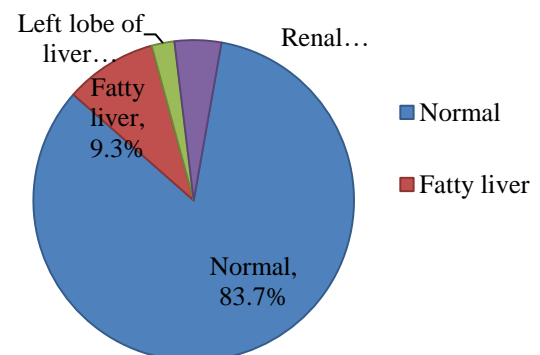
The 20 $\mu\text{L}$  working solution contains DNA, containing 19.5 $\mu\text{L}$  Reaction Mix (Tris-HCl, KCl, MgCl<sub>2</sub>, dNTP, primers, fluorescence) and 0.5 $\mu\text{L}$  Enzyme Mix (Taq polymerase, Uracil-N-Glycosylase) and primer sequences were 5' GTGTCTGCGGCGTTTTATCA 3' (Sense) and 5' GACAAACGGGCA ACATACCTT 3' (Antisense). Respectively added 20 $\mu\text{L}$  extracts of Negative Control, 20 $\mu\text{L}$  of Low Positive Control, 20 $\mu\text{L}$  High Positive Control, and 20 $\mu\text{L}$  of HBV Calibrator 1-4. Cycling conditions was performed  $25^{\circ}\text{C}/10\text{-minute}$ ,  $95^{\circ}\text{C}/3\text{minutes}$ , and 45 cycles followed by  $94^{\circ}\text{C}/15\text{ seconds}$  and  $58^{\circ}\text{C}/30\text{ seconds}$ . The PCR obtained data was interpreted by real-time PCR instrument FAM software by crossing the fluorescence curve with threshold line at  $58^{\circ}\text{C}$  at a specific level. The standard curve cut-off value of HBV DNA detection for this kit is 34 to 35; below this value showed positive results, and above this indicated negative results.

### Data analysis

Statistical analysis was performed using SPSS version 20 and MS excel software. One sample t-test and chi square were used for the significance of P-value of  $< 0.05$ .

### Results

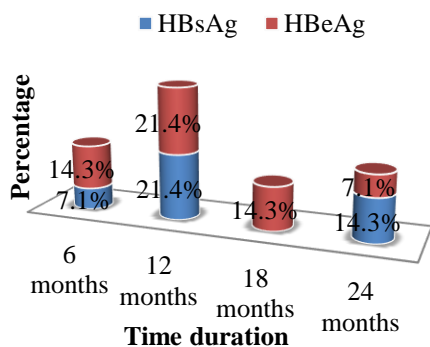
The study designed to investigate HBV patients (43) with antiviral treatment, clinical parameters, and viral load. The HBV patients were analyzed every 6 months or when necessary for clinical parameters and PCR. There were 54.5% (male) and female (45.5%) out of recorded patients. Among these patients, 62.7% were HBsAg positive, and 37.3% were HBeAg positive patients. The patients reported based on ultrasound 83.7% (normal ultrasound), 9.3% (fatty liver), 2.3% (left lobe congenital) and 4.7% (renal impairment) after antiviral drug therapy as in Figure 1.



**Fig.1. Ultrasound base prevalence of HBV patients. Prevalence of Entecavir treatment duration on bases of HBsAg/HBeAg positivity**

The patients under entecavir treatment with different duration were divided on the basis of HBsAg and HBeAg status. Duration of entecavir treatment noted

