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## Evaluation of HBV resistance to tenofovir in patients with chronic hepatitis B using ZNA probe assay in Kerman, southeast of Iran

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## ABSTRACT

**Objective:** To evaluate the mutation rate of polymerase gene and its correlation with tenofovir resistance in patients with chronic hepatitis B.**Methods:** A total of 64 serum samples (36 men and 28 women) were collected from patients with chronic hepatitis B. All of these samples were tested for hepatitis B virus (HBV) DNA level, alanin amino transferase/aspartic amino transferase enzymes and serological markers such as hepatitis B surface antigen (HBsAg)/hepatitis Be antigen (HBeAg) (Electro-chemiluminescence).**Results:** In this study, out of those 64 patients, 13 cases had mutations in the polymerase region (A194T). All mutant cases were HBsAg positive and 5 (38.5%) of them were males and 8 (61.5%) were females, while 6 (46.2%) of the mutants were HBeAg positive and 7 (53.8%) were HBeAg negative and most of the mutants have more than 109 HBV DNA level. Statistical analyses on the 64 samples showed that there was no significant relation between age and HBsAg level but there was a significant relationship between HBV DNA load ( $P = 0.001$ ) and A194T mutation.**Conclusions:** HBeAg had a correlation with A194T mutation ( $P = 0.02$ ) and tenofovir resistance was seen in 13 patients. Real-time PCR with zip nucleic acid probes is a rapid method to detect mutations in the polymerase region of HBV with high sensitivity and specificity. This method could be used for mutation detection in nt-194 position of polymerase gene for tenofovir resistance and other mutations in drug resistance researches.

## 1. Introduction

Hepatitis B virus (HBV) is one of the most important viruses, which infects hepatocytes in livers and leads to hepatocellular carcinoma. HBV is spherical virus with 22 nm diameter and has a partially double-stranded and relaxed circular DNA genome[1]. Four open reading frames encode structural proteins of the virion, the central body of the virus, a small transcription activator (X) and a large polymerase (P), which is responsible for the reverse

transcriptase and Ribo nuclease H[2]. The C gene contains two start codons that encode hepatitis B core antigen and HBe protein, which will be converting to solution hepatitis Be antigen (HBeAg)[3]. HBV is transmitted through blood transfusion, sexual contact and also can cause acute or chronic diseases, especially in infants, and 5%–10% of people suffering from chronic hepatitis B (CHB) after the primary disease[4,5]. Almost one-third of people with chronic active hepatitis show liver failure and cirrhosis. The other two thirds of people having temporary chronic hepatitis and primary hepatocellular carcinoma can be attributed to chronic HBV infection[6]. The World Health Organization estimates that there are about 350 million carriers of chronic HBV in the world that 15%–40% of their lives are in danger due to the possibility of the disease progression to cirrhosis and liver cancer. In Iran, about 1.2%–9.7% of HBV infection has been reported in different areas[7], and 1.5–2.5 million people in Iran suffer from HBV infection[8]. It should be mentioned that the incidence rate of mutations in HBV infection is ten times more than other DNA viruses. According to different studies, anti-retroviral therapy is the most important factor in the creation of mutations in the virus reverse transcription/polymerase gene (the

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most important target for antiviral drugs[9]. A common treatment for patients with CHB is the combination of interferon alpha and the nucleoside/nucleotide analogues such as lamivudine, entecavir, telbivudine, adefovir and tenofovir. The application of these drugs is a big step in the treatment of CHB patients[10]. However, one problem associated with these drugs is the emergence of drug resistance in mutant strains[11]. Different types of mutations in the polymerase region (POL) have been described, such as mutations in tyrosine-methionine-aspartate-aspartate and leucine-leucine-alanine-glutamine regions, rtN236T, rtM204I, rtV84M and rtM194T. The commonest drug resistance is tyrosine-methionine-aspartate-aspartate motif mutation which possesses lamivudine resistance[12]. Mutations in rtA194T can cause resistance to tenofovir disoproxil fumarate (TDF) which is an acyclic nucleotide analogue relating to adefovir dipivoxil[13]. In 2008, tenofovir got the approve for the treatment of CHB, and it is available for the treatment of HIV infection. Tenofovir is superior to adefovir for the HBV DNA suppression, HBeAg seroconversion and the normalization of alanin amino transferase (ALT)[14]. PCR sequencing, restriction fragment length polymorphisms, INNO-LiPA and matrix-assisted laser desorption/ionization time of flight-mass spectrometry are developed to detect resistant mutations. The zip nucleic acid (ZAN) probe assay was used for detecting rtA194T mutation in POL region of HBV that published before[15]. HBV with rtA194T mutation reduces the susceptibility to tenofovir combining with lamivudine resistance rtM204V and rtL180M mutations *in vitro*[16,17]. Also, rtA181T and rtN236T mutations associated with adefovir resistance in *in-vitro* studies have showed the reduction in the susceptibility to tenofovir[18]. In this study, a sensitive real-time fluorescent quantitative PCR using ZNA probes was used to detect tenofovir-resistant mutants in chronic HBV patients with the history of long-term antiviral therapy in Kerman, southeast of Iran.

