VIROLOGY

Hepatitis B Virus Genetic Typing Using Mass-Spectrometry

M. V. Malakhova, E. N. Ilina, V. M. Govorun, S. A. Shutko*, K. R. Dudina*, O. O. Znoyko*, E. A. Klimova*, and N. D. Iushchuk*

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 147, No. 2, pp. 181-186, February, 2009
Original article submitted April 4, 2008

Mini-sequencing with subsequent result registration using MALDI-ToF mass-spectrometry was employed for hepatitis B virus genetic typing in Russian population. This approach was employed for hepatitis B virus genetic typing in HBsAg-positive patients with chronic hepatitis B, hepatitis of combined etiology and hepatic cirrhosis and allowed to show the prevalence of D genotype (83.3%) in all groups of patients. Other hepatitis B virus genotypes: genotype A (5.9%), genotype C (3.6%), and mixed infection with D and C (7.2%) were also found in patients with chronic hepatitis B and hepatic cirrhosis. All genotypes were found in patients with chronic hepatitis B and hepatic cirrhosis. Chronic hepatitis of combined etiology was noted only in patients with genotype D. Possibility of detection of mixed infection with hepatitis B viruses of various genotypes is a distinct advantage of mini-sequencing approach over direct nucleotide sequence evaluation for hepatitis B virus genetic typing.

Key Words: *viral hepatitis B, genetic typing, mini-sequencing, mass-spectrometry*

Hepatitis B virus (HBV) contamination of population remains to be a major problem of modern healthcare. Despite of extensive use of vaccination, amount of patient remains to enlarge. Among immune escape mechanisms the emergence of mutant types might be named in first instance. Epidemiological studies demonstrated possibility of gradual displacement of wild HBV strain [4]. At east 8 genetic groups or genotypes (marked A-H) are currently defined in HBV classification. HBV genotypes possess distinct distribution. Genotypes A and D are spread worldwide, and prevail in countries of South Europe and USA; genotypes B and C are found in countries of South-East Asia. Genotype E is found in West Africa, and Genotype F was seen only in Central and South America [2]. Two HBV genotypes are described: one was named G and found in France, Germany and USA, and Genotype H was found in Nicaragua, Mexico and California [13].

Different HBV genotypes are assumed to produce different hepatic lesions. Genotype C showed to be correlated with more severe acute and chronic hepatitis (CHB) and genotype B might be associated with hepatocellular carcinoma [7]. Emergence of mutations, providing tolerance to lamivudine, is representative for genotype C [9]. Genotype A more than genotypes D and F associated with persistent biochemical remission and HBV DNA elimination [12]. Molecular-genetic investigations also showed the frequency of pre-core muta-

Scientific Research Institute of Physical-Chemical Medicine; *Moscow State Medical-Stomatological University, Moscow, Russia. ***Address for correspondence:** maja_m@mail.ru. M. V. Malakhova

0007-4888/09/1472-0220

tions and core-mutations to be associated with genotype of the virus. These mutations are most common in HBV genotypes B, D and E, and less common in HBV genotypes A, C, F and H [3]. Differences in responses to treatment consider to be possibly connected with virus genotype. Particularly, CHB patients, infected with HBV genotype A, were showed to have better response to interferon treatment [6]. These results indicate that HBV genetic typing have to become an important part of clinical studies.

HBV genetic typing is traditionally based on pre-S1/pre-S2 area nucleotide sequencing, since it is the most variable part of virus genome. This analysis allows determination of all HBV genotypes from A to H. It is no wonder that DNA sequencing provides the most accurate information concerning virus genotype. However, taking into account intrinsic HBV heterogeneity, frequent occurrence of mixed infections can be expected. And in this case sequencing approach is not longer effective.

Among methods, developed for HBV genetic typing, following ones may be emphasized: RFLP — length polymorphism determination among restriction fragments, PCR with genotype-specific primers, real-time PCR and DNA-array technology [8,14,15]. Of these only latter two belong to high-performance analytical methods and suitable for epidemiological screening. However, listed technologies, as a rule, are less accurate in comparison with direct genomic DNA nucleotide sequencing.

