Comparison of INNO-LIPA and TRUGENE Assays for Genotyping and Drug-Resistance Mutations in Chronic Hepatitis B Virus Infection

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Key Words

Hepatitis B virus genotype · Drug-resistance mutation · Line probe assay

Abstract

Objective: Hepatitis B virus (HBV) genotyping and detection of resistance to drugs have become essential in epidemiological and clinical diagnosis. Our main objective was to determine the prevalence of HBV genotypes and drug-resistance mutations in chronic asymptomatic carriers and chronic HBV sufferers comparing 2 detection assays. **Methods:** Serum samples from 28 chronic HBV patients and 22 chronic asymptomatic carriers were analyzed. For HBV genotyping, the INNO-LIPA and TRUGENETM HBV genotyping kits were evaluated. For drug-resistance mutations, INNO-LIPA DR v2 and INNO-LIPA DR v3 prototype and the TRUGENETM HBV genotyping kit were evaluated. **Results:** In HBV genotyping, concordant results were 98% and both assays were able to detect more than one genotype. Different genotypes were detected, the most prevalent being D (46%) and A (26%). In relation to drug-resistance mutations, the sensitivity of the line probe assay was lower than TRUGENE because INNO-LIPA could not detect two mutations

(S202G and V214A). **Conclusions:** Both assays are easy and suitable for detecting HBV genotype and drug-resistance mutations and for routine laboratory use. However, TRUGENE presented better sensitivity in both analyses and it is possible to conduct both on the same sample. This assay is also able to detect primary and secondary mutations.

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Introduction

Hepatitis B virus (HBV) infection is a global health issue and a major cause of acute and chronic liver disease worldwide. More than 350–400 million people are infected with HBV [1], and they are at a very high risk for developing severe liver diseases including liver cirrhosis and hepatocellular carcinoma. Every year, approximately 1 million people die due to HBV infection; 33% of these deaths are caused by hepatocellular carcinoma and the rest by the terminal complications of liver disease [2].

Genomic mutations presented during HBV reverse transcription could explain its genetic diversity and account for the 8 genetically distinct genotypes, designated A–H. These genotypes show distinctive geographical dis-

tribution across the entire world [3]. Moreover, the diversity of HBV genotypes may be related to the clinical patterns of infection, the development of cirrhosis and hepatocellular carcinoma and resistance to antiviral treatment [4].

Nowadays, there are several techniques to detect HBV genotypes: reverse hybridization [5], restriction fragment-length polymorphism [6], genotype-specific PCR assays [7], sequence analysis [8], microarray (DNA-chip) [9] and fluorescence polarization assay [10]. However, a rapid and easy-to-use genotyping assay could become an essential and routinely used tool for disease management.

To date, prolonged therapy with nucleoside/tide analogs usually confers drug resistance because of the presence of mutations in the target gene [11, 12]. These mutations should be detected using rapid and specific methods which allow the identification of all substitutions. These mutations can also appear in patients without any therapy regimen, indicating a natural pressure selection.

The objective of this study was to determine the prevalence of HBV genotypes and drug-resistance mutations in a group of chronic asymptomatic carriers and a group of chronic HBV sufferers comparing 2 detection assays.

Materials and Methods

Serum samples were collected from 28 chronic HBV patients and 22 chronic asymptomatic carriers from the Digestive Service at the Basurto University Hospital in Basque Country in the north of Spain. All participants in this study gave their consent and were tested for liver profile (serum albumin, alanine aminotransferase, alkaline phosphatase and bilirubin levels).

HBV DNA was isolated from serum samples using the commercially available QUIamp DNA Blood minikit (Qiagen, Hilden, Germany). INNO-LIPA genotyping assay was carried out by nested PCR assay using primers from the HBV P gene region as described previously [5]. Amplification products were hybridized using a line probe assay designed to identify HBV genotypes A–H by the detection of type-specific sequences in the HBV polymerase gene domain B–C (INNO-LIPA HBV genotyping assay, Innogenetics, Belgium) according to the manufacturer's instructions. For resistance mutations INNO-LIPA DR v2 and INNO-LIPA DR v3 prototype (Innogenetics) were performed.

TRUGENE™ HBV genotyping kit (Siemens Healthcare Diagnostics, USA) was used simultaneously for HBV genotype and drug-resistance mutations.

