Laboratory Diagnosis for Optimize Therapy of B Hepatitis Virus Infection by Using Biochemical and Molecular Biology Methods

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The optimal management of chronic hepatitis B virus (HBV) infections is known to be related with the genotype of HBV in order to prescribe the most appropriate treatment. In this study, including 19 chronic HBV patients from St. Parascheva Clinical Infectious Disease Hospital, Iaºi, we attempted to double test their samples for DNA/HBV detection and genotyping. DNA/HBV evidenced from19 patients known as being chronic infected by HBV were tested by two molecular techniques: Real Time PCR (Polymerase Chain Reaction) and genotyping by hybridization on strips. We obtained identical results from both methods. The most frequent genotype was D (47.4%), followed by genotype A (10,5%); 9 samples were negative, probably because of virus suppressed replication under treatment. The double testing is accurate, time and cost efficient, and for these reasons we propose this double testing like a possible algorithm for routine diagnostic, being time-efficient and accurate for HBV chronic infected patients' management cases.

Keywords: HBV, PCR classic, Real Time PCR, accurate detection

Chronic HBV affects nearly 350 million patients worldwide and may further progress to cirrhosis and/or hepatocellular carcinoma (HCC) in15–40% of cases. HBV accounts for 30% of cirrhotic and 50% of HCC cases [1]. Over the world, at least ten genotypes of HBV (A through J) have been identified on the basis of more than 8% difference in their genome sequences. Higher rates of HCC have been found in persons infected with genotypes C and F (compared with genotypes B or D), and in those infected with certain subtypes of genotype A found in southern Africa although aflatoxin exposure may play a role in sub-Saharan Africa. Over the past three decades, treatment outcomes for chronic hepatitis B (CHB) have improved, first with IFN-alpha and with nucleos(t)ide analogues (NAs). Currently, seven antiviral agents (six NAs – lamivudine, adefovir, entecavir, telbivudine, tenofovir, emtricitabine, as well as standard and two formulations of PEG-IFN) are approved and widely licensed for the treatment of CHB [1].

Numerous studies have investigated the influence of HBV genotypes on clinical outcomes. Genotype C is associated with rapid fibrosis, high HCC development rate, recurrence, and metastasis compared to genotype B. It appears that genotype D may be associated with more severe disease compared to A, and that genotype F is linked to high mortality rates. Although HBV genotypes do not significantly influence response to nucleoside/nucleotide antiviral therapy, responsiveness to IFN is clearly affected by genotype. HBeAg-positive patients infected with genotype B have a better response compared to those with genotype C, and genotype A responds better than genotype D, especially during short term therapy. Genotype A also responds well to PEG-IFN [2] The response to PEG-IFN has been reported to be influenced by HBV genotypes in many studies. Janssen et al. reported that HBeAg loss as a response to PEG-IFN monotherapy or PEG-IFN plus lamivudine (LAM) was influenced by HBV genotype, and that genotypes A and B had a better response compared to C and D, after 52 weeks of treatment [3]. Genotyping is still used as a research tool in the UK and isn't currently used to guide treatment with interferon based therapies. However, with such a high proportion of the CHB population carrying genotype there is a question as to whether genotyping should now be incorporated. It is possible that the use of routine genotyping could help identify those patients who are at higher risk of liver disease progression so that IFN-based therapies can be targeted earlier [4].

In some studies, DNA/HBV detection with accurate methods is an imperative requirement in daily routine diagnostic of chronic HBV patients. Fast and accurate DNA/HBV detection can be followed by genotyping of HBV, in order to correct choose the antiviral therapy [3, 11].

The aim of presented study was to compare two molecular techniques for detection and genotyping of DNA/HBV: a screening method using Real Time PCR (Amplisens HBV-FRT) technology and INNO LiPA HPV respectively, which involve classical PCR followed by hybridization on strips.

Experimental part

Material and methods

We have tested 19 samples using two molecular biology techniques: first we performed detection of DNA/HBV with a Real Time PCR technique and second we tested the same extracted DNA from each patient, in order to detect the genotype of HBV.