## 2. Materials and methods

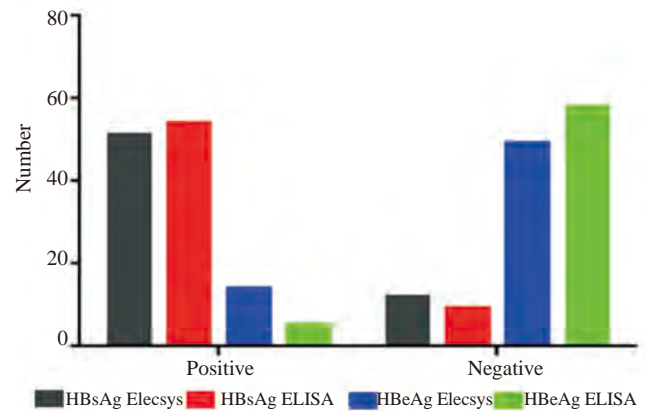
### 2.1. Patients

In retrospective study in our laboratory (Virology Laboratory of the Besat Specialist Clinic, Kerman, Iran), 64 patients with CHB including 36 men and 28 women with the mean age of 42 years ranging from 21–63 years were enrolled from October 2013 to March 2014. The treatment was based on the combination of the serum HBV DNA level, serum ALT level and the histological grade and stage of the underlying liver disease. Exclusion criteria included having severe illness, organ transplantation, treatment with corticosteroids, any liver disease not due to hepatitis B, and seropositivity for HIV or hepatitis C or hepatitis D virus.

### 2.2. Serological analysis

ALT and aspartic amino transferase (AST) were tested in blood samples from the patients with CHB. hepatitis B surface antigen (HBsAg) and HBeAg were tested with immunoassay method and ELISA and then both of them were evaluated with electrochemi-luminescence method (ECL). Our serological markers were analyzed by HBsAgII HBeAg Elecsys and Cobas analyzers (Roche, Germany). ELISA method was carried out with an automatic plate washer (Bioscience, Tian Jin, China) and PETECK96-II Detection System (Bioscience) was used to read relative light units at 450 nm

(Figure 1).



**Figure 1.** Comparison of serological tests in two different methods (Elecsys and ELISA) [ $P < 0.05$ , 95% confidence interval (CI): 25–45].

### 2.3. HBV DNA level

By RIBO-Prep kit (ILS, Russia), HBV DNA was extracted from plasma and quantified via real-time PCR by the commercial kit (ILS, Russia). In our method, the real-time PCR had a lower limit of 30 copies/mL.

### 2.4. Detection of rtA194T mutations

Specific primers and probes designed for HBV polymerase region to determine rtA194T mutation in nt-194 of the POL gene were detected by using Beacon designer software (version 8 primer, Biosoft, USA) (Table 1). Metabion Company (Metabion International AG, Germany) synthesized primers and probes and real time ZNA probe method was developed for tenofovir-resistant mutants. The quantitative real time PCR was done with the Rotor-Gene 6000 (Corbett Research, Australia) and the condition starts with 15 min with hot start Taq DNA polymerase at 95 °C followed by 45 cycles at 95 °C for 10 s and 60 °C for 30 s.

**Table 1**

Sequence primers and probes for the detection of tenofovir resistance.

Name	Sequence
Forward primer	CCGCTGTTACCAATTTTC
Reverse primer	GCCCATGAAATGTAAGA
Wild type probe (A)	FAM-TTGGGTATACATTTAACCCCTACCAA-BHQ1
Mutant probe (T)	JOE-TTGGGTATACATTTAACCCCTACCAA-BHQ1

### 2.5. Statistical analyses

To analyze the data, *Chi*-square and Fisher's exact tests were used by SPSS 15.0 software (SPSS Inc, Chicago; USA) and  $P < 0.05$  were considered significant.

