

Comparison of PCR-based genotyping methods for Hepatitis B Virus

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Abstract

Hepatitis B virus (HBV) is classified into eight genotypes (A to H). In this study, three genotyping methods were compared for their sensitivity and accuracy, namely PCR-RFLP on the S region, PCR-RFLP on the pre-S region and nested PCR with type specific primers. Sixty HBV samples from infected sera were genotyped. The nested PCR with type specific primers was found to be the most sensitive and produced substantial numbers of co-infections by genotypes B and C. The sensitivities for both PCR-RFLP methods were lower and did not reveal co-infections. Generally, the three methods produced consistent genotyping results in samples infected by single genotypes but for co-infections by genotypes B and C, the two PCR-RFLP methods yielded only single genotypic results. For the cases of single genotypic infections, genotypes ascertained by sequencing were in concordance across all three methods. However, when co-infections occurred, PCR analysis on clones revealed only single genotypic infections.

Key words: HBV, Genotypes, Polymerase Chain Reaction, Sequencing

INTRODUCTION

Hepatitis B virus (HBV) is a DNA virus that specifically infects the liver tissue. Infection by HBV could cause acute and chronic hepatitis and has been associated with liver cancer development. HBV is categorized into eight genotypes (A to H). Okamoto *et al.*¹ first introduced the HBV genotyping system with four genotypic groups (A to D) based on 8 % threshold divergence between the genomes of HBV. Currently, eight genotypes are recognized for HBV which include genotypes E, F, G and H.^{2,3,4}

HBV genotypes are found to be geographically distributed. In Northern Europe and Northern America, genotype A is the main genotype. HBV carriers in East Asia regions including China, Japan and South-East Asia are predominantly infected by genotypes B and C.⁵ As for genotype D, it is found mostly in the Mediterranean region. Genotype E is found mainly in parts of Africa.⁶ As for genotype F, it is found so far exclusively in Central and South America.⁷ Genotype G is found in the USA and France while Genotype H is found in the Central America.^{2,4}

Different HBV genotypes could cause varying severities of liver damage. Kao *et al.*⁸ found that genotype C is associated with more severe liver damage while genotype B is linked with development of liver cancer in young HBV carriers. In the Indian sub-continent, genotype D is more associated with severe form of liver disease when compared with genotype A.⁹

As more information on clinical implications associated with different HBV genotypes are being reported, genotyping of HBV becomes increasingly more important. Knowledge on the genotypes of HBV in infected patients could facilitate better diagnosis and more efficient treatment. The most accurate way of genotyping is through direct sequencing but is expensive and impractical.¹⁰ Therefore various genotyping methods such as PCR-RFLP (restriction fragment length polymorphism) methods,^{11,12} nested PCR with type specific primers¹³ and genotype specific probe assay¹⁴ have been developed to facilitate genotyping of HBV.

The genotyping methods based on PCR are popular due to their simplicity and cost efficiency. However, the high variability of the HBV genome and the still many unidentified sequences of HBV

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in various regions might complicate the process of HBV genotyping by the PCR methods. Besides that, co-infections by two HBV genotypes in single individuals reported in various papers have made genotyping by PCR-based methods a more challenging task.¹⁵⁻¹⁸ Thus, the aim of this study was to evaluate the sensitivity and accuracy of three PCR-based genotyping methods, namely the PCR-RFLP on the S region,¹¹ PCR-RFLP on the pre-S region¹² and genotyping using nested PCR with type specific primers.¹³ These methods were designed specifically to detect genotypes A to F. Verification of these results were performed through sequencing of the cloned PCR products and comparison of the sequences with established genotypes deposited in GenBank.

MATERIALS AND METHODS

Samples

Sera from 60 patients tested positive for hepatitis B surface antigen (HBsAg) using AxSYM HBsAg V2® (Abbott Laboratories, North Chicago, IL) immunoassay kit in the Abbott AxSYM® System (Abbott Laboratories, North Chicago, IL) automated blood analyzer were used in this study. Consents for study were obtained from the patients beforehand. Samples were stored at -70 °C until required.

Concentration of HBV DNA

To obtain better sensitivity, HBV DNA in the sera samples were concentrated at medium drying rate for 2 hours using the Integrated SpeedVac™ System (ISS-110) (Savant Technologies, Powys, UK). The sera were concentrated to 400 µL from an initial volume of 1.4 mL. Lipid or protein was separated from the concentrated sera by centrifuging at 20000 g for 10 minutes. HBV DNA samples were extracted from 200 µL concentrated sera using High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Mannheim, Germany).

Genotyping by PCR-RFLP on the S region

Primers designed as well as the PCR profile developed by Lindh *et al.*¹¹ were used to amplify the S region of the HBV genome using PCR Reagent System (Invitrogen, Carlsbad, CA). PCR products were digested with *Hinf*I (New England Biolabs, Ipswich, MA) and *Tsp*509I (New England Biolabs, Ipswich, MA) to produce RFLP patterns. The patterns were compared with patterns of known genotypes (A to F) as observed by Lindh *et al.*¹¹

Genotyping by PCR-RFLP on the pre-S region
PCR amplification of the pre-S region was done by utilizing primers designed by Lindh *et al.*¹² with PCR Reagent System (Invitrogen, Carlsbad, CA). Amplifications of the HBV DNA were performed according to the method as described by Lindh *et al.*¹² The PCR products obtained were subsequently digested separately with *Ava*II (New England Biolabs, Ipswich, MA) and *Dpn*II (New England Biolabs, Ipswich, MA) to produce RFLP patterns. These were compared with patterns of established genotypes (A to F) as observed by Lindh *et al.*¹²

Genotyping by nested PCR with type specific primers

Genotyping with this technique utilized the two primer sets as described by Naito *et al.*¹³ In the first round of PCR amplification, the first set of primers amplified the region around pre-S and S regions of HBV genome. Subsequent amplification of the initial PCR products with the second set of primers consisting of 6 pairs of primers would produce PCR products of six possible sizes, each corresponding to one of six genotypes (A to F). PCR Reagent System (Invitrogen, Carlsbad, CA) was used in this procedure. No changes were made to the PCR profile as described by Naito *et al.*¹³