The subject of this work was the development of new highly-precise method for hepatitis B virus DNA genetic typing, suitable for extensive clinical studies, assessing the relationship between HBV genotype and clinical course of disease (turning into chronic infection, development of complications, *etc.*).

MATERIALS AND METHODS

Serum and blood samples, obtained from CHB patients undergoing examination or treatment in four Moscow clinics: Infection clinical hospital No. 1; Central clinical hospital N1 OAO Russian Railways; City clinical hospital of Ministry of Internal Affairs of the RF; City clinical hospital No. 20.

Before use all samples were stored at -110°C. HBV DNA was extracted using reagent DNA-express-blood (Litech) in accordance with manufacturing firm instructions.

HBV DNA fragment of *preS1*-region was amplified using three primers (Table 1). Reaction was performed in two stages in mixture, contained 66 mM Tris-HCl pH 9.0, 16.6 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 5 pmol of primers and 1 U Taq DNA polymerase (Fermentas) of final volume of 35 ìl.

5'-end phosphate group dephosphorylation of dNTP in amplified reaction mixture was made during incubation with alkaline phosphatase 0.5 U from arctic shrimps (Shrimp Alkaline Phosphatase, Fermentas) for 30 min at 37°C and for 10 min at 85°C.

Thermal cyclic mini-sequencing reaction was performed in reactive mixture: 66 mM Tris-HCl pH 9.0, 16.6 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 mM ddNTP, 20 pmol of every inner primers (Table 2) and 2 U TermiPol DNA Polymerase (Solis Biodyne) in final volume of 13 µl. Amplificated HBV DNA fragments were used as a matrix. Generation of mini-sequencing products was carried out with schedule: 94°C — 20 sec, 58°C — 20 sec, 72°C — 15 sec, 70 cycles in amplificatory DNA Engine Tetrade™ (MJ Research).

Purification of mini-sequencing products was performed using SpectroCLEAN Kit (Sequenom) according to manufacturing firm guidelines.

Sample aliquot (0.2-1.0 ìl), obtained after purification, was laid on the matrix, dried on Anchor-Chip target (400 ì, Bruker Daltonics) and derived from unsaturated solution of 3-hydroxypicolinic acid (Fluka) in 50% acetonitrile (Merck)with addition of ammonium citrate dibasic 10 g/l (Fluka), and air-dried. The vehicles used, including water (Merck), were analytic pure and predesignated for mass-spectrometry.

Spectra were obtained with MALDI-time-of-flight mass-spectrometer Microflex (Bruker Dal-

TABLE 1. Primers and amplification profiles

Step	Primer	Amplification profile
1	HBtype-f: 5'- CGGGTCACCATATTCTTGGG-3' HBtype-r3: 5'- GACTCTGCGGTATTGTGAGG-3'	94°C, 15 sec; 60°C, 10 sec; 72°C, 15 sec; 15 cycles
2	HBtype-f: 5'- CGGGTCACCATATTCTTGGG-3' HBtype-r1: 52 - AGAAAAACCCCGCCTGTAAC-32	94°C, 15 sec; 60°C, 10 sec; 94°C, 15 sec; 60°C, 10 sec; 72°C, 15 sec; 30 cycles

tonics), equipped with nitrogen laser (λ=337 nm) with impulse frequency up to 20 Hz. All measurements were performed in linear conditions with detection of positive ions. For mass-spectra registration, processing and analysis the Bruker Daltonics software was employed: flexControl 2.4 (Build 38) and flexAnalysis 2.4 (Build 11).

HBV genotype was estimated in virtue of presence of ions with distinct molecular weight (Table 2) in mass-spectra of mini-sequencing products.

Nucleotide sequence of amplified HBV DNA fragments was determined by modified Sengler method using ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit and apparatus ABI Prism® 3100 Genetic Analyzer (Applied Biosystems, Hitachi) in accordance with applied guidelines.