## 3. Results

A total of 64 patients with CHB were selected from October 2013 to March 2014. Among them, 36 (56.2%) were men and 28 (43.8%) were women. The age of patients was  $(42.72 \pm 10.90)$  years. The levels of ALT and AST were  $(123.30 \pm 13.80)$  and  $(125.62 \pm 15.06)$  IU/mL, respectively. We categorized HBV DNA load from 30 to more than 109 copies/mL. Twenty-six (40.6%) patients had less than 30 copies/mL DNA load. HBsAg was tested by ELISA method, and

data showed that 54 (83.9%) were positive and 10 (16.1%) were negative. Also, via ECL method, 51 (79.7%) were positive and 13 (20.3%) were negative (Figure 2). HBeAg were tested by ELISA, 5 (7.9%) were positive and 59 (92.1%) were negative but via ECL 14 (21.9%) were positive and 50 (78.1%) were negative. The incidence rate of rtA194T was performed with real-time PCR. The presence of the mutations showed that 13 (20.3%) were mutant and 51 (79.7%) belonged to the wild type (Figure 3). The incidence rate of rtA194T in males and females were 5 (38.5%) and 8 (61.5%), respectively. But there was no significant relation between gender and rtA194T mutation. Serum AST and ALT related to rtA194T mutants showed that the level of ALT was (196 ± 56) IU/mL and in wild variants it was (104 ± 15) IU/mL. Therefore no differences was seen in these category ( $P = 0.48$ ). As for AST, it was (214 ± 59) IU/mL in mutant strains and (102 ± 17) IU/mL in wild type strains. There was no significant relation between AST and rtA194T mutation ( $P = 0.139$ ) (Figure 4). Our results showed that most of mutations were in more than 10<sup>9</sup> HBV DNA loads which may be resistant to the antiviral therapy. Therefore, there was a significant correlation between HBV DNA load and rtA194T mutation. This study showed that all of the mutants were in HBsAg positive samples, therefore rtA194T mutation had significant relation between HBsAg and mutation in rtA194T ( $P = 0.04$ ). Six (46.2%) of the mutations were in HBeAg negative cases ( $P = 0.02$ ). HBeAg status had significant relation with rtA194T mutation.

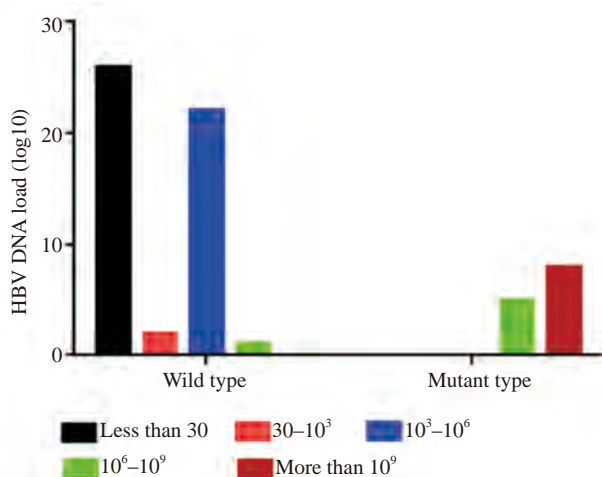


Figure 2. HBV load in mutant and wild type groups ( $P < 0.05$ , 95% CI: 10<sup>1</sup>-10<sup>9</sup>).

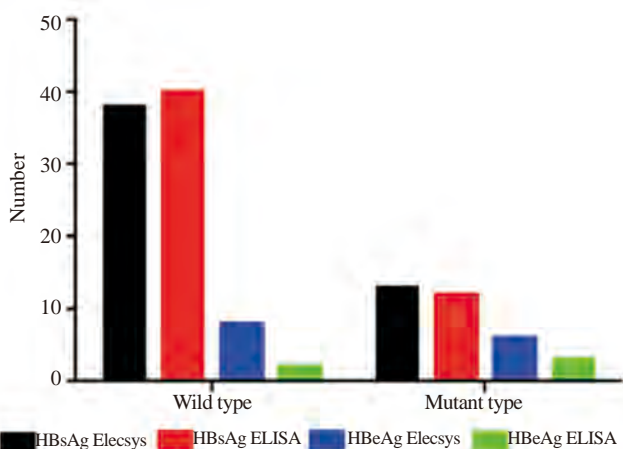


Figure 3. Comparison of HBsAg and HBeAg in mutant and wild type groups ( $P < 0.05$ , 95% CI: 20-55).

