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Journal of Chromatography B, 844 (2006) 32–38
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Isotachophoresis preconcentration integrated microfluidic chip for highly sensitive genotyping of the hepatitis B virus

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Received 18 March 2006; accepted 27 June 2006
Available online 8 August 2006

Abstract

The genotyping of hepatitis B virus (HBV) has become recently a valuable tool not only for epidemiological reasons but also for the clinical practice. Conventional methods for HBV genotyping typically include amplification of the target DNA sequences with a two-round nested PCR followed by separation of the amplified fragments by gel electrophoresis. A microfluidic chip that couples isotachophoresis (ITP) preconcentration and zone electrophoresis (ZE) separation may provide great advantages for sensitive, rapid and cost-effective clinical analysis. In this study, an HBV genotyping method with only one amplification round was developed by the application of the ITP-ZE chip. All the analysis steps of the ITP-ZE separation including sample injection, stacking and separation were performed continuously, controlled by sequential high-voltage switching. A 2.1 cm sample plug was preconcentrated between discontinuous buffers in ITP process, followed by ZE separation. Sensitivity enhancement was obtained through the increase of sample loading volume. The average LOD value of the ITP-ZE microfluidic chip was determined to be 0.0021 pg/μL. In a large-scale HBV genotyping test, single round PCR products were analyzed by ITP-ZE microfluidic chip, and the results were compared with that of the conventional method. Among the 200 cases studied, the classification rate obtained with microfluidic chip was 93%, which was 6% higher than that obtained with the conventional method. Method with ITP-ZE chip analysis provides HBV genotyping information in reduced PCR amplification time with higher detection rate when compared with conventional method. This method holds great potential for extrapolation to the abundance of similar molecular biology-based techniques in clinical diagnosis.

Keywords: Microfluidic chip; Sample preconcentration; Isotachophoresis; Hepatitis virus B; Genotyping

1. Introduction

Hepatitis B virus (HBV) is one of the major causative agents of acute and chronic liver disease worldwide. On the basis of current estimates by the World Health Organization, about 350 million people are carriers of this virus [1]. Carriers of HBV are at an increased risk of developing hepatocirrhosis and hepatocellular carcinoma. HBV has been classified into eight genotypes (A–H) based on the sequence divergence exceeding 8% of the entire genome [2]. As the genotype examination is established on the nucleotide sequence level, it can reflect the real situation of the genotype and genotype's variance and provide more accurate results than serological type examination does, which was established on the protein level. The identification of hepatitis virus genotype has become recently a valuable tool not only for epidemiological reasons but also for the clinical practice. HBV genotype assessment is useful to trace routes of infection, predict disease progression, response to therapy and emergence of antiviral resistance for patients with chronic hepatitis [3–6]. Evidence available suggests that there are notable distinctions in the virological and clinical characteristics of patients infected with different HBV genotypes [7–9].

Abbreviations: HBV, hepatitis virus B; LE, leading electrolyte; TE, terminating electrolyte; HPMC, hydroxyprolymethyl cellulose

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doi:10.1016/j.jchromb.2006.06.041

In recent years, measurement of the circulating HBV DNA level is regarded as the most direct and reliable means of monitoring HBV infection, and has been widely used in pre-treatment evaluation and clinical staging. Due to the varying levels of HBV present in blood, molecular assays for HBV DNA using PCR-based methods have been described in the past decade. Nested PCR was commonly used for genotyping of HBV with two-round amplification, which provided a high sensitivity for the determination of target DNA. However, the process of nested PCR is tedious and time-consuming and also requires an additional time for gel electrophoresis of PCR products.

Microfluidic technology has aroused increasing interest from biological and medical sciences in recent years and revolutionizing the methods for high throughput DNA genotyping and protein analyses as well as diagnostics by enabling the realization of integrated, reliable, low-cost and high-performance devices [10–12]. Rapid separations of DNA fragments have been reported in microfluidic chips [13]. With the development of chip integrated PCR, there is an increasing demand for microfluidic chip-based methods for fast and sensitive DNA analysis [14]. Typical microfluidic chips with cross sampling injectors (zone electrophoresis (ZE) microfluidic chip) were designed to load narrow sample plugs. The chip design provided high resolution, but negatively impacted detection sensitivity. Although detection sensitivity could be improved by increased sample loading capacity, the separation efficiency of microchip with large sampling volumes is usually insufficient [15].

