

Mutation in the Precore Region of HBV in Chronic Hepatitis B PatientsKader O.¹, Metwally D.E.¹, Helaly G. F.¹, El-Batouti G. A.², Hassan M B³ and Elsawaf R.¹¹ Microbiology Department, Medical Research Institute University of Alexandria² Microbiology Department, Faculty of Pharmacy, Pharos University³ Department of Molecular Medicine, University Hospital Düsseldorf, Germany
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Abstract: Background: The prevalence of Hepatitis B surface antigen (HBs-Ag) in Egypt is of intermediate endemicity (2–8%). Nearly 2-3 million Egyptians are chronic carriers of hepatitis B virus (HBV). HBs Ag and HBe Ag are particularly important in the management of CHB. Chronic hepatitis B (CHB) may present either HBe-Ag-positive or HBe-Ag-negative. **Objective:** The aim of the present work is to use the different HBV virological markers and HBV-DNA viral load to evaluate HBV infection, detect mutant forms of HBV. **Methods:** The current study included 52 HBs Ag positive patients; they were investigated for the following: anti- HBe, HBe Ag by ELISA, HBV DNA viral load, detection of precore, core promoter viral mutations in some HBV-DNA positive HBe antigen negative, anti-HBe positive patients by DNA sequencing. **Results:** Among the 52 HBs Ag positive patients only 4 (7.6%) were HBe Ag positive, HBV DNA was detected in 32 (66%) cases out of the 48 HBe Ag negative anti HBe positive patients, with the viral load ranging from 10² to 10⁵ IU/ml. Thirteen (27 %) out of the 48 anti HBe were inactive HBs Ag carriers. Twenty seven (56.25%) cases were anti HBe CHB. Three out of the 5 sequenced strains with the precore mutation were associated with HBe Ag negative CHB. None of these strains developed the triple BCP mutations.

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Key words: HBV, HBe Ag, pre-core, mutation.

1. Introduction

Hepatitis B virus (HBV) is an enveloped DNA virus that belongs to the family *Hepadnaviridae*. The worldwide burden of HBV is enormous.⁽¹⁾ The WHO currently estimates that 2 billion people worldwide have been infected with HBV and that 240 million are chronically infected.⁽²⁾

The global prevalence of HBs Ag varies greatly and countries can be defined as having a high, intermediate and low prevalence of HBV infection based on a prevalence of HBs-Ag carriers of 8%, 2% to 7%, and 2% respectively.⁽¹⁾ The prevalence of HBs Ag in Egypt is of intermediate endemicity (2–8%). Nearly 2-3 million Egyptians are chronic carriers of HBV.⁽³⁾

HBs Ag and HBe Ag are particularly important in the management of chronic hepatitis B (CHB). CHB may present either HBe Ag positive or HBe Ag negative. HBe Ag positive CHB is due to so-called “wild type” HBV which typically represents the early phase of chronic HBV infection, while HBe Ag negative CHB is due to replication of naturally occurring HBV variants with nucleotide substitutions in the precore and/or basic core promoter regions of the genome, resulting in down regulation of HBe Ag production despite ongoing viral replication.^(4,5)

HBe Ag negative patients have a more severe form of chronic HBV, with a poorer prognosis, higher

incidence of cirrhosis and has a higher rate of relapse when therapy, including interferon alfa (IFN α) and lamivudine regimens, is stopped.⁽⁶⁾ Typically, these individuals may develop the anti-HBe antibody but continue to present with elevated liver enzymes as well as persistent HBV-DNA levels, leading to advanced disease.^(7,8) This form of chronic HBV accounts for up to 30% of cases globally and is highly prevalent in the Mediterranean (50–80%).⁽⁶⁾

The aim of the present work was to determine the virological profile of HBs Ag positive chronic hepatitis B patients, detection of mutant forms of HBV characterized by the absence of HBe and correlation of the virological profile with laboratory data.

2. Subjects and Methods**2.1. Subjects**

The current study included 52 HBs Ag positive patients attending the Medical Research Institute outpatient clinics, Alexandria University in the period between 2010- 2011. The study was performed after approval of the ethics committee of the institute. Patients were examined for their demographic data (after taking full consent of patients), HBV related risk factors, radiological and laboratory data were collected.

