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ORIGINAL ARTICLE

An improved method for the isolation of hepatitis B virus DNA from human serum

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Abstract Studies show that hepatitis B virus (HBV) DNA isolation methods vary in their efficiency to extract DNA from serum samples. The purpose of the present study was to develop an improved method for isolation of HBV DNA and compare it with commonly used HBV DNA isolation protocols. In order to develop HBV DNA isolation protocol, serum samples were collected from patients and screened for the presence of hepatitis B surface antigen, hepatitis B e antigen and HBV DNA. Highly viremic samples were pooled and used to compare commonly used HBV DNA isolation methods; namely alkaline lysis, microwave treatment, organic, inorganic with modified inorganic method. DNA isolated by these methods was detected qualitatively by polymerase chain reaction and quantitatively with competitive polymerase chain reaction (cPCR). The modified inorganic method gave maximum yield of HBV DNA followed by inorganic, organic, microwave treatment and alkaline lysis method. Our data also demonstrated a critical role of proteinase K in HBV DNA isolation. DNA isolation method described here, in combination with a reproducible and sensitive quantitative technique would further help in accurate classification of HBV infected patients, designing suitable drug regimen for treatment and monitoring antiviral treatment as well as emergence of drug resistant mutants.

Keywords HBV DNA · Isolation · Quantification · Antiviral treatment · Proteinase K

Introduction

Hepatitis B virus (HBV) belongs to family, *Hepadnaviridae*. Worldwide, more than 350 million people are estimated to be chronically infected with this pathogen that results in more than one million deaths annually [18]. Infection with HBV results in a broad spectrum of liver disease ranging from sub-clinical infection to acute, self-limited/fatal fulminant hepatitis [16]. The presence of hepatitis B surface antigen (HBsAg) in the serum/plasma indicates HBV infection. Various studies conducted to correlate levels of this antigen with HBV DNA load in the serum furnished conflicting results; some showed correlation of HBsAg levels with HBV DNA levels while others did not [8]. Conventionally, HBeAg serves as a marker for active viral replication [16]. The presence or absence of HBeAg is assumed to represent a high or low replicative state of HBV, respectively. However, mutants which do not secrete these antigens (HBsAg and HBeAg), irrespective to their rate of replication, have been reported [12].

In 1989, Kaneko et al. [14] reported that detection of HBV DNA levels in serum of chronic carriers is a better indicator of ongoing viral replication. Currently, hybridization based assays and polymerase chain reaction (PCR) [9] are used for HBV DNA detection. PCR a rapid, specific and sensitive molecular technique is successfully used in the detection of HBV DNA from clinical specimens. The sensitivity of the PCR technique depends mainly on the

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quality of DNA isolated from the pathogen present in the clinical specimen. To evaluate the reliability of HBV detection by various laboratories, a quality control study was conducted by Quint et al. [19] in which 39 laboratories participated and all, except one, used PCR to detect HBV genome. Only 10 laboratories could furnish respectable results and Heermann et al. [13] ascribed such poor performance of various laboratories partially to inefficient DNA extraction protocols used or false amplification.

Various studies suggest that HBV DNA levels are helpful in defining different states of HBV infection and monitoring response to antiviral treatment (AVT) [1, 2]. Correlation of the HBV DNA levels to the stages of histological activity and to AVT response will be important in guiding the therapeutic regimen for the individual patient including, length and dose of a particular drug or use of combination therapy [21, 22]. Furthermore, in a workshop, National Institutes of Health (NIH) suggested a 10^5 copies/ml of serum to differentiate inactive carriers from chronic hepatitis B (CHB) patients (CHB individuals show persistence presence of HBsAg for >6 months and raised alaninie aminotransferse (ALT) levels with progressive disease while asymptomatic carriers are characterized by seroconversion of HBeAg or absence of this antigen and normal ALT levels with minimal or absence of liver fibrosis). The latter forms the high risk group and AVT is recommended for such individuals, while inactive carriers form the low risk group and do not require AVT [17]. Keeping in view the importance of such studies, efficient HBV DNA extraction methods are important, as loss of DNA during its isolation may lead to misclassification of patients and accordingly will affect the AVT outcome. In this study, we present a modified inorganic method for efficient extraction of HBV DNA from human serum, and evaluate its performance in comparison with the commonly used HBV DNA isolation protocols.