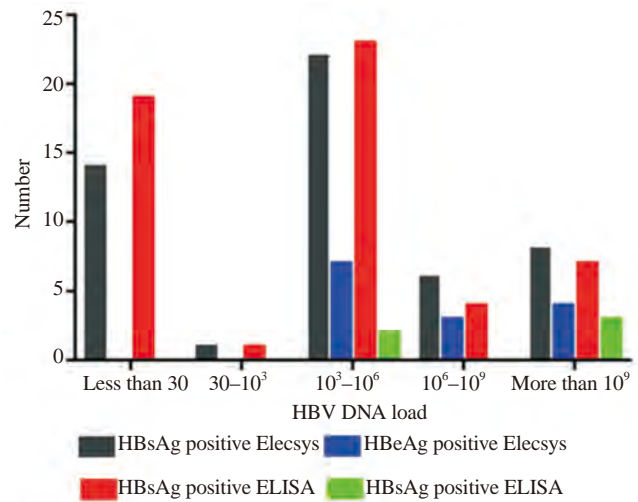


Figure 4. Comparison of HBsAg and HBeAg in different HBV DNA load ( $P < 0.05$ , 95% CI: 10<sup>1</sup>-10<sup>9</sup>).

#### 4. Discussion

Chronic infection with HBV causing liver diseases with morbidity and mortality has become an important global health problem[19]. The drug therapy in HBV-infected patients can limit the progression of the disease through the viral replication[8]. In CHB cases, the rate of spontaneous clearance of HBsAg is 0.5%–2%[20], and 10%–15% of the cases loss HBeAg and serum HBV DNA[21]. In most individuals, long-term antiviral treatment raises the risk of drug-resistant mutants which is associated with polymerase gene mutations. This mutation might be raised by high HBV viral load and ALT levels[4,22]. Different anti-HBV agents may be very important to help patients who are drug-resistant[23]. Quantitative real-time PCR is able to detect any resistant mutants in HBV populations[15,24]. TDF is an acyclic nucleotide analogue that is related to adefovir dipivoxil[25]. It has been shown that tenofovir is better than adefovir in terms of HBV DNA suppression, seroconversion of HBeAg and ALT normalization[26]. Tenofovir resistance is made with mutation in nt-194 location of polymerase gene, an adenine (A) substitution with a thymine (T), therefore a change formation in the polymerase gene cause that tenofovir has no affinity to polymerase and virus continue replication[27]. This is the first study ever done Kerman and more information is not available about HBV resistance to TDF. In the current study, the rate of rtA194T mutation was 20.3%, which is higher than that in the study conducted in Germany showing 4.6% of patients has mutation. Our study also determined that the association of rtA194T mutation with HBV DNA levels is significant. This mutation was seen in higher level of DNA load (10<sup>9</sup> copies/mL). Sheldon *et al.*[28] showed that after treatment, HBV DNA load reduced but after 40 weeks because of the occurrence of mutation and drug resistance, HBV load were increased and ALT level increased suddenly. In our study, the mean of ALT was 196 IU/mL which was higher than that in the wild variants[28]. In the same study, all mutations were seen in HBeAg positive patients, but in our study 71.8% of mutants were seen in HBeAg negative status. In another study, after treatment with TDF, HBeAg remained negative with detectable anti-HBeAg. Patients with HBsAg level < 100 IU/mL during an ALT flare after antiviral discontinuation achieved sustained response, but in our study, all of mutants were seen in HBsAg positive[29,30]. In conclusion, biochemical markers such as HBeAg, serum ALT level and HBV viral load were used in the diagnosis in patients with HBV infection. The sign of response to antiviral therapy is ALT normalization and serum HBV DNA suppression and if virus

is resistant, it can be proposed that some mutations in active site of antiviral drug may happen. In this study, the amount of mutations were evaluated according to the rise of serum HBV DNA titer or ALT level. In addition, high sensitive and specific real-time PCR method for the detection of new mutation of rtA194T to evaluate tenofovir resistance was used for better decision-making and future helpful therapy for patients.