2.2 Methods

2.2.1 Detection of hepatitis B virus serological markers

Blood samples were collected from all patients, Sera were investigated for the following: Detection of hepatitis B virus surface antigen (HBs Ag), antibodies against HB e antigen (anti-HBe), Hepatitis B e antigen (HBe Ag) by ELISA (Abott Murex Diagnostic Division)®,

2.2.2 Quantification of HBV-DNA viral load: ⁽⁹⁾

For detection of HBV DNA, total DNA was extracted from 200 µl of serum using QIAamp viral DNA mini kit (QIAGEN, Valencia, CA) followed by amplification by using Real Artus ® HBV TM RG PCR Assay (Artus-Biotech, Qiagen, Hamburg, Germany). Fifteen µl TaqMan universal PCR master mix 2-fold (Artus ® HBV RG PCR) were added to the reaction mixture. Ten µl of Qiagen extracted DNA were added to bring the reaction to a final volume of twenty five µl.

Real time PCR was performed with the Mx3000P™ (Stratagene) real time PCR system. The reaction was carried under the following thermal profile: AmpliTaq gold activation for 95° C for 10 min, followed by 40 cycles of two PCR-step amplification, denaturation for 95° C for 15 sec, followed by annealing and extension at 60° C for 1 min with end point fluorescence detection.

2.2.3 Detection of precore-core promoter viral mutations in some HBV-DNA positive HBe antigen negative anti-HBe positive patients by DNA sequencing: ⁽¹⁰⁾

Preparation of the template:

HBV DNA was amplified by semi-nested PCR. 10 µl of the qiagen DNA extract were added to a 2x fold PCR master mix (fermentas), Primer pair RMD 26 with the sequence 5'-ATG GAGACC ACC GTG AAC-3' (nt. 1608-1625) and C1 with the sequence 5'-TTC CGG AGA CTC TAAGGC-3' (nt. 2038-2020) at a concentration of 30 picomoles were used for the first amplification round, and primer pair RMD 26 and PC1 with the sequence 5'-GGA AAG AAG TCA GAAGGC-3' (nt. 1974-1957) were used for the second amplification round, at a concentration of 10 picomoles. The first amplification round consisted of denaturation at 94°C for 1 minute, followed by 30 cycles comprising a 30 second denaturation step at 94°C, a 30 second annealing step at 55°C, and a 1 minute extension step at 72°C, each, with a final extension cycle at 72°C for 10 minutes. For the second amplification round 5 µl of the first PCR product were added to the reaction mixture and amplification was performed in a manner identical to the first round.

The PCR product was purified using the Centri-Sep™ spin columns (Applied Biosystems, PN 401763 for 32 columns).

Performing Cycle Sequencing:

For each reaction the following reagents were added to a separate tube: Terminator Ready Reaction Mix* 8.0 µL, template : 3-10 ng of the 366 bp amplicon included between 1608- 1974 bp of the precore –core promoter region of the HBV-DNA, 3.2 pmol Primer and deionized water to a total volume of 20 µL.

Cycle Sequencing was Performed according to the following Thermal profile: an initial denaturation was performed at 96 °C for 1 min followed by 25 cycles of; 96 °C for 10 sec for denaturation, 50 °C for 5 sec for annealing, 60 °C for 4 min for extension.

Electrophoresis and data analysis of samples were performed on the ABI PRISM® 310 Genetic Analyzer and its sequencing analysis software. The strain type was identified by entering the sequencing data into HBV blast in gene bank.

2.3. Statistical analysis

Data were analysed using the Chi Square test

3. Results

3.1. Analysis of demographic data and risk factors

The study group included 52 HBs Ag positive patients 41 males and 11 females with a low male to female ratio of 3.7 and a mean age 41 ± 11 years.

Dental extraction and surgical interference were found to be the major risk factors representing 40 (76.9 %) and 22 (42.3 %) of the cases respectively. While, history of blood transfusion, parenteral anti-schistosomal treatment, and intravenous drug use were not found to be major risk factors (only 1.9 % each).

2.2. Results of Serological tests of HBV

Out of the 52 HBs Ag positive patients 48 (92.3%) were anti-HBe positive, HBe Ag negative, and only 4 (7.6%) were HBe Ag positive.

3.3 Results of HBV DNA testing

Using a sensitive commercial kit (Artus) for HBV-DNA amplification and quantitation. HBV-DNA was detected in 32 (66%) out of the 48 anti-HBe positive patients, with a viral load ranging from 10² to 10⁵ IU/ml. (Table I)

Only 13 cases (27 %) had a viral load below 2000 IU/ ml with normal liver enzymes level and were thus considered as inactive HBs Ag carriers. Twenty seven (56.25%) cases had either viral load more than 20,000 IU/ml and or elevated liver enzymes and were categorized as chronic hepatitis patients. (Table II). No statistically significant difference was found between the 3 groups regarding ultrasound findings of the liver/spleen, bilirubin, prothrombin activity, platelets count. (Table III).