Materials and methods

Samples

Ten highly viremic serum samples were collected from HBV infected patients, who were positive for HBsAg and HBeAg by commercially available kits (Abbott Laboratories, North Chicago, IL) and for HBV DNA by an in-house PCR assay [3]. All the patients or their legal representative gave informed consent. These serum samples were pooled and used for comparison of different HBV DNA extraction protocols and a pool of serum from healthy individuals lacking HBsAg, HBeAg and HBV DNA served as the negative control which was included in each batch of DNA isolation and PCR amplification.

DNA extraction methods

Microwave irradiation

HBV DNA was isolated as described by Costa et al. [5]. Briefly, 50 µl of serum was pipetted into a 1.5 ml microcentrifuge tube and was kept in the rotating platform of a microwave (1200 W, Singer, India) for 3 min till the serum was completely desiccated. Serum water condensed at the top of the centrifuge tubes was spun down by centrifugation and the clear supernatant solution was directly used for PCR amplification or stored at −20 °C for further analysis.

Alkaline lysis

This was done following Kaneko et al. [14]. Briefly, 10 µl serum was pipetted into 0.5 ml microcentirfuge tube and incubated with 1 µl of 1 M NaOH solution at 37 °C for 1 h. The mixture was centrifuged for 15 s in a tabletop microcentrifuge at 13,000 rpm. Subsequently, the solution was neutralized with HCl (final conc. 0.1 M) and the resulting solution was centrifuged and used for amplification.

Organic

Standard protocol by Sambrook and Russel [20] was followed for isolation of HBV DNA. Briefly, 50 µl of serum with 350 µl of TE buffer (pH 8.0) was incubated at 56 °C for 2 h in the presence of 1 mg/ml of proteinase K and 0.66 % SDS. The solution was cooled to room temperature and subsequently extracted with equal amount of PCI (phenol:chloroform:isoamyl alcohol::25:24:1). This step was repeated till the white precipitate at the interphase stopped appearing. Sodium acetate (Fc 0.3 M; pH 5.2) was added to the aqueous layer and the DNA was precipitated with 2.5 volumes of 95 % ethanol in the presence of 2.5 µg glycogen (Invitrogen, NY, USA) and kept at −20 °C for at least 4 h. The DNA was separated by centrifugation at 13,000 rpm for 15 min. and then washed with 70 % ethanol. Finally, the dried pellet was dissolved in 20 µl of TE buffer and stored at −20 °C till further use.

Organic without proteinase K

In this method, all steps were similar to organic method except the samples were not treated with proteinase K enzyme.

Inorganic method

The serum was extracted according to standard inorganic protocol for DNA isolation, briefly, 50 µl of serum was diluted with 350 µl of TE buffer and incubated at 56 °C for

2 h in the presence of proteinase K (1 mg/ml) and SDS (0.66 %). The solution was brought to room temperature, followed by protein precipitation by addition of NH_4OAc (Fc, 3.4 M). The mixture was vortexed and centrifuged (13,000 rpm, 15 min) to extract the DNA from the proteins. Subsequently, the DNA was precipitated and stored as described under the organic section.

Modified inorganic

Serum sample was irradiated by microwave as described by Costa et al. [5]. Subsequently, the DNA was extracted following inorganic method.

Analysis of isolated HBV DNA

Polymerase chain reaction

PCR amplification of HBV DNA was carried out according to Changotra and Sehajpal [3]. Briefly, PCR was performed in 30 µl; containing 10 µl equivalent of serum sample, 2.0 units *Taq* polymerase (Bangalore Genei, India), 150 µM dNTP mixture (dATP, dCTP, dGTP and TTP), 0.1 µM each primer (FW1; 5′-GGT ATG TTG CCC GTT TGT CC-3′; nt 460–480 and RW1; 5′-CCC AAT ACC ACA TCA TCC AT-3′; nt 760–740), 50 mM KCl, 10 mM Tris–HCl (pH 9.0), 1.5 mM $MgCl_2$ and 0.01 % (w/v) gelatin. After thoroughly mixing the contents, the reaction mixture was overlaid with ~30 µl mineral oil. After initial denaturation of PCR mixture for 3 min, the samples were amplified in a programmable thermal cycler (MJR Research Products, USA) for 35 cycles consisting of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s and extension at 72 °C for 30 s, followed with a final extension at 72 °C for 3 min. The primer pair described here amplifies a highly conserved 300 bp region of the surface antigen (S) gene of HBV genome. A positive (known sample of HBV DNA) and a negative control (sample without HBV DNA) were also processed with each batch of amplification. The amplified products were analyzed on agarose gel electrophoresis.