The presence of mixed genotype infection and indeterminate or discordant results were verified by sequencing. The PCR product was purified with QUIAquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced using the BigDye Terminator Cycle sequencing kit on an ABI Prism 3130 Genetic Analyzer (Applied Biosystems, USA).

Table 1. Demographic, serological and risk data of the subjects included in the study

	Chronic HBV ^a (n = 28)	Carriers ^b (n = 22)
Men, n (%)	19 (67.8)	14 (63.6)
Mean median age, years (range)	42.39 (14–63)	41 (18–69)
HBeAg positive, n (%)	9 (32.1)	3 (13.6)
Country of origin, (%):		
China	3 (10.7)	0
Colombia	1 (3.5)	0
Sub-Saharan area	2 (7.1)	3 (13.6)
Pakistan	1 (3.5)	0
Europe	21 (75)	19 (86.4)
Risk factors, n (%):		
Sexual	7 (25)	2 (9.1)
Transfusion	3 (10.7)	3 (13.6)
Maternal transmission	1 (3.5)	6 (27.2)
Interfamilial	1 (3.5)	1 (4.5)
Tattoos	0	1 (4.5)
Unknown	16 (57.1)	9 (40.9)

^a Patients with chronic HBV infection.

^b Chronic asymptomatic carriers.

Table 2. HBV genotype analysis methods comparison

Genotype	Number of samples		INNO-LIPA concordant results, %	TRUGENE concordant results, %
	chronic HBV ^a	carriers ^b		
A	7	6	100	100
B	1	0	100	100
C	2	0	100	100
D	13	10	95.6	100
E	2	2	100	100
F	2	0	100	100
A/F	1	0	100	100
D/H	0	2	100	100
E/F	0	1	100	100
A/D	0	1	100	100
Total	28	22	99.56	100

^a Patients with chronic HBV infection.

^b Chronic asymptomatic carriers.

Results

A total of 50 patients were included in the study: 28 in the chronic HBV patient group and 22 in the chronic asymptomatic carrier group. Demographic, serological and risk data are shown in table 1.

All serum samples were analyzed by using the INNO-LIPA and TRUGENE™ HBV genotyping assays (table 2).

Results were in agreement in 49 cases (98%) but there was an indeterminate result using INNO-LIPA assay that was identified as genotype D using the TRUGENE assay and confirmed by sequencing PCR product. In this case, the sensitivity was 99.56% in INNO-LIPA and 100% in TRUGENE. Moreover, there were no false-positive results so the specificity was 100% in both assays. Both detection assays were able to detect mixed genotypes (10%). In our study population, genotype D was dominant (46%) followed by genotype A (26%). Patients infected with genotypes B and C were from China, those with genotype E from the sub-Saharan area and those with genotype A/F from Colombia.

For the detection of drug-resistance mutations, we treated patients with chronic HBV infection with lamivudine, adefovir and/or entecavir (data not shown). In 28 patients, 7 presented different mutations according to treatment with nucleoside/tide analogs (table 3). The INNO-LIPA assay was able to detect 15 of 16 (93.75%) mutations and TRUGENE detected all of them. The discordant result was sequenced and the result was similar to TRUGENE (S202G entecavir-resistant mutation). In the group of chronic asymptomatic carriers, only 1 patient presented a mutation that was only detected by TRUGENE and confirmed by sequencing (V214A lamivudine-resistant mutation). The specificity in both assays was 100% and the overall sensitivity was 93.75% in the INNO-LIPA assay and 100% in TRUGENE assay.

Analyzing the risk factors for our population, most of our patients were infected by an unknown mechanism (25/50, 50%) followed by sexual (18%) or maternal transmission (14%), transfusion (12%), interfamilial factors (4%) and tattoos (2%).

Characteristics of patients with chronic HBV are shown in table 4.

Discussion

This study describes the comparison of two HBV genotyping and drug-resistance mutations assays (INNO-LIPA and TRUGENE). The genetic variability of this virus is greater than any other DNA virus and its sequence has different types of variability [13]. The HBV genotype or local strain is the first type of variability and is stably transmitted within the host population. INNO-LIPA is a line probe assay able to detect the main HBV genotypes and different drug-resistance mutations using 2 different kits for this purpose. TRUGENE, however, uses direct se-

Table 3. HBV drug-resistance mutations analysis methods comparison

Drug-mutations resistance	Number of samples		INNO-LIPA concordant results, %	TRUGENE concordant results, %
	chronic HBV ^a	carriers ^b		
L180M	4	0	100	100
A181V	2	0	100	100
T184A	1	0	100	100
S202G	2	0	50	100
M204I	2	0	100	100
M204V	1	0	100	100
M204V/I	1	0	100	100
V214A	0	1	0	100
N236T	3	0	100	100
Total	16	1	93.75	100

^a Patients with chronic HBV infection.