Cloning of PCR products

PCR products amplified by either the technique developed by Lindh *et al.*¹² or the first round of nested PCR technique developed by Naito *et al.*¹³ were purified using QIAquick Gel Extraction Kit (Qiagen, Crawley, West Sussex, UK) from agarose gel. The purified PCR products were ligated into pDrive cloning vector (Qiagen, Crawley, West Sussex, UK). The recombinant pDrive vectors were transformed into EZ Competent Cells supplied in the Qiagen PCR Cloning^{plus} Kit (Qiagen, Crawley, West Sussex, UK). The bacteria were grown on Luria Bertani agar plates containing 100 µg/ mL ampicillin (Calbiochem, La Jolla, CA), 80 µg/ mL 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) (MBI Fermentas, St.Leon-Rot, Germany) and 50 µM isopropyl β-D-thiogalactopyranoside (IPTG) (Sigma, St. Louis, MO). Transformed bacteria were isolated and re-cultured for recombinant plasmid extraction.

Genotyping of cloned PCR products

To verify the genotypes of the cloned PCR products, extracted recombinant pDrive vectors

containing PCR products amplified using the PCR on the pre-S region from transformed clones were utilized as template for PCR-RFLP on the pre-S region. Subsequently, PCR products were digested with *Ava*II (New England Biolabs, Ipswich, MA) and *Dpn*II (New England Biolabs, Ipswich, MA) to produce RFLP patterns. For recombinant pDrive vectors containing PCR products amplified using the nested PCR, these were subjected to the nested stage of PCR with the second sets of primers.

PCR products sequencing and phylogenetic analysis

Single clones from selected samples were sequenced. Recombinant plasmids were extracted using QIAprep Spin Miniprep Kit (Qiagen, Crawley, West Sussex, UK) and inserts were verified by *Eco*RI (New England Biolabs, Ipswich, MA) digestion. Sequencing was performed by BigDye® Terminator v3.0 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) in ABI PRISM® 377 DNA Sequencer (Perkin-Elmer, Foster City, CA, USA) with T7 promoter and either SP6 or M13 primers. The sequences obtained were compared with published sequences with established genotypes (A to H) [Accession numbers X70185, X51970, X02763, D00330, D00331, M54923, D00329, AF282918, AB033556, D23681, X75656, X75665, AB03359, M32138, AB090269, X75664, X75657, X75658, X69798, X75663, AF160501, AF405706 and AY090454] in the GenBank database. The generations of the phylogenetic trees were performed using Clustal W 1.83 program with the Neighbor-Joining setting and viewed with TreeView 1.6.6 software program.¹⁹

RESULTS AND DISCUSSION

The results for the three genotyping methods are shown in Table 1. In general, the nested PCR with type specific primers was the most sensitive, as this method successfully amplified HBV DNA in 86.7 % (52/ 60) of the sera. The percentage of HBV DNA amplified was 38.1 % (23/ 60) when PCR-RFLP on the pre-S region was used and 51.7 % (31/ 60) when PCR-RFLP on the S region was used.

We observed that genotyping of the HBV DNA using nested PCR method was better as higher concentration of DNA could be amplified, thus producing clearer DNA bands on agarose gel after electrophoresis. It has been reported by Kaneko *et al.*²⁰ that the minimum amount of DNA template required for successful detection using nested PCR method was found to be 10^{-5} pg of HBV DNA. Besides that, the method did not require restriction digestion and so the PCR products could be clearly observed and genotypes could be determined more easily. In contrast, not all the HBV DNA amplified by both the PCR-RFLP methods could be genotyped due to low PCR product yields and indeterminate RFLP pattern produced. For both PCR-RFLP methods, only one round of PCR amplification was performed thus producing very low yield if the concentrations of template DNA were low initially. The minimum amount of DNA template required for successful detection by single PCR was reported to be at 10^{-2} pg HBV DNA.²¹ The PCR product had to be digested with restriction endonucleases thus producing various smaller DNA bands. At times, these small bands could be difficult to observe especially for the smallest possible digested fragments (about 25 bp).

TABLE 1: Prevalence of genotypes determined by different HBV genotyping methods

Genotypes	Genotyping methods employed		
	PCR-RFLP on the pre-S region ¹²	PCR-RFLP on the S region ¹¹	PCR with type specific primers ¹³
B	12/ 15 (80.0 %)	12/ 13 (92.3 %)	24/ 52 (46.2 %)
C	3/ 15 (20.0 %)	1/ 13 (7.7 %)	6/ 52 (11.5 %)
D	–	–	1/ 52 (1.9 %)
E	–	–	1/ 52 (1.9 %)
B and C	–	–	20/ 52 (38.5 %)
Total	15/ 15 (100.0 %)	13/ 13 (100.0 %)	52/ 52 (100.0 %)

Note: Only samples that were successfully genotyped were included in the data for Table 1. A large number of the samples were unsuccessfully genotyped due to the failure to amplify the HBV DNA during the first round of PCR, especially in the cases for the PCR-RFLP methods of Lindh *et al.*^{11,12}

The dependence of RFLP on determining the genotypes could be sensitive to point mutations within the restriction sites which would result in the changing of RFLP patterns from the expected ones.²² Therefore, these might contribute to emergence of unique RFLP patterns when both the PCR-RFLP methods were used. These unique RFLP patterns might not correspond to the genotypic RFLP patterns as predicted by Lindh *et al.*^{11,12}

The negative results obtained could be due to the absence of HBV DNA in the sera or because of the very low PCR product yield as to make them difficult to observe in agarose gel and be classified as PCR negative. Besides that, mutations or polymorphism on the binding sites for the primers utilized could lead to failure of amplification. A previous study found that 18.3 % of the HBV DNA analyzed had mutations in the pre-S region.²³ Within the same geographic region, mutations and deletions in the S and pre-S regions had been described which could be common to the local geographic HBV strains.^{24,25} This could explain the low detection rate obtained when using the PCR-RFLP on the pre-S region as well as on the S region due to unsuccessful PCR amplification.