Flexible microfluidic systems hold great promise for realizing multidimensional separations in a single integrated system. Several groups have developed microchip-based two-dimensional separation systems, which have integrated micellar electrokinetic chromatography (MEKC), isoelectric focusing (IEF) or isotachophoresis (ITP) with capillary electrophoresis (CE) [16–18]. One method to increase sample loading capacity, in order to improve detection sensitivity without loss of peak resolution, is to preconcentrate the sample by ITP, followed by ZE separation. In ITP process for dsDNA analysis, a long sample plug is injected between discontinuous buffers containing leading electrolyte (LE) and terminating electrolyte (TE) solution. The LE solution contains ions of a higher effective mobility than those of DNA fragments, whereas the TE solution contains ions of lower effective mobility. The separation process of ITP is based on various migration velocities of constituents in the mixed zones, and stacking of DNA can result from a difference in the field strength at different areas of the sample plug. The resulting narrow sample plug was separated without resolution loss in the followed ZE process. Thus, detection sensitivity can be improved by increase of sample loading volume with the integration of ITP and ZE. Analysis of dsDNA with the ITP preconcentration integrated microfluidic chip has been reported [19], providing a desirable platform for highly sensitive clinical DNA analysis.

Microfluidic chips possessing integrated ITP preconcentration may provide great advantages for sensitive, rapid and cost-effective clinical analysis. We describe here a novel, high sensitive assay for HBV DNA genotyping, which combines single round PCR and ITP integrated microfluidic chip analysis. This assay was applied in a large-scale genotyping of HBV, and the results were compared with that of the conventional method.

2. Materials and methods

2.1. Samples

Two hundred and twenty serum samples, collected from 107 asymptomatic HBV carriers, 56 chronic hepatitis, 37 liver cirrhosis patients and 20 healthy controls from Dalian, China, were analyzed. The patients were diagnosed using liver function testing (alanine aminotransferase levels), serological markers (HBsAg, anti-HBs, HBeAg and anti-HBe) and ultrasonography. All serum samples were stored at $-80\,^{\circ}C$ until analysis. DNA was extracted with the phenol–chloroform method [20]. The copy number of HBV DNA was determined with the LightCycler™ real-time PCR amplifier (Roche, Boehringer Mannheim, Germany), using universal commercially available kit (Daangene, Guangzhou, Guangdong, China). The level of virus copy in serum ranged from below 10 copies/μL to 10^8 copies/μL.

2.2. PCR amplification

HBV genotyping was determined with a multiplex PCR technique as previously reported, with which HBV can be classified into six genotypes (A to F) [21]. The sequences of primer are shown in Table 1. In this study, samples for ITP-ZE microfluidic chip analysis were only amplified with the genotype specific primers for 30 cycles. In parallel, conventional methods were also carried out, namely, amplification of the target DNA sequences with a nested PCR, followed by separation of the amplified fragments by gel electrophoresis. The nested PCR technique contains two PCR rounds. The first round amplification used universal outer primers for 30 cycles to generate a long amplicon which then served as target DNA in the second round of another 30 cycles. Negative controls, positive control and standard HBV template were also tested in each run. The PCR reactions were performed with a GeneAmp PCR System 2700 (Applied Biosystems, Singapore). All reagents for PCR were purchased from Takara Biotech (Dalian) Ltd.

2.3. Instrumentation

An in-house developed confocal, single-point laser-induced fluorescence (LIF) detection system was applied to the electrophoresis microchip. An air-cooled laser diode (LD)-pumped solid laser (473 nm; Bangshou, Beijing, China), operating at 10 mW, was used as the excitation source. The beam of the LD solid laser passed through a band pass filter (473 nm; Omega Optical, Brattleboro, VT, USA) and was reflected off a dichroic mirror (505 DRLP 02; Omega Optical) set at 45° to the incident beam and focused into the center of the microchannel by a 20× microscope objective (0.4 numerical aperture). The emitted fluo-

Table 1
Primer sequences used for HBV genotyping

Primer	Sequence	Position	Specificity
First round (universal)			
P1b	5′-TCA CCA TAT TCT TGG GAA CAA GA-3′	nt 2823–2845	Universal, sense
S1-2	5′-CGA ACC ACT GAA CAA ATG GC-3′	nt 685–704	Universal, antisense
Second PCR (genotype specific)			
Mix A			
B2	5′-GGC TCM AGT TCM GGA ACA GT-3′	nt 67–86	Types A to E specific, sense
BA1R	5′-CTC GCG GAG ATT GAC GAG ATG T-3′	nt 113–134	Type A specific, antisense
BB1R	5′-CAG GTT GGT GAG TGA CTG GAG A-3′	nt 324–345	Type B specific, antisense
BC1R	5′-GGT CCT AGG AAT CCT GAT GTT G-3′	nt 165–186	Type C specific, antisense
Mix B			
BD1	5′-GCC AAC AAG GTA GGA GCT-3′	nt 2979–2996	Type D specific, sense
BE1	5′-CAC CAG AAA TCC AGA TTG GGA CCA-3′	nt 2955–2978	Type E specific, sense
BF1	5′-GYT ACG GTC CAG GGT TAC CA-3′	nt 3032–3051	Type F specific, sense
B2R	5′-GGA GGC GGA TYT GCT GGC AA-3′	nt 3078–3097	Types D to F specific, antisense