3.4 Results of sequencing

Sequencing of the basal core promotor, precore and core genes were carried out in five HBV strains. G1896A mutation that lead to precore stop codon was detected in all the five strains. This was found in association with T1858 variant allowing a stable bound with adenine at nucleotide 1896. Three out of the 5 sequenced strains with the precore

mutation G1896A were associated with HBe Ag negative. None of our 5 HBV strains developed the triple BCP mutations A1762T/ G1764, C1766T, however, deletion of the nucleotide 1762 was noticed among the 5 strains. T1753V (C, A or G) substitution at nucleotide 1753 was not detected among our strains. **Figures (1), (2 A,B).**

Table I: HBV- DNA viral load among the 52 HBs Ag positive patients.

Viral load	HBe Ag positive(total= 4)		Anti-HBe positive(total= 48)	
	No	(%)	No	(%)
$> 10^8$	3	(75%)	-	
$10^7 \geq 10^8$	-		-	
$10^6 \geq 10^7$	1	(25%)	-	
$10^5 \geq 10^6$	-		2	(4.16 %)
$10^4 \geq 10^5$	-		8	(16.6%)
$10^3 \geq 10^4$	-		12	(25%)
$10^2 \geq 10^3$	-		7	(14.6%)
$\leq 10^2$	-		3	(6.25%)
Negative	-		16	33.3%

Table II: Classification of the 48 anti HBe Ag positive patients according to ALT/AST and HBV-DNA viral load.

HBV DNA (IU/ml)	Liver enzymes	No of cases	Definition of cases
< 2000	Normal	13(27%)	Inactive HBs Ag carrier
> 2000 to <20,000	Normal	8 (16.6%)	Need follow up
< 20,000	Elevated	22(45.8%)	Chronic HBV
$\geq 20,000$	Normal	2(4.1%)	Chronic HBV
$\geq 20,000$	Elevated	3(6.25%)	Chronic HBV

Table (III): Laboratory and Radiological findings among the 48 anti- HBe positive patients.

Case Definition	No of Cases	Cases with abnormal prothrombin %	Cases with abnormal bilirubin level	Cases with abnormal platelets count	Cases with abnormal U/S liver/spleen	X ² (df)	P-value
Inactive HBs Ag carriers	13	1	-	-	8	x ² (2)= 1.45	0.483
Chronic HBV	27	6	2	-	11	x ² (2)= 1.62	0.444
Cases that need follow up	8	1	-	-	6	x ² (2)= 1.62	0.444