Competitive polymerase chain reaction (cPCR)

This method allows a known amount of a specific sequence (the competitor) to be co-amplified with the target DNA (both are of different sizes), using the same set of primers. The competitor and target DNAs are amplified in the same tube and these compete with each other for amplification components. The amplified products can be resolved on agarose gel and the point where the two DNA fragments are amplified equally (intensity of amplified bands equals) denotes the point of equivalence. This was carried out according to Changotra and Sehajpal [4]. The latter includes the detailed strategy for generation of competitor molecule. Briefly, for synthesis of competitor molecule, a forward primer was designed (modified-FW1) which had sequence of FW1 at its 5′ end and its 3′ end 12 nucleotide sequence was complementary to the HBV DNA template at nt 550–562 of S gene (5′-FW1-CTA TGT TTC CCT-3′). PCR with the modified-FW1 and RW1 primer pair resulted in the amplification of 230 bp competitor molecules with the sequences of FW1 and RW1 at its flanking regions and this was used in the cPCR. PCR with this primer pair amplified both target DNA and competitor molecule of 300 and 230 bp, respectively, when co-amplified. Briefly, for quantification of HBV DNA from the sample, a constant amount of HBV DNA was co-amplified with known dilution series of competitor DNA construct. Amplification was performed in a volume of 30 µl containing 3U of *Taq* polymerase (Bangalore Genei, India), 200 µM of dNTPs mixture (dATP, dCTP, dGTP and dTTP), 0.2 µM of each primer (FW1 and RW1), 50 mM KCl, 10 mM Tris–HCl (pH 9.0), 1.5 mM $MgCl_2$ and 0.01 % (w/v) gelatin. The DNA sample and competitor were added in the last. After mixing thoroughly the contents of the tube, it was overlaid by ~30 µl of mineral oil. The amplification was performed in a programmable thermal cycler (MJR Research Products, USA). After initial 3 min denaturation of samples at 94 °C, 35 cycles of denaturation of samples at 94 °C for 30 s, annealing at 57 °C for 30 s and extension at 72 °C for 30 s was done, with final extension at 72 °C for 3 min. This results in generation of two products (300 bp corresponding to target, and 230 bp corresponding to competitor) and these were analyzed by agarose gel electrophoresis for calculation of amount of unknown HBV DNA from the point of equivalence. Serum used for HBV DNA isolation in microwave treatment and alkaline lysis methods was less, so, this was finally converted to 1 ml equivalent of serum for comparison of HBV DNA extracted with the other methods.

Statistical analysis

Statistical analysis was carried out using the SPSS ver. 7.5 for windows software (SPSS Inc., Chicago, IL, USA). The HBV DNA levels are presented as the mean ± SD. The differences between the groups were analyzed using one way analysis of variance (ANOVA). Results were considered statistically significant at $P < 0.05$.

Results

Qualitative detection of HBV DNA by PCR

HBV DNA isolated by different DNA extraction methods was subjected to amplification by PCR as described.

Figure 1a shows a representative agarose gel electrophoresis of PCR products amplified from HBV DNA isolated employing various methods, showing that the intensity of amplified PCR products in lanes 3, 5 and 6 were comparable and relatively higher as compared to amplified products in lanes 1, 2 and 4. Densitometric analysis of the gel picture (Fig. 1b) revealed that modified inorganic method gave maximum intensity of the expected size PCR products, since the intensity of the amplified product corresponds with the initial amount of DNA in the reaction mixture before amplification i.e. amount of HBV DNA extracted in this case.

HBV DNA yield obtained using modified inorganic method was highest followed by inorganic, organic with proteinase K, organic without proteinase K, microwave treatment and alkaline lysis method. In case of alkaline lysis method, a smear in addition to the expected size product, was also visible that could be due the presence of serum components that are not removed/inactivated in this protocol, as the later involves only the lysis and neutralization of the serum components but not the removal. However, all other protocols gave clean band of expected size with variable intensities (Fig. 1).