rescent light from the sample was collected and collimated by the same microscope objective and focused by a 200 mm lens onto a spatial pinhole filter (400 mm). The fluorescence wavelength was spectrally filtered by a 520 nm band pass filter (Omega Optical), and detected by a photomultiplier tube (PMT; Model R212; Hamamatsu, Japan). An image Charge Coupled Device (CCD, Lanou, Shenzhen, China) was installed for observing the channels. Data acquisition and processing were carried out using an analog/digital (A/D) converter and a laptop computer. The stepper motors were responsible for positioning the detector head, allowing detection at selected channel location. The computer controlled high voltage power supply provided sequential voltage supply. The detection system and the high-voltage system were synchronized by the operation software, which also displayed electropherograms, identified peaks and calculated peak heights.

2.4. Fabrication of microchip

The layouts of the glass microfluidic chip developed for ITP-ZE separation are shown in Fig. 1. The method for fabrication of the glass chip has been described previously [22]. Channel design of the microfluidic chip was drawn with a Macromedia Freehand software. The film mask containing the channel design was prepared on a negative film using an image setter and was then transferred onto a 6.5 cm × 6.5 cm glass wafer (Changsha, Hunan, China) with positive photoresist by UV exposure. The photoresist was then developed to reveal the transferred image. The area defining the channels was exposed in this step. After serially rinsing with distilled water and acetone, the exposed area was etched with a dilute, stirred HF/HNO_3 solution at 0 °C to form channels. The etched plate was thermally bonded to a cover plate in a programmable furnace (Zhonghuan Test Elec-trical Furnace, Tianjin, China). The size of separation channel was 30 μm deep and 80 μm wide. Sample injection segment of the chip was 2.1 cm, corresponding to an approximate sample volume of 40 nL. Holes of 2 mm diameter were made at the end of channels to form sample or buffer pools.

2.5. Microchip electrophoresis

Unless otherwise noted, all chemicals were purchased from Sigma (St. Louis, MO, USA) and all the buffers were prepared with double distilled water (ddH_2O). The surface of micoflu-

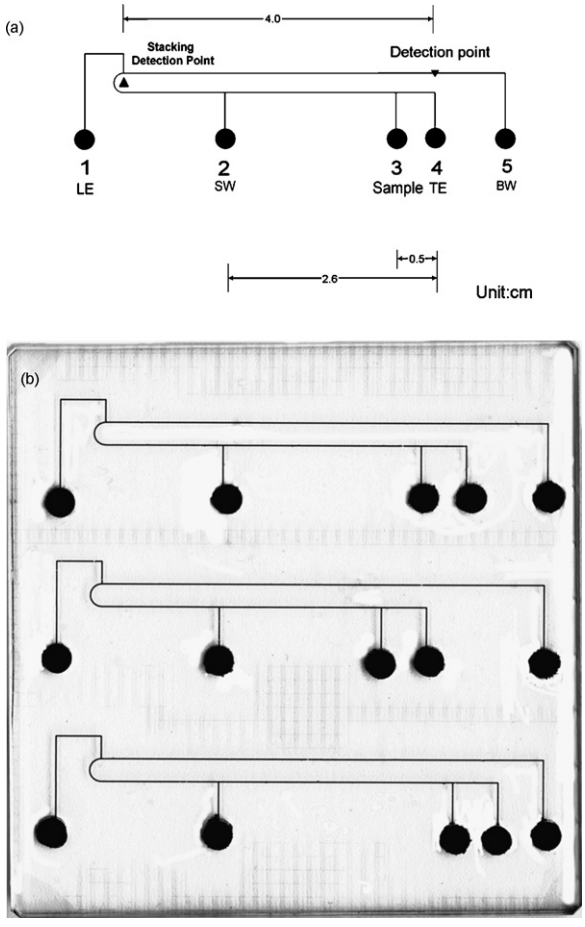

Fig. 1. (a) Layout of the ITP-ZE integrated micofluidic chip; (b) glass chip developed for ITP-ZE incorporated separation. The middle channel with a 2.1 cm sample injection segment was used in this study. Well 1–5 are reservoirs for LE buffer, sample waste (SW), sample, TE buffer and buffer waste (BW).

