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Overestimation of Incidence of Hepatitis B Virus Mixed-Genotype Infections by Use of the New Line Probe INNO-LiPA Genotyping Assay▽

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The new version of the INNO-LiPA HBV genotyping assay (Innogenetics) developed to identify all hepatitis B virus (HBV) genotypes, A to H, has been evaluated in comparison with sequencing of PCR-amplified HBV DNA from 200 samples before or after cloning. The genotyping data obtained with INNO-LiPA were in agreement with those from direct sequencing in the 179 samples characterized by the two methods. INNO-LiPA revealed 28 mixed infections. However, sequencing after molecular cloning confirmed only 15 of them and did not identify any that were of genotype H ($n = 9$). Our study demonstrates that INNO-LiPA overestimates mixed infections as a result of erroneous genotype H detection.

The most accurate method for hepatitis B virus (HBV) genotyping (3, 6) is based on phylogenetic analysis after DNA sequencing of the entire viral genome (11, 13), often restricted to the gene S (12). However, in case of multiple-genotype infection, only the most abundant genotype is identified. Such mixed infections have been identified using a commercial assay based on reverse hybridization on strips carrying genotype-specific probes, the INNO-LiPA HBV genotyping assay (Innogenetics, Ghent, Belgium) (4, 5, 14). The aim of the study was to evaluate the reliability of a new version of INNO-LiPA HBV genotyping assay in the detection of mixed-genotype infections by comparison with sequencing of PCR-amplified HBV DNA, before and after molecular cloning, in a population of 200 HBV surface antigen (HBsAg)- and anti-HBc-HBV-DNA-positive French blood donors who were selected in accordance with the HBV genotype distribution previously described in this population (15). The INNO-LiPA was used according to the manufacturer's instructions using biotinylated primers specific for gene S. The hybridization patterns were interpreted according to the chart provided: (i) a single genotype when at least one genotype-specific probe was reactive; (ii) a mixed-genotype infection when multiple genotype-specific lines were reactive for more than one genotype; (iii) an indeterminate profile when single lines were observed for more than one genotype, except for the combination of two probes (one D probe and one F probe), which was classified as genotype H (this genotype has no specific probes); and (iv) a single genotype (the one with a complete hybridization profile) when an isolated reactivity for a second genotype was observed.

First, the ability of the INNO-LiPA to detect the minor HBV genotype in a dual mixture was evaluated by testing mixtures of two native samples of genotypes A and D, respectively. Five ratios, from 50/50 to 90/10, of genotypes A and D, respectively, were constituted and adjusted to a final viral load (VL) of 1.70×10^5 IU/ml (COBAS TaqMan HBV assay; Roche Diagnostics, Meylan, France). The native samples were added as controls at concentrations of 1.70×10^5 IU/ml and 1.70×10^4 IU/ml for genotypes A and D, respectively. All samples were analyzed by INNO-LiPA, direct sequencing, and molecular cloning as described below.

The INNO-LiPA results obtained in the 200 included samples were compared to those previously obtained by the direct sequencing of the S-PCR product (nt 108 to 552 of the S gene) (15). In the case of mixed infections or indeterminate or single-genotype profiles associated with an isolated reactivity with INNO-LiPA, sequencing after molecular cloning of PCR DNA products was performed in the TOPO-TA vector (Invitrogen, Cergy-Pontoise, France) according to the manufacturer's instructions. For each sample included, cloning was initially performed with 10 recombinant HBV S-gene-specific clones generated from S-PCR DNA products and sequenced with M13 plasmid universal primers (Eurofins MWG Operon, Ebersberg, Germany). When results were inconclusive or discordant with INNO-LiPA, data were completed with 10 additional clones and subsequently by the cloning of the INNO-LiPA-PCR DNA products. The analysis of 20 additional cloned INNO-LiPA sequences was performed to exclude a bias of PCR amplification. Sequences were analyzed as described elsewhere (15) with a set of 68 referenced HBV strains.