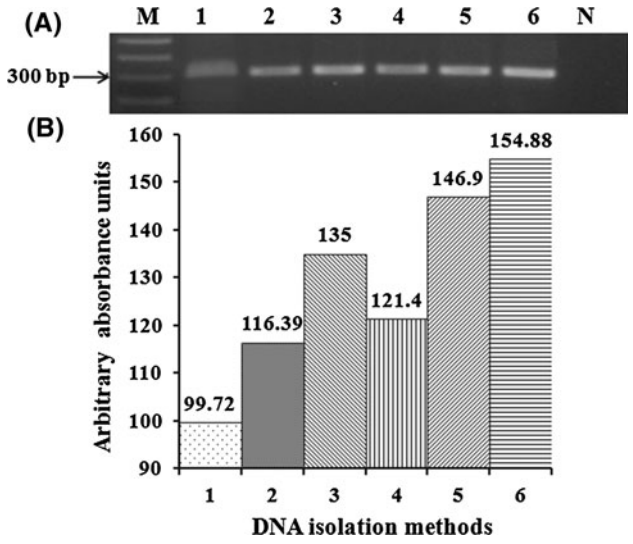

Fig. 1 **a** Representative agarose gel electrophoresis picture showing PCR amplified HBV DNA isolated employing different protocols. *Lanes M & N*, respectively represent 100 bp ladder and negative control. *Lanes 1 through 6* show PCR amplified HBV DNA isolated employing alkaline lysis, microwave irradiation, organic with proteinase K, organic without proteinase K, inorganic and modified inorganic protocols, respectively. **b** Densitometric analysis (arbitrary absorbance unit vs DNA isolation method) of amplified PCR product. *Lane's* assignment is same as in (**a**)

Quantitative detection of HBV DNA by cPCR

Isolated HBV DNA was quantified by cPCR to determine the yield obtained using various methods. Yield of HBV DNA (average amount of HBV DNA isolated in three different extractions) obtained per ml of the serum is shown in the Table 1. It is evident that the modified inorganic method gives the maximum yield of HBV DNA (mean ± SD; 991.00 ± 59.70 ng/ml of the serum, equivalent to 3.92E + 12 ± 2.36E + 11 copies/ml) while it is least in case of alkaline lysis method (0.65 ± 0.24 ng/ml of the serum, equivalent to 2.56E + 9 ± 9.63E + 8 copies/ml). With this assay also, we observed the same order of the DNA quantity as in case of PCR method from the respective isolation method. Statistical significant difference ($P < 0.05$) was observed between the extraction efficiencies of these protocols. HBV DNA isolated by these protocols was also quantified spectrophotometrically (data not shown) and the order of yield was similar to that as in case of cPCR except that alkaline lysis yielded better than microwave.

Discussion

Globally, HBV infection is a major health problem and HBsAg carrier rate in the general population ranges from 2 to 20 % [18]. The term carrier is very important because these are the reservoirs of the infectious agent and are themselves prone to develop severe liver diseases. Diagnosis of hepatitis B is based on serological as well as DNA based methods. HBV DNA is a reliable and better marker of viral replication and infectivity as compared to serological indicators [14].

Various molecular biology techniques have been developed for the detection of HBV DNA, PCR being the most sensitive can detect as little as 10 HBV genomes per ml of the serum. Therefore, PCR is the preferred test for detection of HBV DNA sequences in the clinical specimens for diagnostic purposes [14]. However, the efficacy of PCR amplification may be affected by the presence of number of *Taq* polymerase inhibitors in the serum [7, 23] and thus DNA extraction is required before amplification.

In the present study, we compared five HBV DNA isolation protocols with a modified inorganic method. PCR and cPCR, respectively, were used for detection and quantification of the isolated HBV DNA. We obtained concordant results with both the methods. Table 1 shows the yield of DNA isolated in different protocols by cPCR, clearly alkaline lysis method gave lowest yield (0.65 ± 0.24 ng/ml of the serum) of DNA while it was maximum (991.00 ± 59.70 ng/ml of the serum) with modified inorganic method.

Table 1 HBV DNA quantified using cPCR assay from the equivalent volume of same viremic HBsAg positive sample employing six different protocols

DNA extraction method	Amount of HBV DNA isolated from the equivalent amount of serum (ng/ml) ± SD[a]	Amount of HBV DNA in gE/ml of the serum ± SD[b]
1. Alkaline lysis	0.65 ± 0.24	2.56E + 9 ± 9.63E + 8
2. Microwave treatment	11.60 ± 0.21	4.60E + 10 ± 8.54E + 8
3. Organic (with proteinase K)	80.50 ± 24.90	3.18E + 11 ± 9.80E + 10
4. Organic (without proteinase K)	17.10 ± 13.00	6.75E + 10 ± 5.13E + 10
5. Inorganic	263.83 ± 97.39	1.04E + 12 ± 3.85E + 11
6. Modified inorganic	991.00 ± 59.70	3.92E + 12 ± 2.36E + 11