^b Chronic asymptomatic carriers.

Table 4. Characteristics of patients with chronic HBV

	HBeAg-positive (n = 9)	HBeAg-negative (n = 19)
Men, n (%)	6 (66.6)	13 (68.4)
Mean median age, years (range)	41.22 (14–58)	42.94 (20–63)
HBV-DNA level (log copies/ml)	5.74	4.92
HBV genotype:		
Genotype A	3	4
Genotype B	0	1
Genotype C	1	1
Genotype D	5	8
Genotype E	0	2
Genotype F	0	2
Genotype A/F	0	1
Fibrosis	2	4

quence analysis, detecting the HBV genotype and primary and secondary mutations based in stored reference sequences at the same time.

Genotypes may result from evolutionary drift of the viral genome, from recombination or as a consequence of a long-term adaptation of the virus to genetic determinants of specific host populations. HBV genotyping is an important tool within the diagnostic clinical laboratory. There are many published data showing differences between the clinical response of the infection and implicated genotypes. In this way, patients infected with A or B genotypes usually have a better clinical response than patients infected with C or D genotypes [14]. Moreover,

long-term therapy using nucleoside/tide analogs is also associated with selection of HBV polymerase mutants and the emergence of resistance and it could be a prognostic marker of viral breakthrough [15].

Several technologies are available for identification of HBV genotypes and drug-resistance mutations, but these techniques must be easy and rapid to perform in order to apply them in the laboratory. In our study, both assays were simple and adequate for routine use in a clinical laboratory. Genotype coinfection has been considered a rare event, probably due to suppression of the replication of the second strain, or being hidden by the very high virus load of the first strain, but this information may be valuable in guiding treatment regimens [16–18]. Furthermore, genotype coinfection could raise the possibility of recombination between genotypes. In this study group, there were 5 patients with dual coinfection.

Using INNO-LIPA and TRUGENE assays, we detected different genotypes. Genotypes D and A were the most prevalent (46 and 26%) but others such as E (8%), C (4%), F (4%) and B (2%) were also detected principally in immigrant populations, showing the circulating of unusual HBV genotypes in our population. These findings are in accordance with the specific geographical distribution of HBV genotypes [3, 19, 20], but the presence of unusual HBV genotypes in our area should be monitored in the future for differences in epidemiology or response to antiviral therapy.

Both assays were also able to detect drug-resistance mutations. These mutations could be detected in patients treated with nucleoside/tide analogs but also in an asymptomatic carrier, indicating that patients without treatment would be examined for drug-resistance mutations stage. Moreover, in our study, TRUGENE detected 1 more mutation than INNO-LIPA (V214A) and can also

detect secondary mutations such as Q215S that some studies reveal can occur in chronic HBV patients even without exogenous selection pressures [21].

In general, both assays presented an excellent specificity, but TRUGENE had slightly better sensitivity in both methods than INNO-LIPA (100 vs. 99.56% on genotype detection and 100 vs. 93.75% in detection of drug-resistance mutations). Furthermore, the TRUGENE assay presented the advantage that the same sample could be used in order to detect the genotype and the drug-resistance mutations, while in the INNO-LIPA assay it was necessary to conduct two different detections. So less time was needed for the TRUGENE assay in the routine diagnostic laboratory; this assay does have the disadvantage, however, of having to prepare an acrylamide gel to carry out the sequencing procedure, which is more laborious than a line probe assay.

Different risk factors of HBV acquisition have been described [22]. In our study, unknown transmission was the most prevalent, followed by sexual and maternal transmission. Some studies indicate that genotype A is more efficiently transmitted sexually than genotype D, but that genotype D is more prevalent in intravenous drug users [23, 24]. We could not confirm this report.

In conclusion, INNO-LIPA and TRUGENE are 2 assays that are easy and suitable for detecting HBV genotypes and drug-resistance mutations and are appropriate for routine use in a laboratory. It is clear that HBV genotyping and detection of mutations have a useful place within the diagnostic repertoire of the clinical laboratory.

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