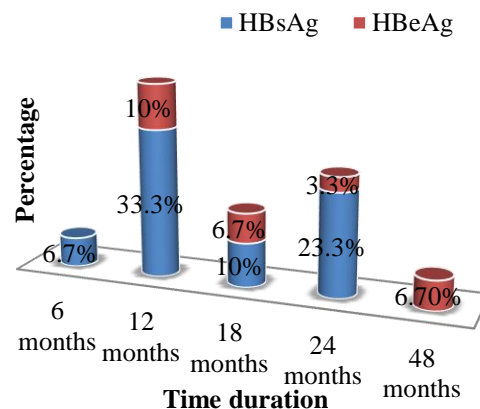
on the basis of HBsAg/HBeAg status, consisting of patients with 6 months of treatment (7.1%) were HBsAg positive and (14.3%) were HBeAg positive. There were patients (21.4%) of HBsAg positive and 21.4% of HBeAg positive under entecavir treatment with 12 months duration. The 18 months of treatment of HBeAg was 14.3% of patients, and 24 months of entecavir therapy was reported 14.3% of patients and 7.1% of HBsAg positive and HBeAg positive, respectively, shown in Figure 2.



**Fig.2. Prevalence of entecavir therapy duration on basis of HBsAg/HBeAg**

**Prevalence of Tenofovir treatment duration on bases of HBsAg/HBeAg positivity**

There were patients under tenofovir treatment with different durations on the basis of HBs/eAg, shown in Figure 3. Among these patients, 6.7% of HBsAg patients recovered after 6 months treatment under tenofovir and 12 months treatment of HBsAg positive found in patients (33.3%) and (10%) of HBeAg positive recovered. HBsAg positive (10%) and HBeAg positive 6.7% patients reported under tenofovir drug with 18 months therapy. Duration of 24 months tenofovir treatment was found 23.3% HBsAg positive and 3.3% HBeAg positive patient. The patients (6.7%) of HBeAg positive were recovered under tenofovir drug with 48 months treatment.



**Fig.3. Prevalence of tenofovir therapy duration on basis of HBsAg/HBeAg.**

**Treatment with 6 months duration**

Table 1 shows the efficacy of tenofovir and entecavir with profile history of patients. With 6 months of treatment, 33.3% were HBsAg positive and 66.7% were HBeAg positive. Patients with tenofovir treatment profile records were each ALT normalization and bilirubin normalization 50%. The normal creatinine, ultrasound, and fatty liver were reported 100, 50 and 50% respectively. Entecavir used patients testified ALT normalization (66.7%), 33.3% raised bilirubin, and 100% creatinine normalization. The ultrasound history of patients recorded 66.7% (normal ultrasound) and 33.3% fatty liver were observed, and the final PCR of all patients was negative with undetected HBV DNA after 6 months therapy.

**TABLE 1. EFFICACY OF TENOFOVIR AND ENTECAVIR WITH 6 MONTHS THERAPY**

No. of variables	Tenofovir treatment				Entecavir treatment			
	No. of patients	Normal	Raised	p.value	No. of patients	Normal	Raised	p.value
HBsAg (+)	100%				33.3%			
HBeAg (+)	0%				66.7%			
ALT		50%	50%	.020		66.7%	33.3%	.020
Bilirubin		50%	50%	.020		100%	0%	.000
Creatinine		100%	0%	.090		100%	0%	.007
Ultrasound		50%	50%	.010		66.7%	33.3%	.056

**Treatment with 12 months duration**

The efficacy of entecavir and tenofovir with a profile history of HBV infected patients with 12 months therapy as shown in Table 2. The patients, including HBsAg positive 76.9% and HBeAg were positive 23.1%. The potency of tenofovir documented with profile records of ALT, bilirubin and creatinine

normalization was 84.6, 92.3 and 92.3% respectively. The ultrasound history of patients recorded 84.6% (normal ultrasound) and 15.4% patients with fatty liver. The efficacy of entecavir was reported with 12 months duration, including 60% of patients were HBsAg positive and 40% HBeAg positive. The normal bilirubin and creatinine were reported 100%,

normal ALT and ultrasound (80%), and 20% of the patient observed with congenital left lobe of the liver.