When the PCR-RFLP method on the pre-S region was used, only 65.2 % (15/ 23) of the HBV DNA amplified could be genotyped. For PCR-RFLP on the S region, only 41.9 % (13/ 31) of HBV DNA amplified could be genotyped. However overall, it is noticeable that from the results obtained, the PCR-RFLP on the S region is more sensitive than the PCR-RFLP on the pre-S region (51.7 % vs. 38.1 %). This could be due to the fact that the S gene sequence is more conserved than the pre-S gene. As the S gene overlaps with the reverse transcriptase site of the viral polymerase reading frame, which is essential to the virus replication, thus it is expected that the S gene to be more well preserved.²⁶ On the other hand, determinations of the genotypes for samples successfully amplified using the nested PCR with type specific primers yielded 100.0 % (52/ 52) success rate. The percentage of the distribution of genotypes identified by the respective methods is summarized in Table 1.

Depending on the genotyping methods used, the prevalence of the genotypes can differ significantly. One such observation of great interest is the substantial numbers of the HBV DNA amplified using the nested PCR with type specific primers producing genotypes B and C co-infections but no co-infection was observed

when both the PCR-RFLP methods were used. No genotypes D and E were detected when PCR-RFLP methods were used but these were detected when nested-PCR was applied. We did not detect any genotype A or F in our samples using all the methods above.

To confirm that the genotypes identified were accurate indeed, we had selected a few of the samples to be sequenced. PCR products amplified using technique developed by Lindh *et al.*¹² that encompassed part of the pre-S region were cloned and sequenced (samples 1, 11, 12, 14 and 23). Sequencing were also performed on cloned PCR products (samples 1 and 15) obtained from the first round of the nested PCR technique developed by Naito *et al.*¹³ that showed single genotypic infections. The region that was sequenced encompassed a region between the pre-S and S sectors of the HBV genome.

Sequence analysis on the PCR products amplified using the technique developed by Lindh *et al.*¹² showed that samples 1, 11, 12 and 23 grouped together with genotype B whereas sample 14 was grouped as genotype C (Figure 3). The genotypes determined by using the PCR-RFLP on the pre-S region were in agreement with genotypes determined through sequencing (Table 2).

Sequence analysis on the PCR products obtained in the first round nested PCR amplification method of Naito *et al.*¹³ showed identity ranges of between 85-99 % with sequences in GenBank. Sample 1 was found to be closest to genotype B while sample 15 was closest to genotype C (Figure 4). The genotypes ascertained were in agreement with other methods used in this study (Table 2).

We also tried to verify the possibility of co-infections in samples 25 and 47 through PCR and sequencing analyses of the recombinant clones. Analysis with the nested PCR with type specific primers on recombinant clones derived from each of the samples (5 clones for sample 25; and 6 clones for sample 47) produced only single similar genotypes. Sequencing analysis of the clones also revealed single genotypic infections only. Sample 25 was determined to be closest to genotype B, having highest identity to M54923 (97 %). Sample 47 was clustered into the genotype C group, having highest identity to AB033556 (97 %) (Figure 4). Thus, it was possible that only PCR products from the predominant genotypic strain were successfully inserted into the pDrive vectors. In co-infections, the minor strain could be present at as low as

TABLE 2: Genotypes in sera as determined by HBV genotyping methods studied

Samples	Genotype determined using			DNA sequencing on clones containing	
	PCR-RFLP on the pre-S region ¹²	PCR-RFLP on the S region ¹¹	PCR with type specific primers ¹³	Products from PCR on the pre-S region	First round products from PCR with type specific primers
1	B	B	B	B	B
2	B	B	B	NA	NA
3	B	B	B	NA	NA
4	B	B	B	NA	NA
5	B	B	B	NA	NA
6	B	B	B	NA	NA
7	B	B	B and C	NA	NA
8	B	B	B and C	NA	NA
9	B	B	B and C	NA	NA
10	B	LOW	B	NA	NA
11	B	-ve	B	B	NA
12	B	-ve	B	B	NA
13	C	C	B and C	NA	NA
14	C	-ve	C	C	NA
15	C	-ve	C	NA	C
16	LOW	B	B	NA	NA
17	LOW	LOW	B and C	NA	NA
18	LOW	LOW	B and C	NA	NA
19	LOW	LOW	B and C	NA	NA
20	LOW	LOW	B and C	NA	NA
21	LOW	LOW	-ve	NA	NA
22	LOW	-ve	C	NA	NA
23	UT	B	B	B	NA
24	-ve	B	B and C	NA	NA
25	-ve	LOW	B and C	NA	B
26	-ve	LOW	B and C	NA	NA
27	-ve	LOW	B and C	NA	NA
28	-ve	LOW	B and C	NA	NA
29	-ve	LOW	B and C	NA	NA
30	-ve	LOW	B and C	NA	NA
31	-ve	LOW	B and C	NA	NA
32	-ve	LOW	B	NA	NA
33	-ve	LOW	B	NA	NA
34	-ve	LOW	B	NA	NA
35	-ve	LOW	-ve	NA	NA
36	-ve	LOW	-ve	NA	NA
37	-ve	-ve	B	NA	NA
38	-ve	-ve	B	NA	NA
39	-ve	-ve	B	NA	NA
40	-ve	-ve	B	NA	NA
41	-ve	-ve	B	NA	NA
42	-ve	-ve	B	NA	NA
43	-ve	-ve	B	NA	NA
44	-ve	-ve	B	NA	NA
45	-ve	-ve	B	NA	NA
46	-ve	-ve	B	NA	NA
47	-ve	-ve	B and C	NA	C
48	-ve	-ve	B and C	NA	NA
49	-ve	-ve	B and C	NA	NA
50	-ve	-ve	B and C	NA	NA
51	-ve	-ve	C	NA	NA
52	-ve	-ve	C	NA	NA
53	-ve	-ve	C	NA	NA
54	-ve	-ve	D	NA	NA
55	-ve	-ve	E	NA	NA
56	-ve	-ve	-ve	NA	NA
57	-ve	-ve	-ve	NA	NA
58	-ve	-ve	-ve	NA	NA
59	-ve	-ve	-ve	NA	NA
60	-ve	-ve	-ve	NA	NA

Note: -ve: PCR negative; LOW: PCR product yield low so genotype untypable; UT: unique RFLP that did not correspond to any known genotyped RFLP pattern; NA: not applicable.