For comparative analysis of *preS1*-region the following HBV DNA sequences, available in Gene Bank, were employed: AJ34415, AY373429, AB116079, AB116081, AY167101, AY167098, AY518556, AB033555, AB106884, AY206393, AB112063, AF157113, AB033552, AB115418, AY167099, AY206388, AB112065, AF461363, AB111946, AJ344116, AY373430, AB116266, AB106564, AB036919, AF405706, AY090460.

Oligonucleotide primers and artificial nucleotide fragments for creation of recombinant constructions were synthesized in RPC Litech. Oligonucleotide fragments with positions for annealing and following intrinsic primer fill-in for determination of genotypes C, B, E, F, H, G were cloned into plasmid pGEM-Teasy (Promega) using restriction endonuclease BamHI and EcoRI and T4 DNA-lygases (Fermentas) according to established protocols [11]. Obtained plasmids were used for transformation of *E. coli* cells DH5a. Plasmid DNA was extracted by previously described procedure [5].

RESULTS

In virtue of performed comparative analysis of *preS1*-region sequences of known HBV genotypes was created the universal primer system, allowing amplification of all 8 HBV genotypes. Amplification was carried out in two stages using three primers (semi-nested PCR), what significantly increased the sensitivity.

Every distinct genotype (A-H) determination was carried out by mini-sequencing with subsequent reaction product detection using MALDI-ToF mass-spectrometry. The technology created is based on reaction of oligonucleotide primer enzymatic fill-in with the participation of deoxy- and dideoxy-nucleotide-triphosphate. Latter provides specific synthesis arresting (according to matrix DNA nucleotide sequence) and obtainment of nucleotide chains, different one from another in 1-4 nucleotides. Measurement of reaction product molecular weight was performed using MALDI-ToF mass-spectrometry, which currently allows to distinguish two DNA molecules with minimal dif-

TABLE 2. Primers, used for HBV genetic typing, and expectative product weights of mini-sequencing.

Genotype	Primer	Primer weight	Mixture content: dNTP/ddNTP	Product weight
A	ForA: 5'-TCTCACATCTCGTCAATCTC-3'	5963 Da		6236 Da (ddC)
C	RevC(1): 5'-TCCGAGGAATCCTGATGTGG-3'	6173 Da	dT+	6791 Da
	RevC(2): 5'-TCCGAGGAATCCTGATGTTG-3'	6148 Da	ddG+ddC	6766 Da (dT+ddG)
D	ForD: 5'-GCCAACAAGGTAGGAGCT-3'	5558 Da		5871 Da (ddG)
B	ForB-E: 5'-CAGAAATCCAGATTGGGACC-3'	6135 Da	dT+dC+ddA.	7026 Da (dT+dC+ddA)
E				6432 Da (ddA)
F	RevF-H: 5'-GTGGTGGTGTAAACCCTGG-3'	5900 Da	dA+ddG+ddC+ddT	6213 Da (ddG)
H				6486 Da (dA+ddC)
G	RevG: 5'-AGGGGTGAAACCGGGTCC-3'	5590 Da		6191 Da (dA+ddT)