**Table 2. Efficacy of tenofovir and entecavir with 12 months therapy**

No. of variables	No. of patients	Tenofovir treatment			Entecavir treatment			
		Normal	Raised	p.value	No. of patients	Normal	Raised	p.value
HBsAg (+)	76.9%				60%			
HBeAg (+)	23.1%				40%			
ALT		84.6%	15.4%	.007		80%	20%	.067
Bilirubin		92.3%	7.7%	.001		100%	0%	.006
Creatinine		92.3%	7.7%	.000		100%	0%	.000
Ultrasound		84.6%	15.4%	.013		80%	20%	.180

**Treatment with 18 months duration**

There were 60% of patients (HBsAg positive) and 40% HBeAg positive under tenofovir with 18 months of therapy as shown in the Table 3. The tenofovir used patients were recorded ALT and bilirubin normalization 80% normal and raised 20%. The patients reported for ultrasound and creatinine

100% normalization after therapy. Patients with entecavir treatment profile records were ALT normalization 100% and bilirubin normalization 50%. The ultrasound and creatinine found 100% normal with entecavir treatment and undetected HBV DNA with final PCR.

**Table 3. Efficacy of tenofovir and entecavir with 18 months therapy**

No. of variables	No. of patients	Tenofovir treatment			Entecavir treatment			
		Normal	Raised	p.value	No. of patients	Normal	Raised	p.value
HBsAg (+)	60%				0%			
HBeAg (+)	40%				100%			
ALT		80%	20%	.000		50%	50%	.070
Bilirubin		80%	20%	.012		50%	50%	.020
Creatinine		100%	0%	.000		100%	0%	.042
Ultrasound		100%	0%	.000		100%	0%	.000

**Treatment with 24 months duration**

Table 4 shows the profile history of patients was recorded ALT and creatinine normalization 85.7% and raised 14.3%. The normal bilirubin, normal ultrasound, and patients with renal impairment were reported 100, 85.7 and 14.3% respectively. The

efficacy of same duration was examined with entecavir drugs of three patients, including patients HBsAg positive 66.7% and HBeAg positive 33.3%. Entecavir used patients were testified each ALT, bilirubin and creatinine normalization 100%.

**Table 4: efficacy of tenofovir and entecavir with 24 months therapy**

No. of variables	No. of patients	Tenofovir Treatment			Entecavir treatment			
		Normal	Raised	p.value	No. of patients	Normal	Raised	p.value
HBsAg (+)	85.7%				66.7%			
HBeAg (+)	14.3%				33.3%			
ALT		85.7%	14.3%	.052		100%	0%	.029
Bilirubin		100%	0%	.002		100%	0%	.057
Creatinine		85.7%	14.3%	.000		100%	0%	.013
Ultrasound		85.7%	14.3%	.059		100%	0%	.000

**Treatment with 48 months duration**

The profile history of these patients reported with ALT (100% normal), creatinine (50% normal), bilirubin (100% normal), 50% (normal ultrasound) and 50% patient renal impairment, shown in Table 5. The raised level of creatinine increases at 48 months

duration with renal impairment, which was not clear, the reason for may renal impairment or long-term use of tenofovir is that the drugs are cleared by kidney. However, none were under entecavir treatment with 48 months of therapy among all reported patients.

**Table 5: efficacy of tenofovir with 48 months duration**

ALT	Creatinine	Bilirubin	Ultrasound
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No. of HBsAg patients	0%	0%	0%	0%
No. of HBeAg patients	100%	100%	100%	100%
Normal	100%	50%	100%	50%
Raised	0%	50%	0%	50%
p.value	0.050	0.040	0.090	.000