< 1 % of the viral population.¹⁶ Therefore, only the prevailing and the predominant strain would be amplified by PCR.²⁷ In order to detect the less prevalent viral population, screening of a larger number of clones (i.e. > 100 clones) might be required.²²

Referring to Table 2, only 10 sera were successfully genotyped by all the three methods used (samples 1 to 9 and 13). The genotypes determined in six of them concurred with each other. For the other four, three produced genotypes B and one produced genotype C when both the PCR-RFLP methods were used. However, all of them produced genotypes B and C simultaneously when nested PCR with type specific primers were used.

Nevertheless, it could be deduced that the nested PCR with type specific primers was more robust in detecting co-infections. To provide a better insight if co-infections were detectable by PCR-RFLP on the pre-S region and the nested PCR technique, we artificially created co-infection controls by mixing HBV DNA templates containing genotypes B and C and subjecting the mixture to PCR analyses. Given that quantification of HBV DNA in serum could not be measured accurately, thus we had mixed equal volumes of serum that were confirmed to contain HBV of genotypes B and C respectively to constitute a co-infection control, designated as X1. Besides that, a co-infection control was prepared by mixing equal volumes of extracted DNA from the sera used for the X1 co-infection

control, which is henceforth designated as X2. Similarly, another co-infection control, which is designated as X3, was prepared containing mixed equal volumes of extracted DNA from another two samples verified to be of genotypes B and C respectively. From the PCR analyses of these controls, only X3 was positive for co-infection results while X1 and X2 were only successfully determined to have co-infections using the nested PCR technique developed by Naito *et al.*¹³ but not using the PCR-RFLP technique of Lindh *et al.*¹² (Figure 2a to 2e). We found that the ability for detecting co-infections by the PCR-RFLP technique was dependent on the concentrations of the initial templates found in the genotyped samples in the controls. The statement on the concentrations above was based on the intensities of the undigested PCR bands (as observed in the agarose gel in Figure 2a), which we postulated to be proportional to the initial DNA templates found in the control samples prior to PCR amplification. Given that the same sets of control samples were used, we observed that the nested PCR technique was better able to detect co-infections irrespective of the relative concentrations of the initial templates of the two genotypes (Figure 2d and 2e). Thus, this control procedure raised the likelihood that co-infections observed in this study could be accurate indeed. The masking of the less prevalent genotypic strain by the predominant genotype could have caused the detection failures of co-infections by the PCR-RFLP technique.¹⁷

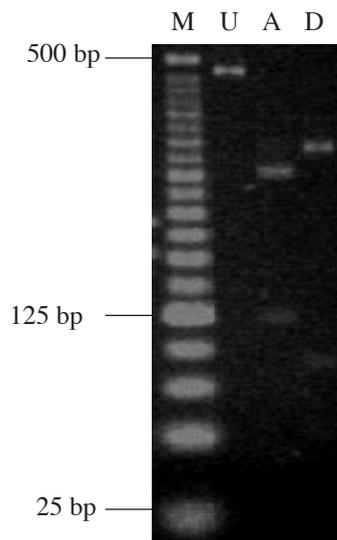


FIG. 1: Gel electrophoresis analysis of digested PCR products amplified from sample 23 using technique developed by Lindh *et al.*¹² The RFLP produced did not correspond to any genotype as predicted by Lindh *et al.*¹² However, this sample was determined to be genotype B when PCR-RFLP on the S region and nested PCR with type specific primers were used. U: undigested PCR product; A: *Ava*II digested PCR product; D: *Dpn*II digested PCR product; M: marker lane.

However, we observed possible co-infections by two different genotype B substrains in sample 12 when PCR-RFLP on the pre-S region was used. By substrains, we meant that two distinct RFLP patterns were observed but both of these belonged to genotype B. The two patterns observed corresponded to the B3 and B5 RFLP patterns as described by Lindh *et al.*¹² PCR analysis on seven clones revealed that all contained PCR product inserts with the B5 RFLP pattern, suggesting that this was the predominant substrain. Although nested PCR might be robust in detecting single genotypes and co-infections, the PCR-RFLP methods might prove superior in the detection of substrains as indicated by the existence of various RFLP patterns for every genotype. There is an increased interest in research on genotypic substrains.^{7,28}

One unique RFLP pattern that could not be genotyped was seen when PCR-RFLP on the pre-S region was used (sample 23). Restriction digestion of this PCR product with *Ava*II produced DNA band sizes of about 125 bp and 275 bp whereas DNA band sizes of about 90 bp and 320 bp were observed when digested with *Dpn*II (Figure 1). This RFLP pattern did not correspond to any of the RFLP patterns observed by Lindh *et al.*¹² This sample was subsequently found to be genotype B when PCR-RFLP on the S region and nested PCR with type specific primers were used (Table 2). Sequencing and phylogenetic analysis revealed this sample to be closest to genotype B, with highest identity to AF282918 (Table 2 and Figure 3).