When tested in mixtures, both genotypes A and D were identified by INNO-LiPA when the minor genotype represented at least 20% of the total VL. Identical results were obtained by S-PCR DNA product sequencing after molecular cloning, whereas direct sequencing of the S-PCR DNA products identified only the major genotype when this genotype represented more than 70% of the mixture. Indeed, when the genotype represented less than 60% of the mixture, the geno-

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type could not be determined due to ambiguities at genotype-specific nucleotides.

Of 200 blood donors, 187 were successfully genotyped by direct sequencing and 191 by INNO-LiPA (Table 1). Of these 191 samples, 163 (85.3%) carried a single genotype, whereas 28 (14.7%) had mixed-genotype infections. Samples identified as single genotype were not always reactive to the full set of genotype-specific probes. This was especially true for genotypes B and D: none of these samples hybridized to the second B-specific probe, and only 69% hybridized to the second D-specific probe. Moreover, even though not taken into account according to the interpretation chart, additional isolated positive signals with non-A probes were observed for 10 of the 48 genotype A samples. Of 28 mixed infections (Table 1), 25 were double infections and 3 were triple infections. The most frequent double infections were D + H (n = 6), A + D (n = 4), and A + E (n = 4). Finally, eight samples had an indeterminate profile with inconclusive hybridizations (six hybridized to one D probe and one H/F probe; one sample hybridized to one C probe and one E probe; and one sample hybridized to one B probe and one D probe).

Identical results were obtained with both INNO-LiPA and direct sequencing for 179 samples when one of the genotypes identified by INNO-LiPA in mixed-genotype infections was also identified by direct sequencing (n = 22) (Table 1). Of the 13 samples that were not genotyped by direct sequencing, 6 were double-genotype infections with INNO-LiPA, 6 were single genotype, and 1 was indeterminate (Table 1). Nine samples that were not genotyped by INNO-LiPA were genotyped by direct sequencing as D (n = 6) and C (n = 1). Finally, INNO-LiPA identified genotype H in 9 samples, in association with one or more genotypes. This was not confirmed by direct sequencing.

Of the 28 INNO-LiPA mixed infections investigated by sequencing of 10 S-PCR DNA clones (Table 2), 11 (39%) were confirmed, including a complete concordance for 9 samples and a partial concordance for 2 samples. In the latter 2 samples, one additional genotype (D or B) was identified by cloning; that result was in agreement with the INNO-LiPA isolated reactivity observed in one D probe and one B probe, respectively. The 17 remaining samples showing a single genotype after investigating 10 additional S-PCR clones were analyzed after the molecular cloning of the INNO-LiPA-PCR DNA products. As shown in Table 2, the sequence analysis of 20 clones per sample revealed (i) the presence of a second minor genotype in accordance with the INNO-LiPA profiles for 4 samples and (ii) the presence of a single genotype for 13 samples. Genotype H was never identified.

Sequencing of S-DNA clones derived from the 8 INNO-LiPA indeterminate profiles identified genotype D only (n = 6) or C only (n = 1) and a mixed infection of B + D (n = 1; note that INNO-LiPA revealed in this case a hybridization with one B-specific probe and one D-specific probe).

As confirmed here, direct sequencing, as opposed to the INNO-LiPA HBV genotyping assay, is not suitable for genotype identification in cases of mixed infection. In our hands, INNO-LiPA was capable of identifying more than one genotype in a given sample as long as its VL was greater than 20% of the total VL. The INNO-LiPA assay could detect genotypes A to G, with an excellent correlation with the direct sequencing

TABLE 1. Comparison of HBV genotyping results obtained with direct sequencing and INNO-LiPA HBV genotyping assay[a]