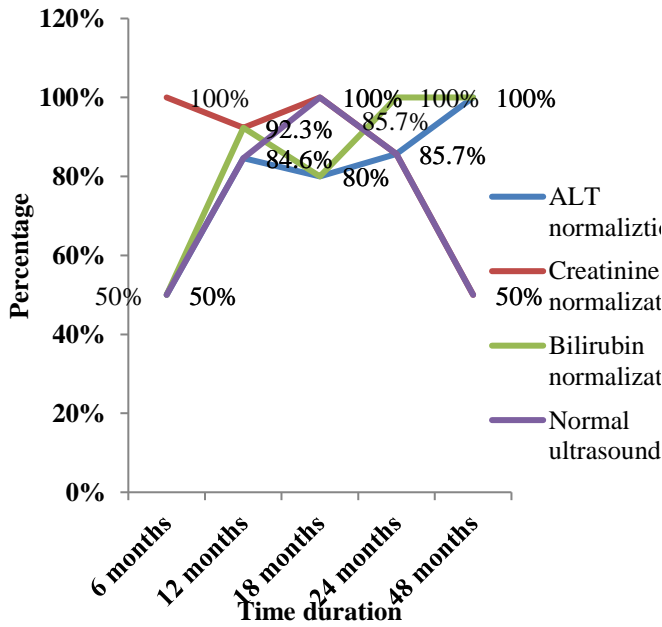
**Comparison between tenofovir and entecavir treatment**

The HBV patients in our data with tenofovir and entecavir treatment reported efficacy under different

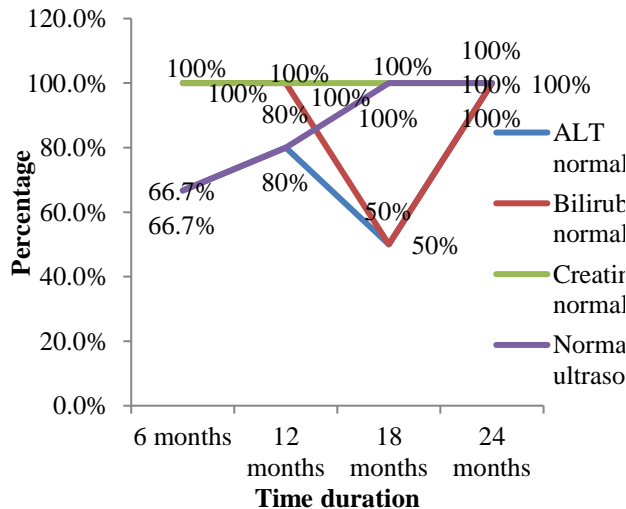
monthly durations, shown in (Figure 4 & 5). The pregnancy and mortality base prevalence of HBV patients are shown in Table 6 and Figure 6.

**Table 6. Efficacy of antiviral drugs in treatment of pregnant hbv patients**

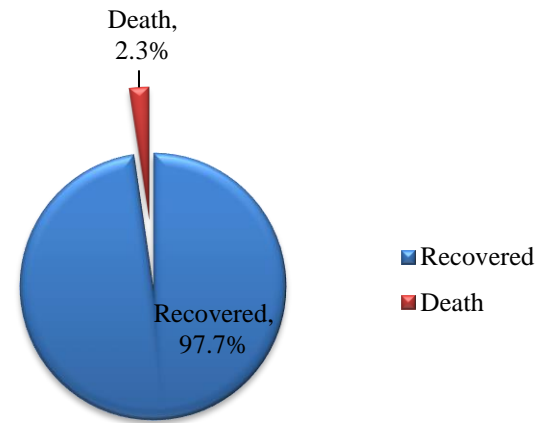
No. of pregnant patients	Ag-Status	Treatment	Normal ALT	Normal Bilirubin	Normal Creatinine	Normal Ultrasound	Mean	SD	P.value
10%	HBs-Ag	Entecavir	50%	100%	100%	100%	1.901	0.308	.000