Generally the genotypes determined using the above methods were all consistent if the sera contained only single genotype but they were inconsistent if the samples had co-infections. These were also supported by PCR analysis on the clones and sequencing of the PCR products inserts where no contradictory results of genotypes was observed. Studies by Lindh *et al.*^{11,12} on the genotyping methods using the PCR-RFLP on the S and pre-S regions showed some discrepancy between the two methods. One sample in their studies was determined as genotype A when based on the S region but as genotype D when on the pre-S region. It was suggested that the latter was probably accurate as the sample contained a 33 bp deletion in the pre-S region which is typical for genotype D.¹² However, it should be noted that this discrepancy might be due to genotypes A/ D recombinant. Owiredu *et al.*²⁹ reported a HBV strain of genotypes A/ D recombinant where the

recombinant breakpoints occurred within the S and pre-S regions. Moreover, in their report, the genotypes of two HBV samples, which were untypable when PCR-RFLP on the S region was used, were successfully genotyped when PCR-RFLP on the pre-S region was used. Conversely, some HBV samples found untypable based on the pre-S region were successfully genotyped based on the S region.^{11,12} In this study, the genotypes of HBV determined by the two methods involving PCR-RFLP concurred with each other.

In our case, the genotyping results obtained using the PCR-RFLP methods of Lindh *et al.*^{11,12} did not yield an optimum result as one hope to get. The genotyping result obtained using PCR-RFLP on the S gene here is quite comparable to the result obtained by Kao *et al.*³⁰ where in their study, only about 66 % of the samples were successfully genotyped when the PCR-RFLP method developed by Mizokami *et al.*³¹ was used. This is despite the fact that the PCR-RFLP method developed by Mizokami *et al.*³¹ utilized the nested PCR technique which in theory should be more sensitive. It was reported that the use of the PCR-based methods could sometimes fail in genotyping all the samples studied where genotypes of some samples were found to be untypable.^{22,32} Besides that, there would be difficulty in interpreting samples co-infected by two different genotypes when PCR-RFLP methods were used to genotype the HBV strains.³³

Despite the disadvantages of the PCR-based genotyping methods as observed in this study, they are still preferred when compared to other methods such as commercial assays or real-time PCR methods due to their simplicity and cost-effectiveness. The commercial assays for HBV genotyping that are currently available are like enzyme immunoassay (EIA) with monoclonal antibodies specific to the epitopes found in the pre-S2 regions which differed between different genotypes, the genotype-specific probes assay (GSPA) kit and the genotyping kit utilizing line probe assays.^{14,18,34} Although more expensive, these commercial kits were found to be more sensitive when compared with the PCR-based genotyping methods. In a study by Chen *et al.*,³⁵ about 98.8 % of their HBV samples were successfully genotyped using the line probe assay. Also, the commercial kits were found to be more superior in detecting sera samples with mixed genotypic infections.^{14,34,35} As for real-time PCR-based genotyping methods, several examples have been developed.^{36,37} However, due to their higher costs, these other methods