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The DNA/HBV was purified from 200µL blood from each patients, using Invisorb Spin Virus DNA Mini Kit. The kit is using the classical buffers for binding, washing, ellution, and use also proteinase K, RNA-ase free water; for testing with Amplisens HBV-FRT a negative control (PCE) and Positive Control-1-HBV were necessary too. The DNA purification involved one step of incubation at 56°C for 15 min in Biosan thermobloc, and 4 steps of centrifugation (Beckman Coulter microcentrifuge) and washing steps. The DNA/HBV was eluted in 100µL ellution buffer and it was used imediately for PCR amplifications.

The Real Time PCR detection used Amplisens HBV-FRT, AccuPower PyroHotStart Taq PCR PreMix and MX3005P thermocycler, Stratagene. All the Real Time PCR reagents were unfrozen, mixed and centrifuges from the PCR kit. The reaction mix contained: 10 µL PCR-mix-1-FL HBV + 5µL PCR-mix-2-FRT + 0.5µL Polymerase TaqF/sample. After mixing of these reagents were added the DNA samples (10 µL each) and also 10µL of each of the controls: Positive Control-1-HBV, *C-* purified from Negative Control, *C+* from DNA calibrator PIC2 HBV and NCA (water for negative control of amplification). The DNA isolated from the patients were pippeted in the plate in row A from 1 to 12 and in row B from 1 to 7. The negative controls were in row H, from 9 to 12 (fig. 1). The thermal profile can be seen in table 1 and in figure 2.

The HBV genotyping was performed using INNO-LiPA HBV Genotyping kit Innogenetics N.V., Ghent, Belgium and AccuPower PyroHotStart Taq PCR PreMix. This method involved a first step of outer amplification which supposed that in the Eppendorf tubes with premixed *AccuPower PyroHotStart Taq* PCR PreMix was added 38µL water + 2µL Outer Primer Mix + 10µL ADN or water for negative control. The PCR amplification was realized in Gene Amp 9700 termocycler and has used the amplification protocol detailed in table 2.

The second amplification was the nested one, in which the total reaction volume was 50µL: 46 µL water + 2µL Nested Primer Mix + 2µL amplicons or water for negative control. In this step, amplification was similar with the outer amplification protocol, with single difference of 35 cycles instead of 40 cycles. The products of nested amplification were then hybridized on the strip, using TwinCubator device, using the steps from the kit protocol.

Results and discussions

The analysis of Real Time amplification had used the chanels FAM and JOE, and the exactly distribution of each of them for samples and control type can be seen in table 3.

Temperature°C	Time	No of cycles
50	15 min	1
95	15 min	1
95	5 s	5
60	20 s	
72	15 s	
95	5 s	40
60	30 s*fluorescence reading FAM, JOE	
72	15 s	

Table 1
THE STEPS OF THERMAL REAL TIME PCR AMPLIFICATION

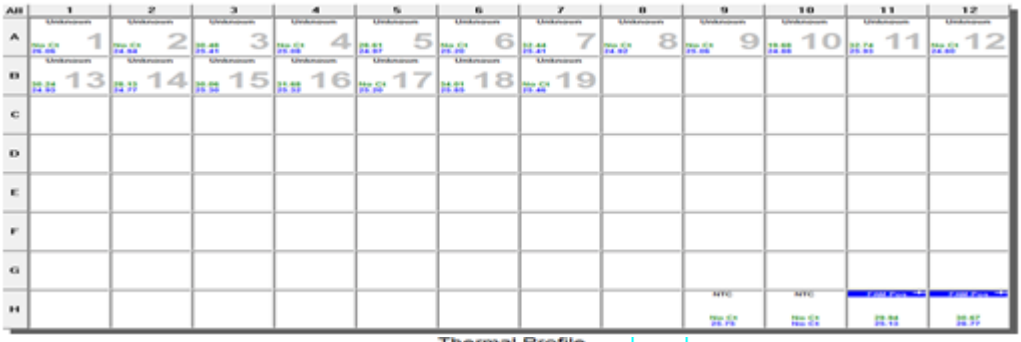

Fig. 1. Plate setup for Real Time PCR: 19 unknown samples and 4 controls: 2 positive and 2 negative