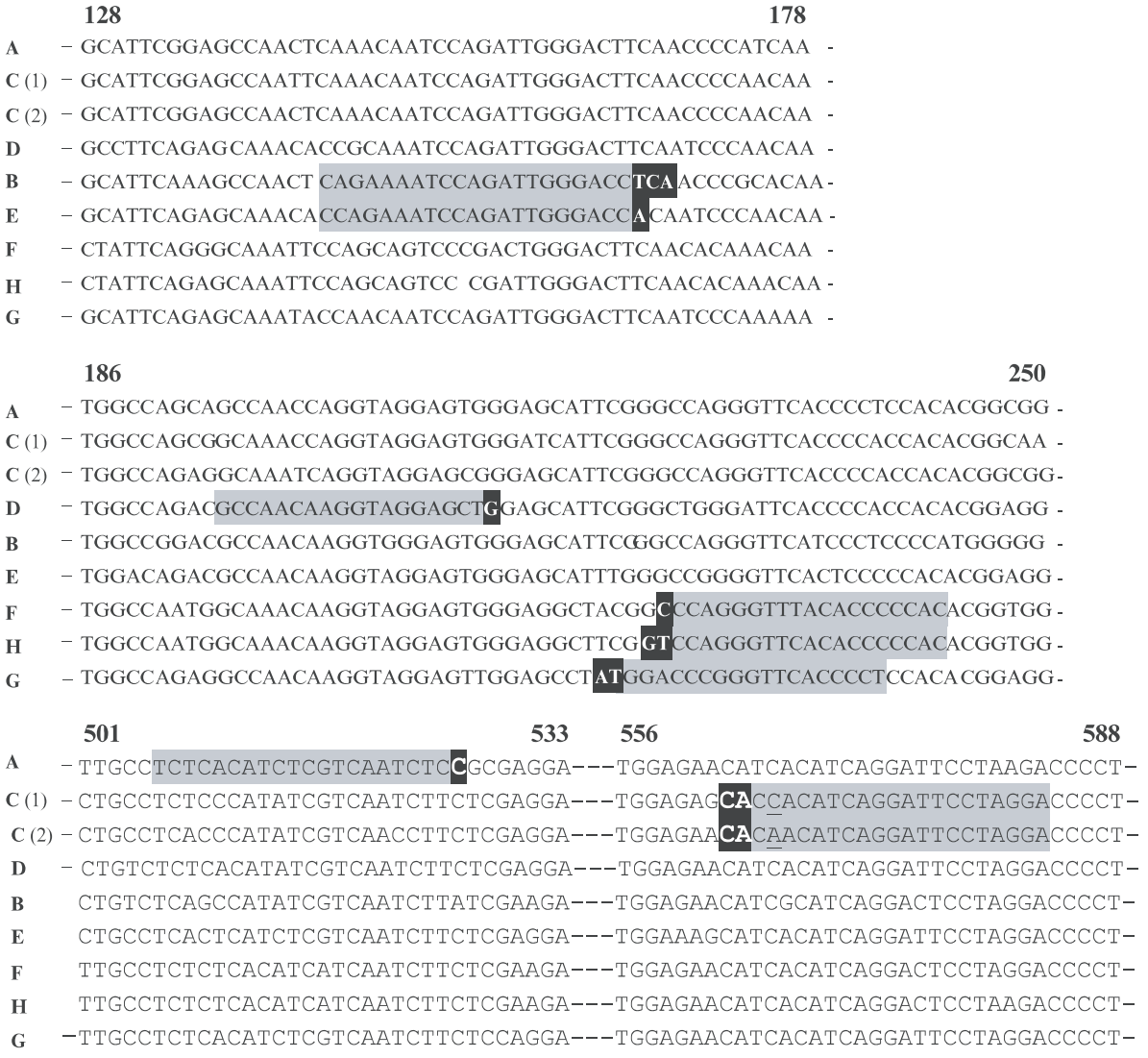

Fig. 1. Fragments of comparative analysis of HBV DNA preS1-region sequences for genotypes A-H. Positions of intrinsic primers, used for genetic typing, are marked with fray color. Nucleotides, which fill-in at the process of mini-sequencing, maked with black color.

ferences in weight of 2-4 Da. Previously this method was successfully employed for genetic typing of hepatitis C virus [1].

According to comparative analysis of HBV DNA *preS1*-region, 7 oligonucleotide primers were selected, which add on from 3'-end allows to determine genotype-specific feature of HBV DNA (Fig. 1). For simultaneous determination of A, C, and D genotypes (most widespread in Euro-Asian region), B and E genotypes and F, H, and G genotypes the details of multiprimer sequencing were optimized. As an illustration, the multiprimer sequencing system employment for determination of genotypes A, C and D is provided (Fig. 2).