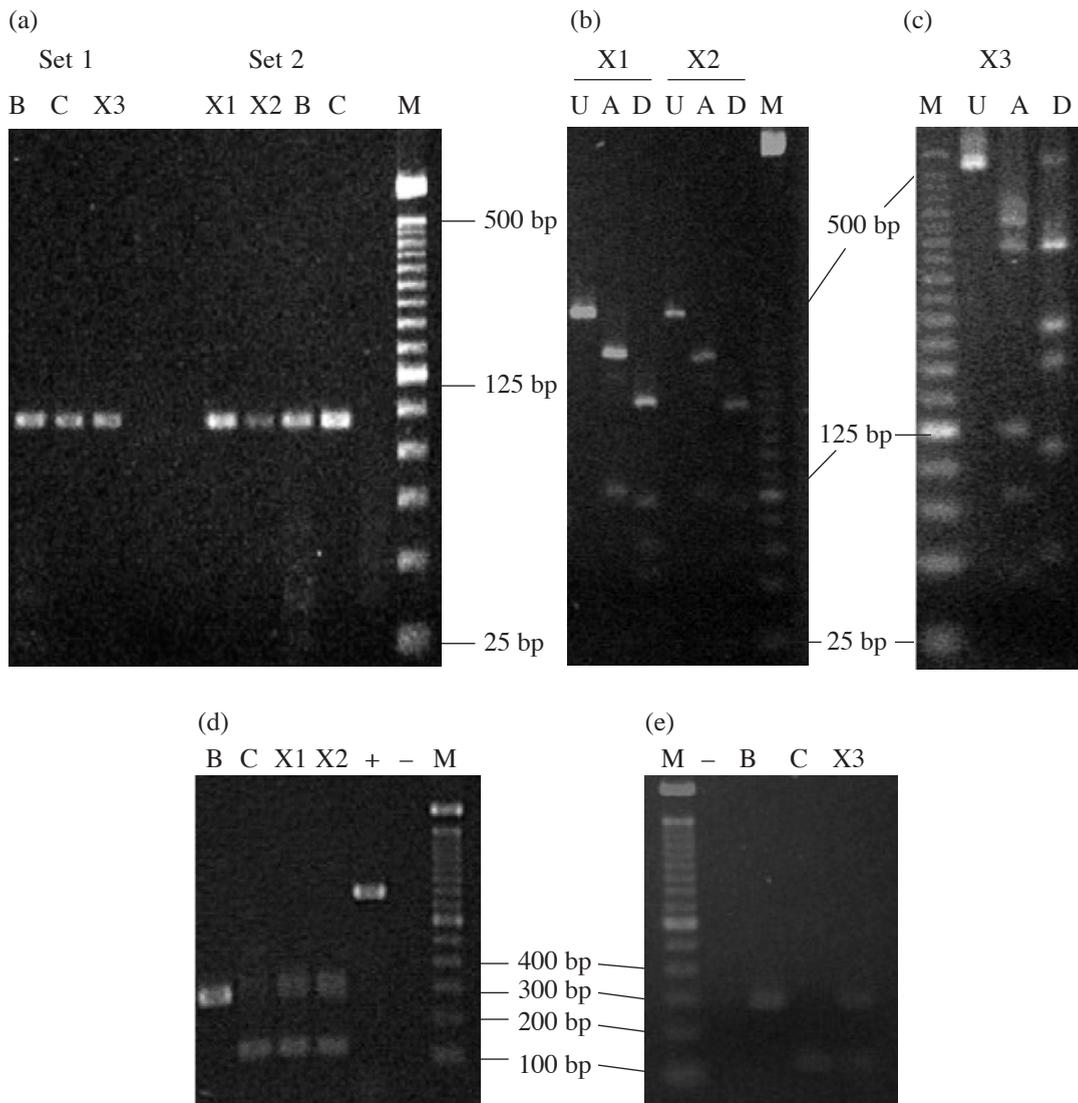


FIG. 2: Gel electrophoresis analyses of co-infection (X1, X2 and X3). (a) Undigested PCR products of HBV-DNA using PCR-RFLP as described by Lindh *et al.*¹² Note the higher intensity of DNA band of lane C against lane B at the right hand side. (b) RFLP of digested PCR products amplified from PCR-RFLP as described by Lindh *et al.*¹² of X1 and X2. Both RFLP patterns correspond to genotype C. (c) RFLP of digested PCR products amplified from PCR-RFLP of Lindh *et al.*¹² of X3. (d) Nested PCR products amplified using technique developed by Naito *et al.*¹³ of X1 and X2 samples. (e) Nested PCR products amplified using technique developed by Naito *et al.*¹³ of X3 sample. B: genotype B; C: genotype C; U: undigested PCR product; A: *Ava*II digested PCR product; D: *Dpn*II digested PCR product; M: marker lane; +: positive control provided by the PCR Reagent System (Invitrogen, Carlsbad, CA); -: negative control; X1: co-infection control derived by mixing equal volumes of sera determined to be infected by genotypes B and C respectively; X2 and X3: co-infection controls derived by mixing equal volumes of extracted DNA from sera with genotypes B and C respectively.

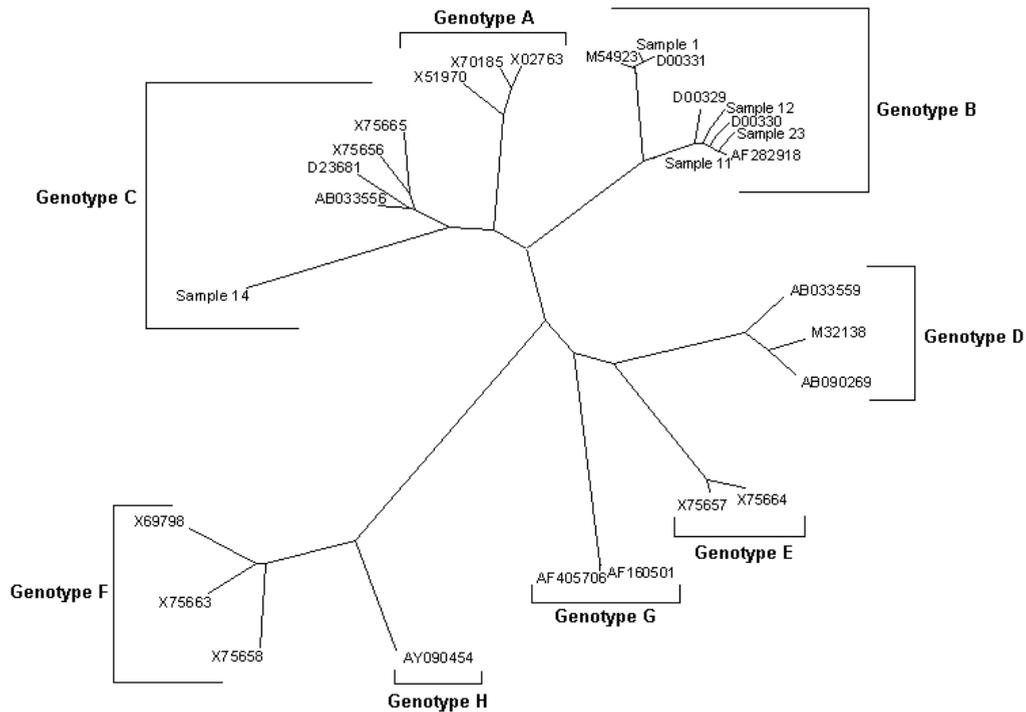


FIG. 3: Unrooted phylogenetic analysis of the cloned PCR products of samples amplified using technique developed by Lindh *et al.*¹² in relationship with genotyped sequences from the GenBank database. Analysis was performed with Clustal W 1.83 program with the Neighbor-Joining setting and the tree diagram was generated by the TreeView 1.6.6.

might be impractical in the studies on HBV which involve large sample sizes. Besides that, they could be less robust in accommodating newly discovered genotypes such as genotypes G and H or other as yet unknown genotypes. This is true for the case of the EIA technique as it relies on only a few amino acids to determine the genotypes.²² When used in tandem with the other methods, the PCR-based genotyping methods can still be useful. It is interesting to note that in the study by Kao *et al.*²⁹ as mentioned above, the remaining unsuccessful genotyped samples (0.2 %) using the line probe assay were successfully identified with the PCR-based method developed by Mizokami *et al.*³⁰

On the limitations of our study, we must admit that we did not have a complete data on the HBeAg/ anti-HBe statuses of our samples. This might be important as it has been shown in some studies HBeAg-positive samples have higher HBV DNA levels than HBeAg-negative samples.^{38,39} Besides that, some of the failure of PCR amplifications encountered in our study could be due to suboptimal assay conditions. Standard PCR profiles as described by Lindh *et*

al.^{11,12} and Naito *et al.*¹³ were used in our study. Due to the large number of samples involved, it was impractical to optimize every of the PCR assay. Another limitation in our study is that we were unable to have all the samples sequenced due to financial constraints. As the nested-PCR is more sensitive in amplifying HBV DNA, it could also mean that the assay is more susceptible to contaminations. In our approach in determining the genotypes through sequencing of the PCR products (which consisted of just a small segments of the whole HBV genome), we can only give an indication of the closeness of our sequenced samples to a particular genotypic group.