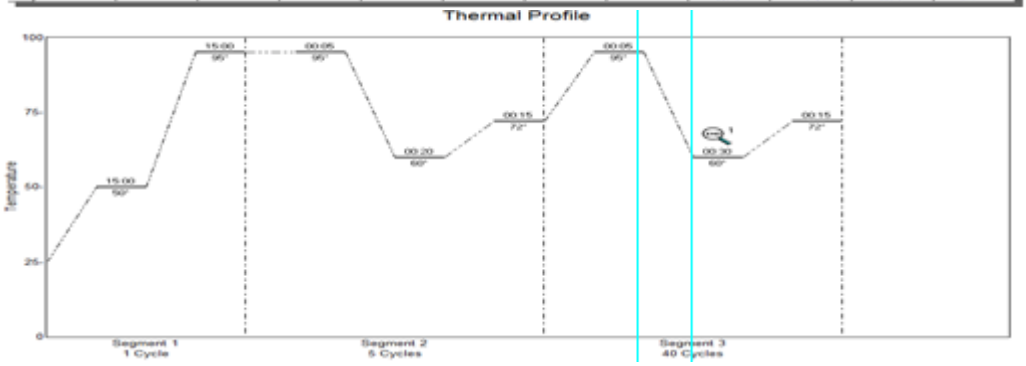

Fig. 2. The thermal profile using Amplisens HBV-FRT kit

Temperature °C	Time	No of Cycles
94	4 min	1
94	30 s	40
45	30 s	
72	30 s	
72	10 min	1

Table 2
THE AMPLIFICATION PROTOCOL FOR OUTER AMPLIFICATION

Amplification product	Channel
Internal control (IC)	FAM
DNA/ HBV	JOE
PCE	FAM, JOE
C-	FAM/absent
C+	FAM, JOE
NCA	absent

Table 3
THE ANALYSIS OF RESULTS

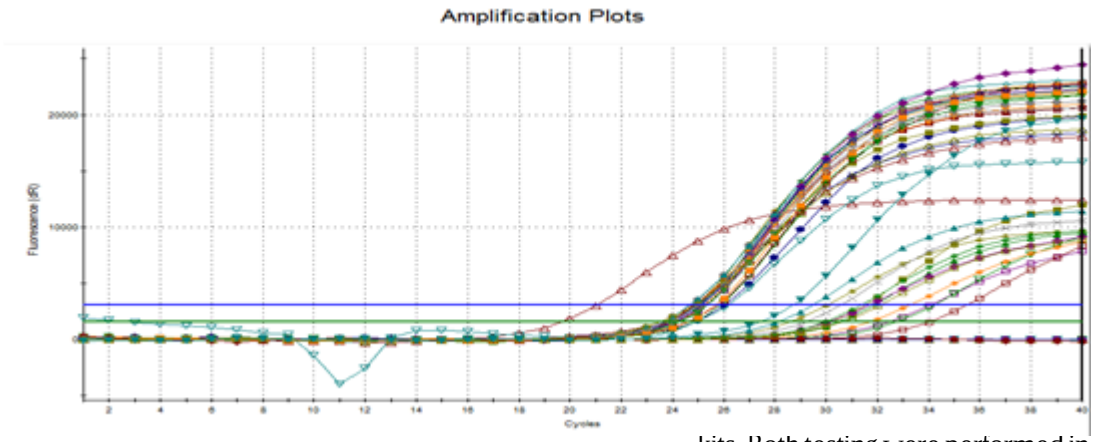

Fig. 3. The amplification plots for the unknown samples and for the positive controls