Using Chi Square test

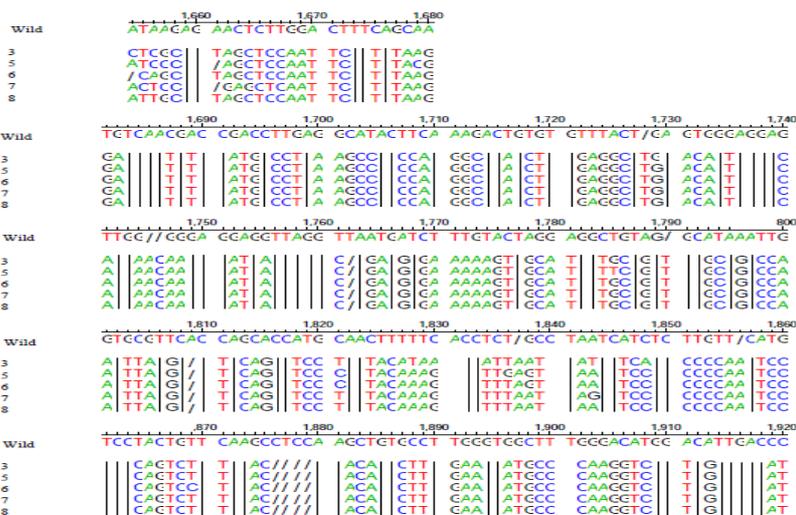
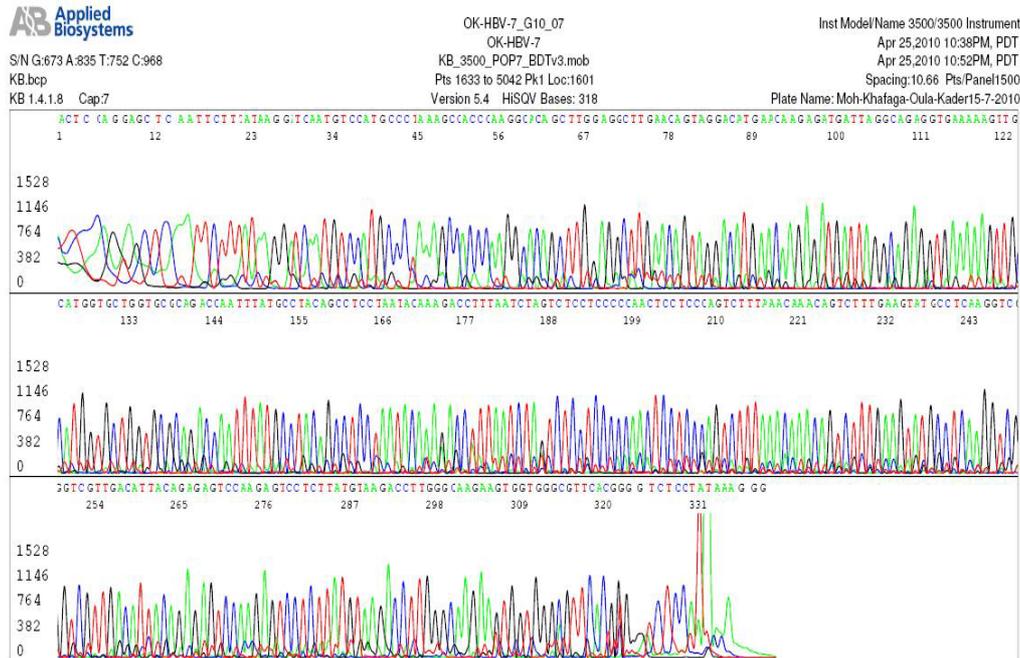


Figure (1) G-----A Mutation



Figures (2 A)

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> gb|AY161156.1| Hepatitis B virus clone S6.7, complete genome
Length=3134

Score = 569 bits (308), Expect = 3e-159
Identities = 322/328 (98%), Gaps = 3/328 (0%)
Strand=Plus/Minus

Query 5 CAGGAGCT-C-AATTCTTTATAAGGATCAATGTCATGCCCTAAAGCCACCCAAGGCACA 62
      ||| |||| | |||||||||||||||||||||||||||||||||||||||||||
Sbjct 1937 CAGTAGCTCCAAATTCTTTATAAGGATCAATGTCATGCCCTAAAGCCACCCAAGGCACA 1878

Query 63 GCTTGGAGGCTTGAACAGTAGGACATGAACAAGAGATGATTAGGCAGAGGTGAAAAAGTT 122
      |||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1877 GCTTGGAGGCTTGAACAGTAGGACATGAACAAGAGATGATTAGGCAGAGGTGAAAAAGTT 1818

Query 123 GCATGGTCTGGTGGCGACACCAATTTATGCCTACAGCCTCCTAATACAAAGACCTTTAA 182
      |||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1817 GCATGGTCTGGTGGCGACACCAATTTATGCCTACAGCCTCCTAATACAAAGACCTTTAA 1758

Query 183 TCTAGTCTCCTCCCCCAACTCCTCCAGTCTTTAAACAAACAGTCTTTGAAGTATGCCTC 242
      ||| |||||||||||||||||||||||||||||||||||||||||||
Sbjct 1757 TCTAATCTCCTCCCCCAACTCCTCCAGTCTTTAAACAAACAGTCTTTGAAGTATGCCTC 1698

Query 243 AAGGTCGGTCTGTGACATTACAGAGAGTCCAAGAGTCTCTTATGTAAGACCTTGGGCAA 302
      |||||||||||||||||||||||||||||||||||||||||||
Sbjct 1697 AAGGTCGGTCTGTGACATTACAGAGAGTCCAAGAGTCTCTTATGTAAGACCTTGGGCAA 1638

Query 303 GAAGTGGTGGGCGTTCACGG-GGICTCC 329
      |||||||||||||||
Sbjct 1637 GAAGTGGTGGGCGTTCACGGTGGICTCC 1610
    
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Figure (2B)

Figures (2 A,B): Sequencing of HBV genome in case number 7 shows that mutation at nucleotide 1896 was detected in case number 7, who had a viral load of 6×10^3 IU/ml, with an HBe Ag negative, anti HBe positive viral profile, normal liver enzymes tests, decreased prothrombin time. U/S spleen was normal, but U/S liver showed periportal fibrosis.