In conclusion, the nested PCR developed by Naito *et al.*¹³ was found to be the most sensitive in our study. Given the fact that the prevalence of genotypes identified could vary significantly when using different genotyping methods, thus it is suggested to perform genotyping studies with a several genotyping procedures to complement each other. Since the development of these methods, new genotypes have been discovered (genotypes G and H) that could be identified

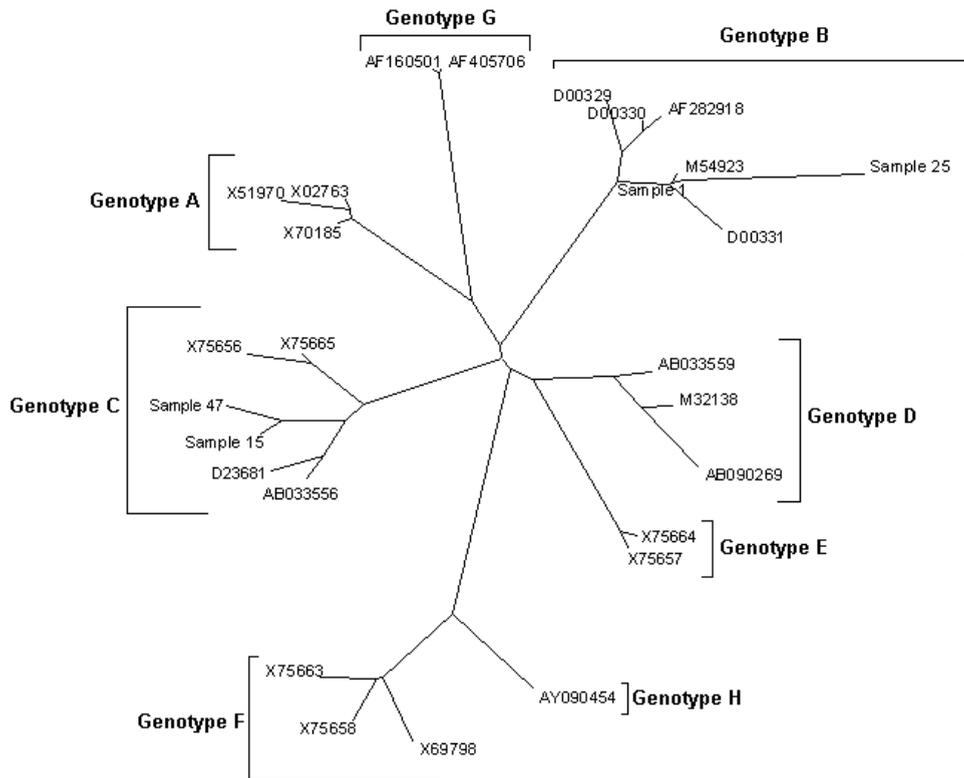


FIG. 4: Unrooted phylogenetic analysis of the cloned first-round PCR products of samples amplified using technique developed by Naito *et al.*¹³ in relationship with genotyped sequences from the GenBank database. Analysis was performed with Clustal W 1.83 program with the Neighbor-Joining setting and the tree diagram was generated by the TreeView 1.6.6.

by other PCR-based methods.^{2,40} Although the methods studied here were only able to detect genotypes A to F, they were still widely used by the scientific community in the current studies of HBV.⁴¹⁻⁴³ It is with hope that the evaluation study of the PCR-based genotyping methods here might provide useful information to investigators researching on HBV, especially in places where the HBV strains of genotypes B and C are prevalent.

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REFERENCES

1. Okamoto H, Tsuda F, Sakugawa H, *et al.* Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. *J Gen Virol* 1988; 69: 2575-83.
2. Arauz-Ruiz P, Norder H, Robertson BH, Magnius LO. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. *J Gen Virol* 2002; 83: 2059-73.
3. Norder H, Hammas B, Lee SD, *et al.* Genetic relatedness of hepatitis B viral strains of diverse geographical origin and natural variations in the primary structure of the surface antigen. *J Gen Virol* 1993; 74: 1341-8.
4. Stuyver L, De Gendt S, Van Geyt C, *et al.* A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. *J Gen Virol* 2000; 81: 67-74.
5. Magnius LO, Norder H. Subtypes, genotypes and molecular epidemiology of the hepatitis B virus as reflected by sequence variability of the S-gene. *Intervirology* 1995; 38: 24-34.
6. Kidd-Ljunggren K, Miyakawa Y, Kidd AH. Genetic variability in hepatitis B viruses. *J Gen Virol* 2002; 83: 1267-80.