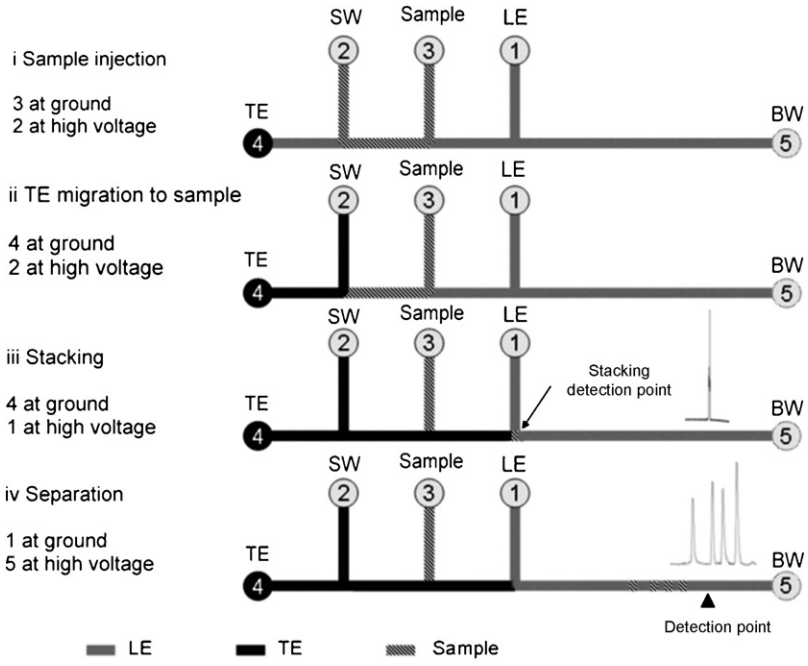

Fig. 2. Sequential steps for the ITP-ZE microfluidic chip analysis: (i) sample injection-3 at ground; 2 at high voltage, (ii) TE migration to sample-4 at ground; 2 at high voltage, (iii) stacking-4 at ground; 1 at high voltage and (iv) separation-1 at ground; 5 at high voltage. Well 1–5 are reservoirs for LE buffer, sample waste (SW), sample, TE buffer and buffer waste (BW).

idic channel was pretreated by a previous reported method [23] with some modification to reduce adsorption. The channels were first conditioned with 10 mol/L HNO_3, deionized water, 1 mol/L NaOH and ethanol each for 30 min, followed by successively flushing with silanization solution (50 μLH_2O, 1 μL acetic acid, 30 μL 3-methacryloylpropylmethoxysilane in 950 μL methanol) for 1 h, ethanol for 10 min and polymerized network solution (75 mg acrylamide, 15 mg ammonium persulfate and 1 μL *N*,*N*,*N*′,*N*′-tetramethylethylenediamine in 2 mL deionized water) for 4 h.

The LE buffer was 15 mM HCl/36 mM imidazole, pH7.0, and the TE buffer was 20 mM HEPES/40 mM imidazole, pH7.2. The LE buffer contained 1× GeneFinder™ (Biovision, Xiamen, Fujian, China) as intercalating DNA dye and a sieving polymer solution of 2% hydroxyprolymethyl cellulose (HPMC). Lower pH values could decrease the efficiency of the fluorescence detection and higher buffer pH may jeopardize the stacking efficiency because of the increase in the terminating ion mobility. Higher polymer concentration facilitated high resolution but resulted in lower DNA net-mobilities, which negatively impacted the stacking efficiency. For instance, the stacking efficiency was insufficient when LE buffer contained 2.5% HPMC was used.

In the analysis of clinical samples, single round PCR products, 0.5 μL diluted in 4.5 μL sterile ddH_2O, were analyzed with the ITP-ZE microfluidic chip. Before each run, separation channels of the microfluidic chip were washed with ddH_2O and infused with LE buffer from the buffer reservoir with a vacuum pump. According to the well number showed in Fig. 1a, the sequential steps were as follows: (i) sample injection—well 3 (sample) at ground; well 2 (sample waste, SW) at high voltage, (ii) TE migration to sample—well 4 (TE buffer) at ground; well 2 at high voltage, (iii) stacking—well 4 at ground; well 1 (LE buffer) at high voltage and (iv) separation—well 1 at ground; well 5 (buffer waste, BW) at high voltage (Fig. 2). The effective separation length was set to 40 mm. Stacking time was determined by monitoring the ITP stack plug reaching to the stacking detection point, and the determined time was added to the program for continuous operation.

In parallel tests with the conventional method, 9 μL of nested PCR product mixed with 1 μL 10× loading buffer was applied for electrophoresis on 2% slab agarose gel (6 cm × 5.6 cm × 0.8 cm), followed by 30 min separation in TAE buffer (40 mM Tris-acetate, 1 mM EDTA) under 110 V, and viewed under UV light with ethidium bromide staining.