4. Discussion

Among our 52 HBs Ag positive patients only 4 (7.6%) were HBe Ag positive with a mean age of 28.5 years (ranging from 23-38 years). *Chu et al.*,⁽¹¹⁾ reported that persistent HBe Ag seropositivity beyond 40 years of age is relatively uncommon and is associated with a higher risk of cirrhosis and hepatocellular carcinoma (HCC) and therefore should be considered as 'delayed' HBe Ag seroconversion.

Patients with HBs Ag positive, HBe Ag negative, anti-HBe positive and high level of HBV-DNA with intermittent or persistent increase in ALT are diagnosed as HBe Ag negative variants, affecting older age groups (36-45 years) with male predominance, severe necro-inflammation and/or cirrhosis at the time of presentation.⁽¹²⁾

In the present study 27 (56.25 %) out of the 48 anti-HBe positive patients had a viral load \geq 20,000 IU/ml and/or elevated liver enzymes and thereby, were considered as anti-HBe CHB. On the other hand, patients who are HBs Ag positive, HBe Ag negative, anti-HBe positive, with persistently normal ALT levels and HBV-DNA $<$ 2,000 IU/ml were diagnosed as inactive HBs Ag carriers.⁽¹³⁾ In this study 13 (27 %) out of the 48 anti-HBe positive cases had negative HBV-DNA or had a viral load $<$ 2000 IU/ml, and normal ALT/AST values.

Both HBe Ag negative variants and inactive HBs Ag carrier state are more frequently encountered in clinical practice and are difficult to differentiate. Differentiation between both phases of natural history is mandatory before embarking on antiviral therapy for HBe Ag negative patients.⁽¹⁴⁾ Eight (16.6%) of our anti-HBe positive cases had a viral load ranging in between 2000-20,000 IU/ml and normal ALT/AST values. Such individuals should be followed-up clinically, have frequent ALT assays and in case of increasing ALT levels be further tested for serum HBV-DNA levels.⁽¹⁵⁾

Progression to HBe Ag negative disease involves the replication of HBV that no longer has the ability to produce the precore protein HBe Ag. The emergence of this altered HBV has been linked to two categories of mutations that result in reduced or blocked HBe Ag expression.⁽¹⁶⁾ In the present study, sequencing of the basal core promoter, precore and core genes were carried out in five HBV strains. G1896A mutation that leads to precore stop codon was detected in all the five strains. This was found in association with T1858 variant allowing a stable bond with adenine at nucleotide 1896. In HBV genotypes B, D, E, and G and in some strains of genotype C, the 1858 nt is a thymidine (T). Thus, the stop-codon mutation created by G1896A (T-A) stabilizes the ϵ structure. In contrast, the precore stop-codon mutation is rarely detected in HBV genotypes

A or F or in certain strains of HBV genotype C because the nt at position 1858 is a cytidine (C), maintaining the preferred Watson-Crick (G-C) base pairing.⁽¹⁷⁾

This is consistent with our findings, as 3 out of the 5 sequenced strains with the precore mutation G1896A were associated with HBe Ag negative CHB, with either viral load more than 20,000 IU/ml and/or elevated liver enzymes while the other 2 strains were associated with HBe Ag negative carriers, with a viral load less than 20,000 IU/ml but more than 2000 IU/ml and normal liver enzymes. These individuals should be followed-up clinically, by frequent ALT testing and in case of increasing ALT levels, be further tested for serum HBV-DNA levels.

HBV genotype D is reported to be the prevalent genotype in Egypt. The clinical impact of HBV genotype D has been studied less extensively. However, initial studies have found that it may be associated with lower rates of sustained remission and HBs Ag clearance and more severe liver disease compared with genotype A.⁽¹⁷⁾ A study from India indicated that genotype D is more often associated with HBe Ag negative CHB, more severe diseases and may predict the occurrence of HCC in young patients.⁽¹⁸⁾

In conclusion, the majority (92.3%) of our HBs Ag positive were HBe Ag negative, anti HBe positive, however, these patients did not represent a homogenous group regarding ALT level and HBV-DNA viral load. Some of them shared some of the characteristics that have been associated with adverse long term outcomes, also our findings highlighted the fact that the distinction is not always clear-cut between inactive carriers and patients with HBe Ag negative hepatitis. The precore stop codon G1896A mutations detected in all of our five strains were not reported to be associated with the risk of HCC, regardless of HBe Ag status.

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