**Fig.4. Efficacy of tenofovir treatment on basis of duration**



**Fig.5. Efficacy of entecavir treatment on base of duration**



**Mortality rate of HBV patients**

**Fig.6. Mortality basis prevalence of HBV patients**

**Discussion**

Hepatitis B virus (HBV) is an infectious agent responsible for causing liver parenchyma cell inflammation. Pakistan has been ranked with high threat of HBV infection globally (Manzoor et al., 2011). Presently, two antiviral drugs are approved for CHB treatment such as NAs and pegylated interferon-α (IFN-α). Both drugs are capable to eradicate the virus and efficiently control the infection. The NAs are long-term, well-tolerated therapies but have a high risk of drug resistance development in some NAs that restrict their extended use. The antiviral drugs show progression toward HBV DNA loss, decrease cirrhosis and HCC and normalization of ALT and ultrasound as well as renal impairment.

This cross-sectional study represents the different antiviral agent for CHB treatment in Pakistan. A number of studies reported the various therapy strategies and clinical outcomes worldwide and few studies are reported from Pakistan considering various covariates. The study performed prevalence of HBV patients with entecavir or tenofovir treatment and assessed medical course. The



male and female were included in our research investigation, 23 out of 43 (54%) male and female found with HBV infection 46% (20 patients). This study closely relates to Toka *et al.*, (2020), who conducted a study on the highest incidence of HBV infection in males than females (Toka *et al.*, 2020). In our study, total 43 HBV patients, there were 62.7% of patients with HBsAg positive and 37.3% patients were found with HBeAg positive. Our study showed two patients (4.7%) were co-infection with HCV. Similarly, study has conducted for incidence of HBV and HCV. Amongst total, there were 10.71% found HBV in which 9.64% were HBs Ag positive. No patient was found with HBV and HCV co-infection (Kalim *et al.*, 2017). The variation in our study was due to dissimilar study area or sample size.

The patients with HBsAg positive had expired after 18 months treatment with entecavir. The patient found with normal bilirubin but raised ALT and creatinine with renal impairment during antiviral treatment. The study estimated the death rate with less frequency is only 2.3% found among all reported data. There is contrast in our study from previous study due to associated renal or liver problems to patients. The mortality rate showed that the patient had acute liver failure due to renal failure and the previous one. This study closely related to the Toka *et al.*, (2020) who conducted to compare entecavir or tenofovir concerning liver associated mortality (Toka *et al.*, 2020).

Antiviral drugs (tenofovir and entecavir) used for treating HBV patients. There were 30 patients under tenofovir treatment and 14 patients reported under entecavir treatment. The 30 patients under tenofovir treatment with different duration also estimated on basis of HBs/eAg. The result showed that 6.7% of HBsAg patients recovered after 6 months of treatment under tenofovir and 12 months of HBsAg positive found in patients (33.3%) and (10%) of HBeAg positive recovered. HBsAg positive (10%) and HBeAg positive (6.7%) patients were reported under tenofovir drug with 18 months therapy. Duration of 24 months tenofovir treatment was found 23.3% HBsAg positive and 3.3% HBeAg positive patient. The patients (6.7%) of HBeAg positive were recovered under tenofovir drug with 48 months treatment. The prevalence of entecavir treatment with duration was noted based on HBsAg/HBeAg status. The result showed 6 months of treatment in which 7.7% of patients were HBsAg positive and 15.4% were HBeAg positive. There were patients (21.3%) of HBsAg positive and (15.4%) of HBeAg positive under entecavir treatment with 12 months duration. The patients reported 15.4% (HBsAg) and 7.7% (HBeAg) positive under 24 months therapy with entecavir drug. There is no study in difference prevalence according to used tenofovir and entecavir in contrast to Toka *et al.*, (2020). It was found from both study, high number of patients were treated with tenofovir therapy than entecavir therapy.