3. Results and discussion

3.1. Effect of applied electric field strength on stacking efficiency

Stacking plugs were monitored at the stacking detection point with a series of applied electric field strengths ranging from 280 V/cm to 500 V/cm (Fig. 3a). Higher field strength facilitates fast and highly efficient stacking. In the discontinuous ITP buffer, difference in field strength exists at different areas of the sample plug. Suppose Δv and Δt denote the difference of migration velocities and retention time of DNA molecules at the beginning and the ending point of the stacking plug, respectively. If L is the effective ITP separation distance (in this test the value of L was 2.2 cm), $\Delta v = L/\Delta t$. As shown in Fig. 3b, Δv increase with applied field strength within the tested

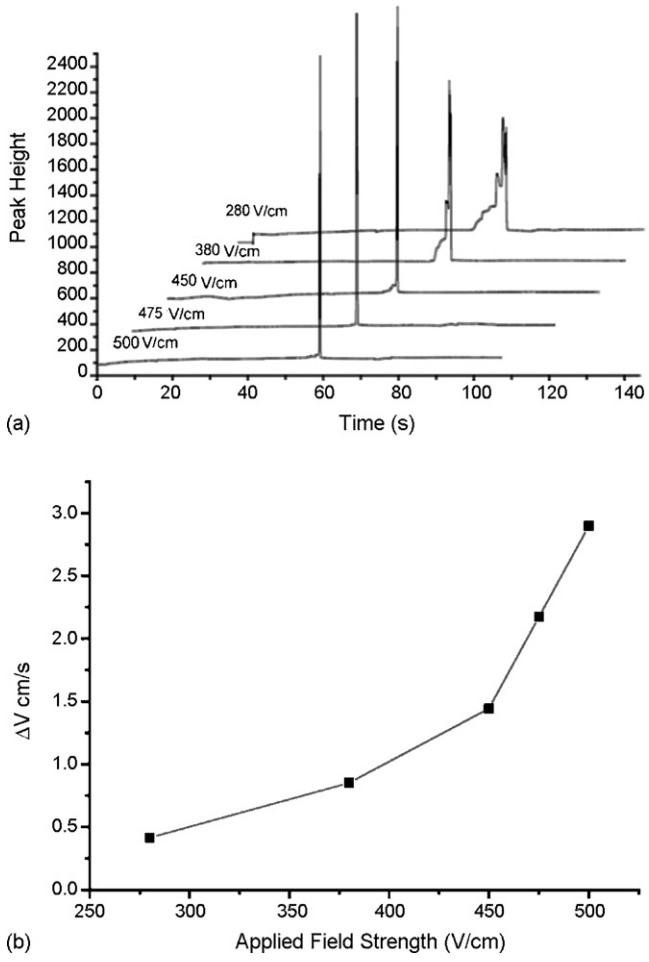

Fig. 3. Stacked plug obtained under different applied electric field strengths. (a) The applied field strength between well 1 and 4 was adjusted to 280, 380, 450, 475 and 500 V/cm, respectively. The sequential steps were as follow: 600 V/cm between well 2 and 3, 30 s for sample injection; 400 V/cm between well 3 and 4, 10 s for terminating electrolyte migration, and desired field strength was applied between well 1 and 4 for stacking. Signals were detected at the stacking detection point. (b) Δv and Δt denote the difference of migration velocities and retention time of DNA molecules at the beginning and ending points of sample zone, respectively. Within the tested voltage range, Δv increase with applied field strength.

voltage range. Higher applied field strength increased the difference of the migration velocities, which contributed to higher stacking efficiency. In this test, satisfactory stacking efficiency could be obtained with applied electric field strength higher than 475 V/cm, and 500 V/cm applied field strength was used for sample stacking in subsequent tests.

3.2. Determination of LOD of the ITP-ZE microfluidic chip

The limit of detection (LOD) of the ITP-chip was estimated prior to the analysis of clinical samples. $\phi \times 174$-*Hae*III digest (Takara Biotechnology, Dalian) with known concentration was analyzed with the ITP-ZE microfluidic chip. The original 50 ng/μL $\phi \times 174$-*Hae*III digest was diluted to 2.5 pg/μL with 0.1× PCR buffer. The average value of LOD of digested DNA on the ITP-ZE microfluidic chip was calculated to be 0.0021 pg/μL (S/N = 3). In comparison with a ZE glass chip with the same channel size, the LOD was decreased by 300-fold (0.0021 pg/μL versus 0.65 pg/μL).

In the polymeric sieving buffer, net-mobility of dsDNA fragments was lower than that of the leading ion—Cl^- and higher than that of the terminating ion—HEPES. The long sample zone was stacked into narrow plug between the leading ion and the terminating ion in the ITP process, followed by ZE separation. Thus, sensitivity enhancement could be achieved through the increase of sample loading volume without resolution loss.