Genotype determined by direct sequencing[b]	No. of samples with result in INNO-LiPA HBV genotyping assay																						
	Successfully genotyped (n = 191)																				IND	PCR Neg	Total
	Single genotype (n = 163)							Mixed infection (n = 28)															
	A	B	C	D	E	F	G	A + C	A + D	A + E	B + D	B+ E	C + D	D + E	D + G	D + H	F + H	A + D + E	A + D + H	B + D + H			
A	46								1	1								1	1				50
B		8																					8
C			10										1								1		12
D				61					1		1		1		1	6				1	6	1	79
E					30					1		1		3									35
F						1											1						2
CGA rec							1																1
ND	2			4				1	2	2			1								1		13
Total (n = 200)	48	8	10	65	30	1	1	1	4	4	1	1	3	3	1	6	1	1	1	1	8	1	200

[a] CGA rec, CGA recombinant genotype; ND, not determined; IND, indeterminate; PCR Neg, PCR negative.
[b] A total of 187 genotypes were identified as A to G; 13 genotypes were not determined.

TABLE 2. Results of molecular cloning in the 28 INNO-LiPA HBV mixed-infection samples

Genotype (no. of isolates)	Molecular cloning result (no. confirmed/no. tested)[a]	
	S-PCR product	INNO-LiPA-PCR product
A + C (1)	1[c]/1	NT
A + D (4)	3[d]/4	0/1
A + E (4)	1/4	2/3
B + D (1)	1/1	NT
B + E (1)	1/1	NT
C + D (3)	2/3	0/2
D + E (3)	1/3	1/1
D + G (1)	0/1	0/1
D + H (6)	0/6	0/6
F + H (1)	0/1	0/1
A + D + E (1)	1/1	NT
A + D + H (1)	0/1	0/1
B + D + H (1)	0/1	1[e]/1
Total[b]	11/28	4/17

[a] NT, not tested.
[b] Molecular cloning was initially performed in 10 S-PCR product clones in all INNO-LiPA mixed-infection samples ($n = 28$) and subsequently in 20 INNO-LiPA-PCR product clones in unconfirmed mixed-infection samples ($n = 17$).
[c] Identified as A + C + D.
[d] One sample identified as A + B + D.
[e] Identified as B + D.

analysis. Unfortunately, the detection of genotype H could not be tested due to the lack of such samples. Based on INNO-LiPA, and in accordance with a previous report (14), our study showed a 14.1% frequency (28 of 199) of mixed-genotype infections. However, this rate could have been overestimated, since the molecular cloning technique confirmed only 54% of them, even though this does not totally exclude the presence in minor amounts of an additional genotype that would have been identified by sequencing of a large number of clones or, alternatively, by using a more powerful sequencing technique, such as ultradeep pyrosequencing (8, 9, 16). Moreover, the high proportion of genotype H infection observed with INNO-LiPA and never confirmed by molecular cloning appears erroneous and has widely contributed to the overestimation of mixed infection. Indeed, genotype H is restricted to South and Central America (1, 2) and was not detected in a set of 940 French blood donors (15). If the 9 genotype H isolates are excluded, the frequency of mixed-genotype infection decreases from 14.1 to 10.6%, which is more in agreement with the 8% prevalence (7 of 93) observed in blood donors in Belgium (10). Interestingly, the proportion of mixed-genotype infection in the blood donor population, which mainly consists of asymptomatic HBV carriers, appears to be lower than those reported in patients suffering from chronic HBV infection (16% [41 of 262] [5]; 22% [23 of 103] [7]) or in intravenous drug users (16% [53 of 325] [4]). In addition to the overestimation of mixed infection, especially with regard to genotype H, INNO-LiPA probably also underestimates genotype mixtures, due to genotype-specific probe reactions which are not taken into account when isolated.

In conclusion, despite minimal significant improvement over the previous version, the modified version of INNO-LiPA remains a convenient and simple method for HBV genotyping. Our findings should convince the manufacturer to include genotype H-specific probes and to revise the interpretation criteria.

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