This approach was employed for investigation of serum samples obtained from 154 HBsAg-positive patients: 131 (85%) with CHB, 16 (10.4%) with HBV-induced hepatic cirrhosis and 7 (4.6%) with CHB of mixed etiology. HBV DNA was obtained from 84 (54.5%) patients, among which in 70 (83.3%) was established genotype D, in 3 (3.6%) — genotype C, and in 5 (5.9%) — genotype A, for 6 (7.2%) — mixed genotype D and C. Genetic typing data was confirmed by the method of direct sequencing (except cases of mixed infection).

CHB was diagnosed in 87.4% of patients with genotype D, in 66.7% with genotype C, in 80% with genotype A and in 66.7% with genotype C and D. Hepatic cirrhosis was found in 8.6% of patients with genotype D, in 33% (1 patient) with genotype C, in 20% (1 patient) with genotype A and in 33.3% (2 patients) with genotypes C and D. Chronic viral hepatitis of mixed etiology was found only in 7 (10%) patients with genotype D.

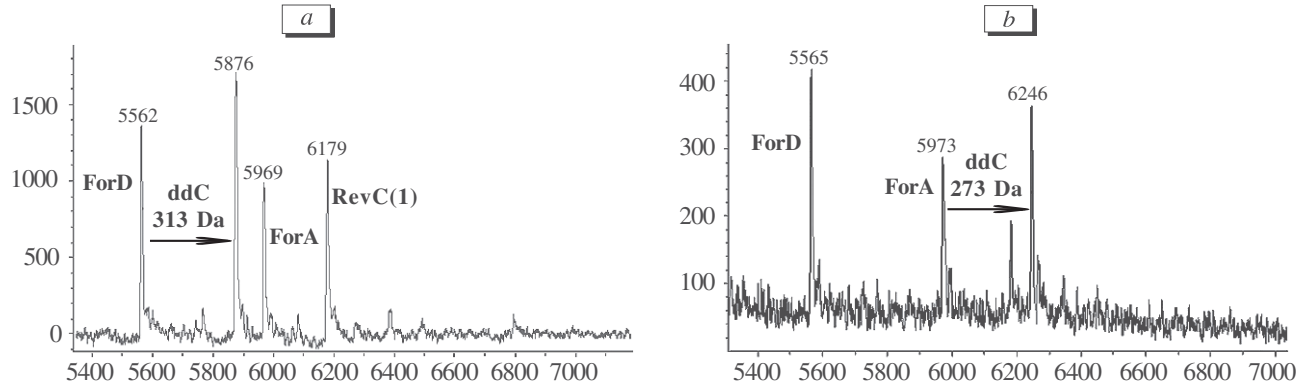

Fig. 2. Examples of mini-sequencing using multiprimer system for detection of genotypes A, C and D. The peaks marked, corresponding to primers ForA, ForD and RevC(1), employed for detection of genotypes A, D and C. *a* — example of mass-spectrum, obtained at genotype D detection; *b* — example of mass-spectrum, obtained at genotype A detection.

Since that in first clinical sample were not found HBV genotypes C, B, E, F, H, G, to confirm efficiency of all mini-sequencing systems the recombinant constructions were artificially obtained in plasmid vector pGEM. Each of them contained nucleotide fragment with positions for annealing and following intrinsic primer fill-in for determination of genotypes B, F, G, C (sequence complementary for primer C(1) and genotypes E, H, C (sequence complementary for primer C(2). These constructions may be used as matrix for mini-sequencing with intrinsic primers for detection of genotypes C, B, E, F, H, G. Using MALDI-ToF mass-spectrometry was demonstrated that fill-in of these primers is going according to context by relevant number of deoxy- and dideoxy-nucleotide-triphosphates and products with predetermined molecular weight appear.

Mini-sequencing with following registration of results with MALDI-ToF mass-spectrometry was successfully employed for HBV genotype determination in serum samples from patients with chronic hepatitis. Although this approach was performed with plates, it allows using of robotic systems to carry samples and reagents, automatic algorithms of result registration. Promoting of this method, allowing simultaneous testing of 96, 384, or 1536 samples, may significantly accelerate massive investigations, focused on monitoring of HBV circulation in our country. Mini-sequencing method possesses distinct advantages over direct nucleotide sequencing, because it allows detecting the cases of mixed HBV infection with several genotypes.