3.3. Determination of clinical samples

The sequence of voltages applied for the analysis of clinical samples was: (i) 600 V/cm between well 2 and 3, 30 s for sample injection; (ii) 400 V/cm between well 3 and 4, 10 s for TE migration; (iii) 500 V/cm between well 1 and 4, 61 s for stacking; and (iv) 200 V/cm between well 5 and 1, 250 s for separation.

Expected PCR product was detected from standard HBV template and no PCR product was detected from the healthy controls on the microfluidic chip and on agarose gel electrophoresis. Among the 200 cases, the distribution of HBV genotype identified by ITP-ZE microfluidic chip was: genotype A, 2%; genotype B, 8.5%; genotype B and C, 5.5%; genotype C, 77%; unclassified 7%.

Our results show that genotypes A, B and C exist in Dalian, China. Genotype C is the major genotype in this area, which is similar to the result from other reports [24], while genotype B is the next major genotype and genotype A is rare. Mixed infection of genotype B and C also exist in this area. Genotypes D, E and F were not found in this study. The pattern of HBV genotype distribution is also in agreement with the distribution pattern of serological types in China, where the HBV adr serological type (a serological type related to genotype C) has high prevalence.

The genotyping results by microfluidic chip-based method and conventional method are compared in Table 2. The classifi-

Table 2
Comparison of the genotyping results of 200 HBV carriers obtained with the ITP-ZE chip-based method and the conventional method

Method	Genotyping results		
	Classified	Non-classified	
Microfluidic chip coupled with single round PCR	186 (93%)	14 (7%)	10 (5%) <10 copy/μL 4 (2%) >10^3 copy/μL
Gel electrophoresis coupled with nested PCR	174 (87%)	26 (13%)	10 (5%) <10 copy/μL 12 (6%) 10–10^3 copy/μL 4 (2%) >10^3 copy/μL

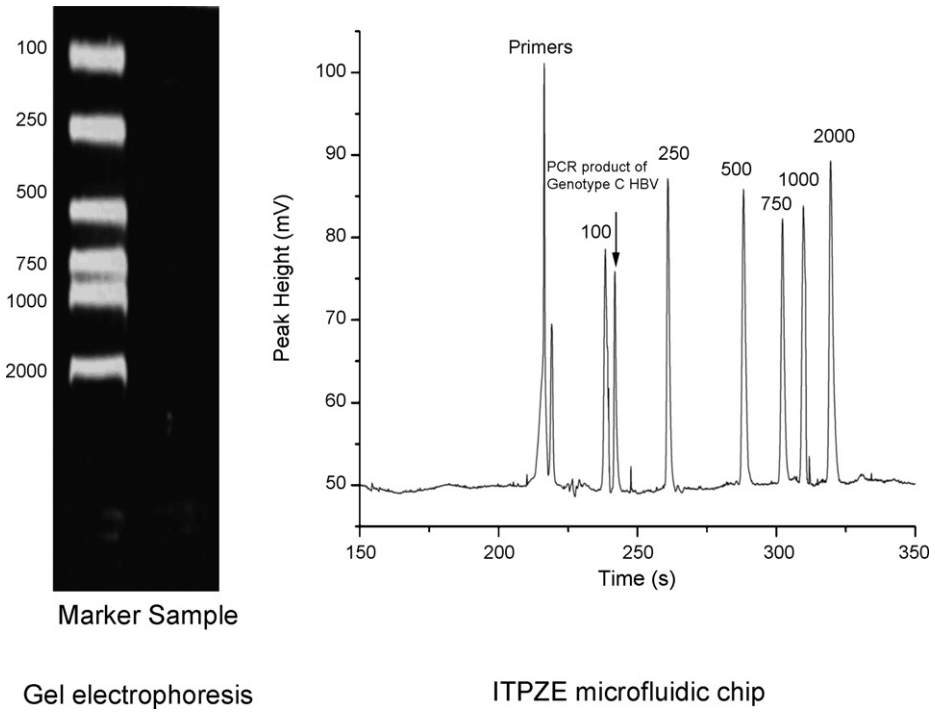

Fig. 4. Electropherograms of one case obtained with gel electrophoresis and micofluidic chip single round and two-round PCR products of a sample with 1.16×10 copy/μL HBV were analyzed with ITP-ZE chip and gel electrophoresis, respectively. The case was identified as genotype C (122 bp) with the ITP-ZE microfluidic chip but not detected with gel electrophoresis. Sample was analyzed against DL2000 DNA marker, numbers on the electropherograms corresponded the size of DNA fragments in bp.