7. Norder H, Courouce AM, Magnius LO. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. *Virology* 1994; 198: 489-503.
8. Kao JH, Chen PJ, Lai MY, Chen DS. Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. *Gastroenterology* 2000; 118: 554-9.
9. Thakur V, Guptan RC, Kazim SN, Malhotra V, Sarin SK. Profile, spectrum and significance of HBV genotypes in chronic liver disease patients in the Indian subcontinent. *J Gastroenterol Hepatol* 2002; 17: 165-70.
10. Hussain M, Chu CJ, Sablon E, Lok AS. Rapid and sensitive assays for determination of hepatitis B virus (HBV) genotypes and detection of HBV precore and core promoter variants. *J Clin Microbiol* 2003; 41: 3699-705.
11. Lindh M, Andersson AS, Gusdal A. Genotypes, nt 1858 variants, and geographic origin of hepatitis B virus--large-scale analysis using a new genotyping method. *J Infect* 1997; 175: 1285-93.
12. Lindh M, Gonzalez JE, Norkrans G, Horal P. Genotyping of hepatitis B virus by restriction pattern analysis of a pre-S amplicon. *J Virol Methods* 1998; 72: 163-74.
13. Naito H, Hayashi S, Abe K. Rapid and specific genotyping system for hepatitis B virus corresponding to six major genotypes by PCR using type-specific primers. *J Clin Microbiol* 2001; 39: 362-4.
14. Kato H, Orito E, Sugauchi F, *et al.* Frequent coinfection with hepatitis B virus strains of distinct genotypes detected by hybridization with type-specific probes immobilized on a solid-phase support. *J Virol Methods* 2003; 110: 29-35.
15. Ding X, Mizokami M, Ge X, *et al.* Different hepatitis B virus genotype distributions among asymptomatic carriers and patients with liver diseases in Nanning, southern China. *Hepatol Res* 2002; 22: 37-44.
16. Hannoun C, Krogsgaard K, Horal P, Lindh M. Interpret trial group. Genotype mixtures of hepatitis B virus in patients treated with interferon. *J Infect Dis* 2002; 186: 752-9.
17. Kao JH, Chen PJ, Lai MY, Chen DS. Acute exacerbations of chronic hepatitis B are rarely associated with superinfection of hepatitis B virus. *Hepatology* 2001; 34: 817-23.
18. Usuda S, Okamoto H, Iwanari H, *et al.* Serological detection of hepatitis B virus genotypes by ELISA with monoclonal antibodies to type-specific epitopes in the preS2-region product. *J Virol Methods* 1999; 80: 97-112.
19. Chenna R, Sugawara H, Koike T, *et al.* Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res* 2003; 31: 3497-500.
20. 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.
21. Kaneko S, Miller RH, Feinstone SM, *et al.* Detection of serum hepatitis B virus DNA in patients with chronic hepatitis using the polymerase chain reaction assay. *Proc Natl Acad Sci U S A* 1989; 86: 312-6.
22. Bartholomeusz A, Schaefer S. Hepatitis B virus genotypes: comparison of genotyping methods. *Rev Med Virol* 2004; 14: 3-16.
23. Huy TT, Ushijima H, Win KM, *et al.* High prevalence of hepatitis B virus pre-s mutant in countries where it is endemic and its relationship with genotype and chronicity. *J Clin Microbiol* 2003; 41: 5449-55.
24. Chen WN, Oon CJ. Pre-S deletion mutants co-exist with wild-type virus at low viral replication stage in Singapore chronic hepatitis B virus carriers. *Curr Microbiol* 2002; 44: 145-7.
25. Ong HT, Duraisamy G, Kee Peng N, Wen Siang T, Seow HF. Genotyping of hepatitis B virus in Malaysia based on the nucleotide sequence of preS and S genes. *Microbes Infect* 2005; 7: 494-500.
26. Mizokami M, Orito E, Ohba K, Ikee K, Lau JYN, Gojobori T. Constrained evolution with respect to gene overlap of hepatitis B virus. *J Mol Evol* 1997; 44: S83-S90.
27. Schroter M, Feucht HH, Zollner B, Schafer P, Laufs R. Multiple infections with different HCV genotypes: prevalence and clinical impact. *J Clin Virol* 2003; 27: 200-4.
28. Mbayed VA, Barbini L, Lopez JL, Campos RH. Phylogenetic analysis of the hepatitis B virus (HBV) genotype F including Argentine isolates. *Arch Virol* 2001; 146: 1803-10.
29. Owiredu WK, Kramvis A, Kew MC. Hepatitis B virus DNA in serum of healthy black African adults positive for hepatitis B surface antibody alone: possible association with recombination between genotypes A and D. *J Med Virol* 2001; 64: 441-54.
30. Kao JH, Chen PJ, Lai MY, Chen DS. Hepatitis D virus genotypes in intravenous drug users in Taiwan: decreasing prevalence and lack of correlation with hepatitis B virus genotypes. *J Clin Microbiol* 2002; 40: 3047-9.
31. Mizokami M, Nakano T, Orito E, *et al.* Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. *FEBS Letters* 1999; 450: 66-71.
32. Sanchez-Tapias JM, Costa J, Mas A, Bruguera M, Rodes J. Influence of hepatitis B virus genotype on the long-term outcome of chronic hepatitis B in western patients. *Gastroenterology* 2002; 123: 1848-56.
33. Kato H, Ruzibakiev R, Yuldasheva N, *et al.* Hepatitis B virus genotypes in Uzbekistan and validity of two different systems for genotyping. *J Med Virol* 2002; 67: 477-83.
34. Osiowy C, Giles E. Evaluation of INNO-LiPA HBV genotyping assay for determination of hepatitis B virus genotype. *J Clin Microbiol* 2003; 41: 5473-7.
35. Chen BF, Chen PJ, Jow GM, *et al.* High prevalence of mixed genotype infections in hepatitis B virus infected intravenous drug users. *J Med Virol* 2004; 74: 536-42.
36. Yeh SH, Tsai CY, Kao JH, *et al.* Quantification and genotyping of hepatitis B virus in a single reaction by real-time PCR and melting curve analysis. *J Hepatol* 2004; 41: 659-66.

37. Liu WC, Mizokami M, Buti M, *et al.* Simultaneous quantification and genotyping of hepatitis B virus for genotypes A to G by real-time PCR and two-step melting curve analysis. *J Clin Microbiol* 2006; 44: 4491-7.
38. Chu CJ, Hussain M, Lok ASF. Quantitative serum HBV DNA levels during different stages of chronic hepatitis B infection. *Hepatology* 2002; 36: 1408 -15.
39. Chen CH, Lee CM, Lu SN *et al.* Clinical significance of hepatitis B virus (HBV) genotypes and precore and core promoter mutations affecting HBV e antigen expression in Taiwan. *J Clin Microbiol* 2005; 12: 6000-6.
40. Kato H, Orito E, Sugauchi F, *et al.* Determination of hepatitis B virus genotype G by polymerase chain reaction with hemi-nested primers. *J Virol Methods* 2001; 98: 153-9.
41. Chattopadhyay S, Das BC, Hussain Z, Kar P. Hepatitis B virus genotypes in acute and fulminant hepatitis patients from north India using two different molecular genotyping approaches. *Hepato Res* 2006; 35: 79–82.
42. Eroglu C, Leblebicioglu H, Gunaydin M, *et al.* Distinguishing hepatitis B virus (HBV) genotype D from non-D by a simple PCR. *J Virol Methods* 2004; 119: 183–7.
43. Jutavijittum P, Jiviriyawat Y, Yousukh A, Kunachiwa W, Toriyama K. Genotypes of hepatitis B virus among voluntary blood donors in northern Thailand. *Hepato Res* 2006; 35: 263–6.