The present study documented the potency of tenofovir with profile record of ALT, bilirubin and creatinine normalization was 84.6%, 92.3% and 92.3% respectively under tenofovir treatment with 12 months. The report of normal ultrasound also examined of 84.6% patients and with undetected HBV DNA by last negative PCR. Whereas 5 patients were under entecavir treatment with 12 months and result showed ALT 80% normalization and bilirubin, creatinine and ultrasound was reported 100% normalization with undetected HBV DNA. The study showed the efficacy of tenofovir, ALT 80% normalization and 20% raised ALT and 80% normal bilirubin and 20% raised. The patients were also reported 100% normal ultrasound and creatinine. HBV DNA found undetected with negative PCR after 18 months treatment of tenofovir. The efficacy of entecavir with 18 months duration also recorded from profile history of HBV patients with undetected HBV DNA diagnosed by PCR amplification after 18 months treatment. The data showed ALT and bilirubin 50% normal, 100% creatinine and ultrasound found normal with entecavir treatment. Similarly, the study performed efficacy of patients with entecavir or tenofovir and assessed medical course. No potential significant was found between TDF and ETV groups concerning treatment duration (Toka *et al.*, 2020). Compared to previous studies, there was a significant difference between the efficacy of tenofovir and entecavir due to severity of infection.

Another outcome of the study, the efficacy of the same duration was also examined with entecavir drugs, including 66.7% HBsAg positive and 33.3% HBeAg positive. The study exposed ALT normalization and normal bilirubin and creatinine with 100%. The result showed 66.7% found with normal ultrasound and 33.3% found with renal impairment and HBV DNA of all patients was undetected by last PCR. Antiviral efficacy was checked with profile history of ALT, bilirubin, creatinine and ultrasound of HBV patients. The HBsAg positive patients under tenofovir treatment and recovered with 24 months therapy. The profile parameters of patients was recorded after 24 months, ALT and bilirubin 85.7% normalization and 14% patient raised bilirubin and creatinine was found 100% normal. The ultrasound of these patients found 85.7% normal and 14.3% patient recorded with congenital left lobe of liver and last PCR was detected negative with loss of HBV DNA. Similar study was conducted to evaluate the efficacy of Entecavir for chronic HBeAg patients. The creatinine normalization at 12 and 24 months of entecavir treatment were 88.9% and 97.8%, respectively. HBV DNA level decreased after 24 months of entecavir therapy (Xu *et al.*, 2013).

### Conclusions

HBV management is an art that need careful assessment of the likelihood of morbidity and mortality related to liver and treatment response.

Treatment responses include prevention and reduction of adverse clinical outcomes in patients. The current study concluded the treatment response of patients to various antiviral drugs such as entecavir and tenofovir for long treatment duration by comparing several clinical parameters. However, this study emphasizes that further studies are required to completely elucidate the response to treatments and risk factors of therapies and possible resistance mechanism of long-term treatment. Additionally, molecular based research is required to shed light on treatment response to numerous antiviral agents in different HBV genotypes. Studies involving different genotypes will help to understand that specific treatments were effective against some HBV genotypes and not on others. Other factors that should be considered during treatment are age of patient, liver disease severity, immunity level, cross infection etc. The current work will enable the microbiologist/virologist to focus drug designing for viral infection in Pakistan in future.

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**Declaration**

**Data Availability statement**

All data generated or analyzed during the study have been included in the manuscript.

**Consent for publication**

Not applicable

**Funding**

There were no sources providing support, for this research.

**Conflict of interest**

The authors assure that there were no financial relationships involved that could be perceived as a conflict of interest.

**Acknowledgment**

The authors are very thankful to the tertiary care hospitals of Peshawar, Khyber Pakhtunkhwa.

**Author, Disclosure Statement**

**Ethics approval and consent to participate**

The study permitted by the Ethical Committee of Abasyn University Peshawar and written informed consent was obtained from all the patients participated in the study.

**Author Contributions**

NH did the experiments and wrote the manuscript. AU and FA conceived the study and design, MSK, AU and MAK reviewed the manuscript. All the authors participated in the experimentation & optimizations.



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