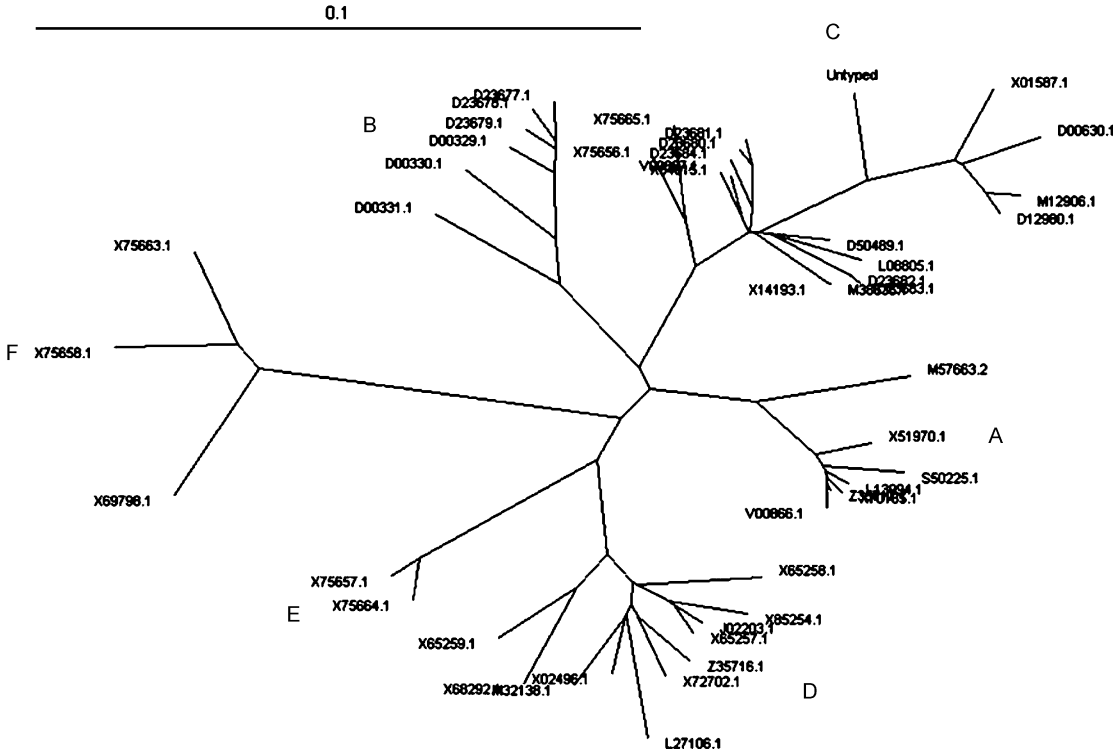

Fig. 5. Phylogenetic analysis of the case showed in Fig. 4. Genome sequenced of sample shown in Fig. 4 was aligned with HBV sequences of known genotype for phylogenetic analysis. As displayed with the treeview software, the case (marked "untyped" in the figure) was verified to be genotype C on the basis of phylogenetic analysis of pre-S region.

cation rate obtained by ITP-ZE microfluidic chip-based method was 93% (186/200), which was six percentage points higher than that obtained by the conventional method. Of the 14 cases that were not detected with the ITP-ZE microfluidic chip, 10 samples had HBV less than 10 copy per μL; the remaining four samples that had HBV copy number more than 10^3/μL, were not detected by either microfluidic chip or gel electrophoresis. This could be explained by the fact that our genotyping system is unavailable for these four cases, as the multiplex PCR-based genotyping method cannot classify all cases. Besides these 14 cases, another 12 cases with HBV copy number ranging from 10 to 10^3/μL were also not classified with the conventional method.

For HBV template higher than 10^3 copy/μL, detection rate of the microfluidic chip-based method and the conventional one is equivalent. The microfluidic chip-based method had higher detection rate for HBV template lower than 10^3 copy/μL. In asymptomatic HBV carriers or in chronic hepatitis patients receiving antivirus therapy, the level of hepatitis B virus in blood could be as low as 10 copy/μL. Genotyping method with ITP-ZE chip analysis showed the ability to detect single round amplification fragments of target HBV DNA ranging from 10 to 10^8 copy/μL. Thus, the applicability of PCR-based HBV genotyping method was enhanced using the ITP-ZE chip. For example, the electropherogram of a sample with low HBV copy (1.16×10 copy/μL) is shown in Fig. 4. The case was identified as genotype C (122 bp) by the microfluidic chip-based method but not detected with the conventional method. Genotype of the case was verified by sequencing. Multiple sequence alignment was performed to align the sequence with other HBV sequences of known genotype [25] using CLUSTAL W software [26]. The case was verified to be genotype C on the basis of phylogenetic analysis of pre-S region (Fig. 5).