### Conflict of interest statement

We declare that we have no conflict of interest.

### Acknowledgments

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### References

- [1] Boyd A, Maylin S, Gozlan J, Delaugerre C, Simon F, Girard PM, et al. Use of hepatitis B surface and “e” antigen quantification during extensive treatment with tenofovir in patients co-infected with HIV-HBV. *Liver Int* 2015; **35**(3): 795-804.
- [2] Sablon E, Shapiro F. Advances in molecular diagnosis of HBV infection and drug resistance. *Int J Med Sci* 2005; **2**(1): 8-16.
- [3] Seto WK, Liu K, Wong DK, Fung J, Huang FY, Hung IF, et al. Patterns of hepatitis B surface antigen decline and HBV DNA suppression in Asian treatment-experienced chronic hepatitis B patients after three years of tenofovir treatment. *J Hepatol* 2013; **59**(4): 709-16.
- [4] Sharon A, Chu CK. Understanding the molecular basis of HBV drug resistance by molecular modeling. *Antiviral Res* 2008; **80**(3): 339-53.
- [5] Shaw T, Bartholomeusz A, Locarnini S. HBV drug resistance: mechanisms, detection and interpretation. *J Hepatol* 2006; **44**(3): 593-606.
- [6] Song ZL, Cui YJ, Zheng WP, Teng DH, Zheng H. Diagnostic and therapeutic progress of multi-drug resistance with anti-HBV nucleos(t)ide analogues. *World J Gastroenterol* 2012; **18**(48): 7149-57.
- [7] Tuma P, Pineda JA, Labarga P, Vidal F, Rodriguez C, Poveda E, et al. HBV primary drug resistance in newly diagnosed HIV-HBV-coinfected individuals in Spain. *Antivir Ther* 2011; **16**(4): 585-9.
- [8] Virine B, Osiowy C, Gao S, Wang T, Castillo E, Martin SR, et al. Correction: hepatitis B virus (HBV) variants in untreated and tenofovir treated chronic hepatitis B (CHB) patients during pregnancy and post-partum follow-up. *PLoS One* 2015; **10**(12): e0145898.
- [9] Wang J, Liu J, Qi C, Yan T, Cao F, Jin L, et al. Efficacy of tenofovir disoproxil fumarate to prevent vertical transmission in mothers with lamivudine-resistant HBV. *Antivir Ther* 2015; **20**(7): 681-7.
- [10] Zhang Q, Liao Y, Chen J, Cai B, Su Z, Ying B, et al. Epidemiology study of HBV genotypes and antiviral drug resistance in multi-ethnic regions from Western China. *Sci Rep* 2015; **5**: 17413.
- [11] Zhao J, Liu J. [The drug resistance mutation detection and relevant factors analysis of HBV P region in chronic hepatitis B patients in Weifang City, Shandong Province]. *Bing Du Xue Bao* 2015; **31**(1): 14-7. Chinese.
- [12] Zhou BP, Zhang HM, Li XH, Yuan J, Li W, Xu LM, et al. [High throughput detection of drug-resistance gene mutations in HBV using MALDI-TOF mass spectrometry]. *Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi* 2008; **22**(5): 351-3. Chinese.
- [13] Rawal RK, Konreddy AK, Chu CK. Mechanism of adefovir, tenofovir and entecavir resistance: molecular modeling studies of how a novel anti-HBV agent (FMCA) can overcome the drug resistance. *Curr Med Chem* 2015; **22**(34): 3922-32.
- [14] Cheng YC, Ying CX, Leung CH, Li Y. New targets and inhibitors of HBV replication to combat drug resistance. *J Clin Virol* 2005; **34**(Suppl 1): S147-50.
- [15] Afshar RM, Mollaie HR. Detection of HBV resistance to lamivudine in patients with chronic hepatitis B using zip nucleic acid probes in Kerman, southeast of Iran. *Asian Pac J Cancer Prev* 2012; **13**(8): 3657-61.
- [16] Etiebet MA, Shepherd J, Nowak RG, Charurat M, Chang H, Ajayi S, et al. Tenofovir-based regimens associated with less drug resistance in HIV-1-infected Nigerians failing first-line antiretroviral therapy. *AIDS* 2013; **27**(4): 553-61.