P value < 0.05, ANOVA for these methods

[a] Shows the average amount of HBV DNA isolated three times and quantified each time

[b] Amount of HBV DNA quantified in genome equivalents (gE)

Alkaline lysis and organic method are two most frequently used methods for HBV DNA isolation; the former is convenient, as it does not require multiple steps and can be accomplished in a single microfuge tube. With alkaline lysis method, we got lower yield of DNA compared to organic method, a finding supported by Costa et al. [5] but is contrary to the observation of Kaneko et al. [14] who reported slightly better results using alkaline lysis method. Interestingly, these results were opposite when we quantified HBV DNA spectrophotometrically (alkaline lysis method gave more DNA yield compared to organic protocol, data not shown). This may be due to (1) shearing of DNA in alkaline lysis protocol could lead to increased absorbance, thus giving erroneous quantification results, (2) presence of *Taq* polymerase inhibitors in case of DNA isolated by alkaline lysis method which adversely affects the PCR protocol.

Organic method avoids use of expensive enzymes to separate proteins from nucleic acids, as buffered phenol has capability of separating the two, with DNA going into aqueous phase and proteins in the interphase. Gerlich and Ribinson [10] reported that virion derived HBV DNA contains a covalently linked protein to the 5′ end of the long strand of the HBV genome. Consequently, upon phenol–chloroform treatment alone, HBV DNA will be linked with the protein and go to the organic phase, thus leading to inefficient extraction of HBV DNA. However, upon proteinase K treatment, the protein gets separated from the HBV genome and results in efficient separation of the HBV DNA into the aqueous phase. Similar observations have also been made by other workers [6]. In addition to this, when Kramvis et al. [15] compared QIA Amp Blood Kit and GeneReleaser with phonol:chloroform (organic) method, the later was found to be most sensitive extraction method. However, Gobbers et al. [11] reported high sensitivity without involving proteinase K treatment; who extracted HBV DNA by guanidinium thiocyanate. When we compared organic HBV DNA isolation protocols with and without proteinase K to analyze the effect of later, a stark difference in HBV DNA yield was observed. On addition of protease, the yield of extracted DNA was increased to approximately five times than the protocol without involving proteinase K (difference was ~100 times in case of inorganic protocols with and without proteinase K, data not shown). In the modified inorganic method, a step involving microwave irradiation was added before processing the serum sample following inorganic protocol. Microwave irradiation somehow inactivates the *Taq* DNA polymerase inhibitors and creates unfavorable energy conditions for HBV DNA and 5′-linked protein resulting in their dissociation hence, increased yield [5].

The yield after inorganic protocols (inorganic and modified inorganic) was much higher as compared to organic ones (with and without proteinase K). Moreover, organic method is complicated, uses hazardous chemicals and there are chances of loss of DNA at interphase of organic and aqueous phases as evident from the data. In addition to this, when we compared averages of the HBV DNA isolated and quantified following different protocols, a statistically significant difference ($P < 0.05$) was observed between the extraction efficiencies of these methods. This apparently demonstrates that minor variations incorporated in the individual protocols for HBV DNA detection method could lead to significant variation in the results. This could be one of the reasons for poor performance of different laboratories [13] in the quality control study conducted by Quint et al. [19].

In summary, new modified inorganic HBV DNA extraction method gives maximum yield of DNA and the data of this study suggest that proteinase K plays a pivotal role in HBV DNA extraction. DNA isolation method described here, in combination with a reproducible and

sensitive quantitative technique would further help in accurate classification of HBV infected patients, designing suitable drug regimen for treatment and monitoring AVT as well as emergence of drug resistant mutants.