Employment of this approach for analysis of HBV genotypes in small group of CHB patients demonstrated the prevalence of genotype D (83.3%), but other HBV genotypes — genotype A (5.9%) and C (3.6%), and also cases of mixed infection with D and C (7.2%) were also found. The data obtained is a little different from the results of analogue study, where the prevalence of genotype D in Russian Federation was demonstrated, whereas over genotypes — A and C — constituted only 1% [10]. Thus, epidemiological situation had changed in few years. It can be explained by extensive migration from Yakutia, Caucasian and Asian regions, where ratio of A and C genotypes to genotype D is well above, to central regions of Russian Federation.

However, insufficient number of patients in investigated population does not permit to make statistically significant conclusions concerning associations of certain HBV genotype, detectable in our country, with clinical particularities of viral hepatitis, nature of hepatic lesions, occurrence of distinct consequences, *etc*. Further investigation with lager amount of clinical groups will permit indubitable assessment of HBV genotype magnitude as a factor, influencing the course of disease.

Authors express thanks to employee of Infection clinical hospital No. 1, Central clinical hospital No. 1 OAO Russian Railways, City clinical hospital of Ministry of Internal Affairs of the RF, City clinical hospital No. 20, to D. S. Cheshik, E. S. Vjuchnova, T. B. Penkina, I. B. Korshunov for providing of clinical material.

REFERENCES

1. E. N. Ilina, M. V. Malakhova, E. V. Generozov, *et al.*, *Biomed. Chim.*, **51**, No. 1, 41-47 (2005).
2. P. Araus-Ruiz, H. Norder, K. A. Visona, and L. O. Magnius, *J. Med. Virol.*, **51**, No. 4, 305-312 (1997).
3. H. L. Chan, M. Hussain, and A. S. Loc, *Hepatology*, **29**, No. 3, 976-984 (1999).
4. G. Francois, M. Kew, P. Van Damme, *et al.*, *Vaccine*, **19**, No. 28-29, 3799-3815 (2001).
5. D. Holmes and M. Quigley, *Anal. Biochem.*, **114**, No. 1, 193-197 (1981).

6. H. L. Janssen, M. van Zonneveld, H. Senturk, *et al.*, *Lancet.*, **365**, 123-129 (2005).
7. J. H. Kao, P. J. Chen, M. Y. Lai, and D. S. Chen, *Gastroenterology*, **118**, No. 3, 554-559 (2000).
8. M. Lindh, J. E. Gonzalez, G. Norkrans, and P. Horal, *J. Virol. Method.*, **72**, No. 2, 163-174 (1998).
9. C. M. Lo, C. K. Cheung, G. K. Lau, *et al.*, *Am. J. Transplant.*, **5**, No. 8, 1893-1900 (2005).
10. V. M. Morozov, M. M. Pisareva, and M. P. Groudinin, *Gene*, **260**, Nos. 1-2, 55-65 (2000).
11. J. Sambrook, E. F. Fritsch, and T. Maniatis, *Molecular cloning: a laboratory manual*, N. Y. (1989).
12. J. M. Sanchez-Tapias, J. Costa, A. Mas, *et al.*, *Gastroenterology*, **123**, No. 6, 1848-1856 (2002).
13. L. Stuyver, S. De Gendt, C. Van Geyt, *et al.*, *J. Gen. Virol.*, **81**, Pt. 1, 67-74 (2000).
14. S. Payungporn, P. Tangkijvanich, P. Jantaradsamee, *et al.*, *J. Virol. Methods*, **120**, No. 2, 131-140 (2004).
15. Y. Song, E. Dai, J. Wang, *et al.*, *Mol. Cell. Probes*, **20**, No. 2, 121-127 (2006).