- [17] Frangeul A, Barral K, Alvarez K, Canard B. *In vitro* suppression of K65R reverse transcriptase-mediated tenofovir- and adefovir-5'-diphosphate resistance conferred by the boranophosphonate derivatives. *Antimicrob Agents Chemother* 2007; **51**(9): 3162-7.
- [18] Furco A, Gosrani B, Nicholas S, Williams A, Braithwaite W, Pozniak A, et al. Successful use of darunavir, etravirine, enfuvirtide and tenofovir/emtricitabine in pregnant woman with multiclass HIV resistance. *AIDS* 2009; **23**(3): 434-5.
- [19] Stockdale AJ, Phillips RO, Geretti AM, HEPIK Study Group. The gamma-glutamyl transpeptidase to platelet ratio (GPR) shows poor correlation with transient elastography measurements of liver fibrosis in HIV-positive patients with chronic hepatitis B in West Africa. Response to: 'The gamma-glutamyl transpeptidase to platelet ratio (GPR) predicts significant liver fibrosis and cirrhosis in patients with chronic HBV infection in West Africa' by Lemoine et al. *Gut* 2016; **65**(5): 882-4.
- [20] Ha M, Xia W, Tang D, Wu J, Sun L, Shen W, et al. Hepatitis B e antigen-positive and high levels of alanine aminotransferase are associated with prevalence of metabolic syndrome in chronic HBV patients. *Obes Res Clin Pract* 2015; doi: 10.1016/j.orcp.2015.10.004.
- [21] Roberts H, Kruszon-Moran D, Ly KN, Hughes E, Iqbal K, Jiles RB, et al. Prevalence of chronic hepatitis B virus (HBV) infection in U.S. households: National Health and Nutrition Examination Survey (NHANES), 1988–2012. *Hepatology* 2016; **63**(2): 388-97.
- [22] Shimakawa Y, Lemoine M, Njai HF, Bottomley C, Ndow G, Goldin RD, et al. Natural history of chronic HBV infection in West Africa: a longitudinal population-based study from the Gambia. *Gut* 2015; doi: 10.1136/gutjnl-2015-309892.
- [23] Afshar RM, Mollaie HR. Use of ALLGIO probe assays for detection of HBV resistance to adefovir in patients with chronic hepatitis B, Kerman, Iran. *Asian Pac J Cancer Prev* 2012; **13**(11): 5463-7.
- [24] Antinori A, Trotta MP, Nasta P, Bini T, Bonora S, Castagna A, et al. Antiviral efficacy and genotypic resistance patterns of combination therapy with stavudine/tenofovir in highly active antiretroviral therapy experienced patients. *Antivir Ther* 2006; **11**(2): 233-43.
- [25] Bihl F, Martinetti G, Wandeler G, Weber R, Ledergerber B, Calmy A, et al. HBV genotypes and response to tenofovir disoproxil fumarate in HIV/HBV-coinfected persons. *BMC Gastroenterol* 2015; **15**: 79.
- [26] Larsson SB, Malmström S, Hannoun C, Norkrans G, Lindh M. Mechanisms downstream of reverse transcription reduce serum levels of HBV DNA but not of HBsAg in chronic hepatitis B virus infection. *Virol J* 2015; **12**: 213.
- [27] Lacombe K, Ollivet A, Gozlan J, Durantel S, Tran N, Girard PM, et al. A novel hepatitis B virus mutation with resistance to adefovir but not to tenofovir in an HIV-hepatitis B virus-co-infected patient. *AIDS* 2006; **20**(17): 2229-31.
- [28] Sheldon J, Camino N, Rodés B, Bartholomeusz A, Kuiper M, Tacke F, et al. Selection of hepatitis B virus polymerase mutations in HIV-coinfected patients treated with tenofovir. *Antivir Ther* 2005; **10**(6): 727-34.
- [29] Uribe LA, Nguyen N, Kim L, Trinh HN, Wong C, Wong C, et al. Rates of treatment eligibility in follow-up of patients with chronic hepatitis B (CHB) across various clinical settings who were initially ineligible at presentation. *Dig Dis Sci* 2015; **61**(2): 618-25.
- [30] Lu J, Li J, Liu Y, Ren S, Cao Z, Jin Y, et al. Study on post-treatment relapse in HBeAg positive CHB patients. *PLoS One* 2015; **10**(11): e0141072.