4. Concluding remarks

Perhaps the greatest advantage of the microfluidic chip platform over the slab gel and the capillary formats is the potential for function integration. In this study, the ITP-ZE integrated microfluidic chip was used in large-scale genotyping of HBV and showed higher detection sensitivity than the gel electrophoresis with one PCR round fewer. In comparison with the conventional method, the microfluidic chip-based method simplified the operation with reduced amplification time and reagent consumption. Decreasing the time needed for PCR amplification has obvious implications for clinical diagnostic testing. In addition, the experiments became more convenient with fast separation and simple operation. These results, although preliminary in nature, illustrate the feasibility for this methodology in clinical diagnosis.

Acknowledgement

This project was supported by NSFC of China (Grant Nos. 20275067 and 20299035). The authors gratefully acknowledge the financial support.

References

[1] WHO 2002, Hepatitis B, www.who.int/csr/disease/hepatitis/whocdscsrlyo20022/en/, Access at 17 March 2006.
[2] S. Schaefer, J. Viral Hepat. 12 (2005) 111.
[3] A. Erhardt, D. Blondin, K. Hauck, A. Sagir, T. Kohnle, T. Heintges, D. Haussinger, Gut 54 (2005) 1009.
[4] C.H. Chen, H.L. Eng, C.M. Lee, F.Y. Kuo, S.N. Lu, C.M. Huang, H.D. Tung, C.L. Chen, C.S. Changchien, Hepatogastroenterology 51 (2004) 552.
[5] N. Akuta, H. Kumada, J. Antimicrob. Chemother. 55 (2005) 139.
[6] T. Sakai, K. Shiraki, H. Inoue, H. Okano, M. Deguchi, K. Sugimoto, S. Ohmori, K. Murata, H. Fujioka, K. Takase, Y. Tameda, T. Nakano, J. Med. Virol. 68 (2002) 175.
[7] M. Kobayashi, N. Akuta, F. Suzuki, Y. Suzuki, Y. Arase, K. Ikeda, T. Hosaka, S. Saitoh, M. Kobayashi, T. Someya, J. Sato, S. Watabiki, Y. Miyakawa, H. Kumada, J. Med. Virol. 78 (2006) 60.
[8] C.M. Chu, Y.F. Liaw, J. Hepatol. 43 (2005) 411.
[9] H.L. Chan, A.Y. Hui, M.L. Wong, A.M. Tse, L.C. Hung, V.W. Wong, J.J. Sung, Gut 53 (2004) 1494.
[10] D.J. Harrison, A Manz, Z.H. Fan, Anal. Chem. 64 (1992) 1926.
[11] R.H. Stephen, C. Jacobson, L.B. Koutny, R.J. Warmack, J.M. Ramsey, Anal. Chem. 66 (1994) 1107.
[12] A.T. Woolley, R.A. Mathies, Anal. Chem. 67 (1995) 3676.
[13] D.Y. Liu, X.M. Zhou, R.T. Zhong, N.N. Ye, G.H. Chang, W. Xiong, X.D. Mei, B.C. Lin, Talanta 68 (2006) 616.
[14] M.U. Kopp, A.J. Mello, A. Manz, Science 280 (1998) 1046.
[15] G. Hempel, Electrophoresis 21 (2000) 691.
[16] R.D. Rocklin, R.S. Ramsey, J.M. Ramsey, Anal. Chem. 72 (2000) 5244.
[17] Y.C. Wang, M.H. Choi, J. Han, Anal. Chem. 76 (2004) 4426.
[18] A. Wainright, S.J. Williams, G. Ciambrone, Q. Xue, J. Wei, D. Harris, J. Chromatogr. A 979 (2002) 69.
[19] A. Wainright, U.T. Nguyen, T. Bjornson, T.D. Boone, Electrophoresis 24 (2003) 3784.
[20] A. Kramvis, S. Bukofzer, M.C. Kew, J. Clin. Microbiol. 34 (1996) 2731.
[21] H. Naito, S. Hayashi, K. Abe, J. Clin. Microbiol. 39 (2001) 362.
[22] H. Huang, F. Xu, Z. Dai, B. Lin, Electrophoresis 26 (2005) 2254.
[23] S. Hjertén, J. Chromatogr. 347 (1985) 191.
[24] X. Ding, H. Gu, Z.H. Zhong, X. Zilong, H.T. Tran, Y. Iwaki, T.C. Li, T. Sata, K. Abe, Jpn. J. Infect. Dis. 56 (2003) 19.
[25] M. Lindh, J.E. Gonzalez, G. Norkrans, P. Horal, J. Virol. Methods 72 (1998) 163.
[26] J.D. Thompson, D.G. Higgins, T.J. Gibson, Nucleic Acids Res. 22 (1994) 4673.