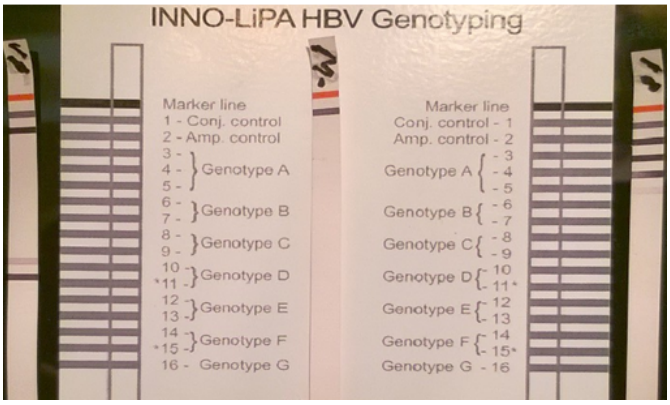

Fig. 4. The reading card of INNO LiPA kit with a strip positive for D genotye, the negative control and a strip positive for A genotype of HBV

In figure 3 can be seen the amplification plots of Real Time PCR amplification for unknown samples and for positive controls. The Ct values for the patients (JOE/HEX) varied from 19.68 to 34.01. The Ct values for internal extraction control (FAM) varied from 24.88 to 25.93.

The results of INNO LiPA Genotyping method: from 20 samples, one was the negative control, and the samples from 19 patients had the next results: 8 were negative, 9 had genotype D and 2 were detected having genotype A of HBV. In figure 4 can be observed the negative control strip, a strip with genotype D and a strip with genotype A.

The results of both method can be observed in table 4. All the results obtained by both methods were validated with the help of positive and negative controls from the kits. Both testing were performed in 2 working days, which easily can be seen like an advantage in daily routine testing. The Real Time PCR amplification is less than 2 h, shorter than the classical ELISA methods (3.5 h) used for HBsAg detection, for example. The INNO LiPA assay is take longer because needs two PCR amplification, but is having the advantage of genotype detection, which can be used to optimize the antiviral therapy. The possible explanation for the negative results is that these patients are receving antiviral therapy and is possible that in the moment of testing, the DNA not to be detectable because of virus replication supression.

INNO-LiPA is a reverse hybridization method that has been developed by Innogenetics and is commercially available as INNO-LiPA. The overall success rate for the detection of all eight HBV genotypes by this method was 98% using 100 clinical specimens with the majority having viral titers ranging from 10^5 to 10^7 IU/mL.Like advantages of this method we can mention that is easier and cheaper than sequencing, overall success rate for the detection of all HBV genotypes was recently found to be 98% and it is highly specific. Like disadvantage, is relatively expensive compared to PCR-based and serological techniques [2]. Amplisens HBV-FRT assay is recognized being a sensitive and specific assay [12].

Although HBV genotyping before anti-viral therapy is not recommended by current guidelines from three regional liver associations, the American Association for the Study of Liver Disease (AASLD), the European Association for the Study of Liver (EASL), and Asian Pacific Association for the study of liver (APASL), the impact of HBV genotype on therapeutic response to both interferon-based and

Patients and negative control	Real Time PCR	INNO LiPA
1.	NEG	NEG
2.	NEG	NEG
3.	POSITIV	GENOTYPE A
4.	NEG	NEG
5.	POSITIV	GENOTYPE D
6.	NEG	NEG
7.	POSITIV	GENOTYPE D
8.	NEG	NEG
9.	NEG	NEG
10.	POSITIV	GENOTYPE D
11.	POSITIV	GENOTYPE D
12.	NEG	NEG
13.	POSITIV	GENOTYPE D
14.	POSITIV	GENOTYPE D
15.	POSITIV	GENOTYPE D
16.	POSITIV	GENOTYPE D
17.	NEG	NEG
18.	POSITIV	GENOTYPE A
19.	POSITIV	GENOTYPE D
Negative Control	NEG	NEG

Table 4
THE CORRELATION OF THE RESULTS OBTAINED BY THE TWO PCR METHODS

nucleos(t)ide analogues has been increasingly recognized [13-21].

Conclusions

In the North-East region of Romania, the D genotype is the most frequent. According with these results, even in a smal patients group, we propose a posible diagnosis algorithm which can be used for management of chronic HBV cases: DNA/HBV detection by Real Time PCR followed by HBV genotyping. The algorithm is having the advantage of being cost and time efficient for optimal management of HBV positive patients. Further studies are needed on larger groups of patients to confirm these preliminary results.

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