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References

1. Buti M, Sanchez F, Cotrina M. Quantitative hepatitis B virus DNA testing for the early prediction of the maintenance of response during lamivudine therapy in patients with chronic hepatitis B. J Infect Dis. 2001;183:1277–80.
2. Changotra H, Dwivedi A, Nayyar AK, Sehajpal PK. Diagnosing different stages of hepatitis B infection using a competitive polymerase chain reaction assay. Indian J Med Microbiol. 2008;26:138–42.
3. Changotra H, Sehajpal PK. Development of S-gene based PCR for detection of hepatitis B virus. Indian J Vet Pathol. 2004;28:4–6.
4. Changotra H, Sehajpal PK. Quantitative detection of serum HBV DNA levels employing a new S gene based cPCR assay. Arch Virol. 2005;150:481–91.
5. Costa J, Lopez-Labrador FX, Sanchez-Tapias JM, Mas A, Vilella A, Olmedo E, et al. Microwave treatment of serum facilitates detection of hepatitis B virus DNA by the polymerase chain reaction. Results of a study in anti-HBe positive chronic hepatitis B. J Hepatol. 1995;22:35–42.
6. Drosten C, Weber M, Seifried E, Roth WK. Evaluation of a new PCR assay with competitive internal control sequence for blood donor screening. Transfusion. 2000;40:718–24.
7. Frickhofen N, Young NS. A rapid method of sample preparation for detection of DNA viruses in human serum by polymerase chain reaction. J Virol Methods. 1991;35:65–72.
8. Ganji A, Esmaeilzadeh A, Ghafarzadegan K, Helalat H, Rafatpanah H, Mokhtarifar A. Correlation between HBsAg quantitative assay results and HBV DNA levels in chronic HBV. Hepat Mon. 2011;11:342–5.
9. Garbuglia AR, Angeletti C, Lauria FN, Zaccaro P, Cocca M, Pisciotta M, et al. Comparison of Versant HBV DNA 3.0 and COBAS AmpliPrep-COBAS TaqMan assays for hepatitis B DNA quantitation: possible clinical implications. J Virol Methods. 2007;146:274–80.
10. Gerlich WH, Robinson WS. Hepatitis B virus contains protein attached to the 5′ terminus of its complete DNA strand. Cell. 1980;21:801–9.
11. Gobbers E, Oosterlaken TA, van Bussel MJ, Melsert R, Kroes AC, Claas EC. Efficient extraction of virus DNA by Nuclisens Extractor allows sensitive detection of hepatitis B virus by PCR. J Clin Microbiol. 2001;39:4339–43.
12. Hawkins AE, Gilson RJ, Gilbert N, Wreghitt TG, Gray JJ, Ahlers-de Boer I, et al. Hepatitis B virus surface mutations associated with infection after liver transplantation. J Hepatol. 1996;24:8–14.
13. Heermann KH, Gerlich WH, Chudy M, Schaefer S, Thomssen R. Quantitative detection of hepatitis B virus DNA in two international reference plasma preparations. Eurohep Pathobiology Group. J Clin Microbiol. 1999;37:68–73.
14. Kaneko S, Feinstone SM, Miller RH. Rapid and sensitive method for the detection of serum hepatitis B virus DNA using the polymerase chain reaction technique. J Clin Microbiol. 1989;27:1930–3.
15. Kramvis A, Bukofzer S, Kew MC. Comparison of hepatitis B virus DNA extractions from serum by the QIAamp blood kit, GeneReleaser, and the phenol-chloroform method. J Clin Microbiol. 1996;34:2731–3.
16. Lee WM. Hepatitis B virus infection. N Engl J Med. 1997;337:1733–45.
17. Lok AS, Heathcote EJ, Hoofnagle JH. Management of hepatitis B: 2000—summary of a workshop. Gastroenterology. 2001;120:1828–53.
18. Parkin DM. The global health burden of infection-associated cancers in the year 2002. Int J Cancer. 2006;118:3030–44.
19. Quint WG, Heijtink RA, Schirm J, Gerlich WH, Niesters HG. Reliability of methods for hepatitis B virus DNA detection. J Clin Microbiol. 1995;33:225–8.
20. Sambrook J, Russell DW. Molecular cloning. A laboratory manual. 3rd ed. Cold Spring Harbor: Cold Spring Harbor Laboratory Press; 2001.
21. Wong DK, Yuen MF, Tse E. Detection of intrahepatic hepatitis B virus DNA and correlation with hepatic necroinflammation and fibrosis. J Clin Microbiol. 2004;42:3920–4.
22. Yuan HJ, Yuen MF, Ka-Ho WD, Sum SM, Sablon E, IO NG, et al. Impact of precore and core promoter mutations on hepatic histology in patients with chronic hepatitis B. Aliment Pharmacol Ther. 2005;22:301–7.
23. Zeldis JB, Lee JH, Mamish D, Finegold DJ, Sircar R, Ling Q, et al. Direct method for detecting small quantities of hepatitis B virus DNA in serum and plasma using the polymerase chain reaction. J Clin Invest. 1989;84:1503–8.