

Novel Hepatitis B Virus Genotype A Subtyping Assay That Distinguishes Subtype Aa from Ae and Its Application in Epidemiological Studies

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The eight genotypes of hepatitis B virus (HBV) have different geographical distributions, virological characteristics, and clinical manifestations. A unique subtype of HBV genotype A (HBV/A) was reported in sub-Saharan Africa, raising the possibility that patients infected with this subtype (HBV/Aa ["a" for African and Asian]) may have different clinical outcomes than other HBV/A isolates (HBV/Ae ["e" for European]). Comparison between 30 HBV/Aa and 30 HBV/Ae isolates indicated that almost all HBV/Ae isolates had G at nucleotide (nt) 1809 and C at nt 1812, whereas HBV/Aa isolates had T1809/T1812. Taking advantage of these two single nucleotide polymorphisms (SNPs), a novel subtype-specific PCR assay in the X/precore/core region was developed. This assay was combined with a restriction fragment length polymorphism assay using BglII in a different region (nt 1984 to 1989), which has a SNP distinguishing HBV/Aa from HBV/Ae, resulting in 100% specificity for the combined assay. Application of the subtyping assay using sera from 109 paid donors in the United States indicated significantly different distributions of HBV/A subtypes among races; African-Americans, Caucasians, and Hispanics had HBV/Ae, whereas Asians had mainly HBV/Aa, suggesting that the HBV/Aa isolates may have been imported by recent immigration from Asia. In conclusion, the specificity and sensitivity of the combined subtyping assay were confirmed, and its usefulness was demonstrated in a practical context.

Hepatitis B virus (HBV) is one of the most important causes of acute and chronic liver disease, such as fulminant hepatitis, cirrhosis, and hepatocellular carcinoma, throughout the world, especially in Asian and African countries. Eight genotypes of HBV, classified A to H (HBV/A to HBV/H), are distinguished by intergenotypic differences of more than 8% in the entire nucleotide sequence of approximately 3,200 nucleotides (nt) (3, 21, 22, 25). These genotypes have different geographical distributions, virological characteristics, and clinical manifestations (18, 20). Moreover, among the HBV isolates of the same genotype, there might also be differences of virological characteristics.

Recently, in Asian countries, two subtypes of HBV genotype B, designated Ba ("a" standing for Asia) and Bj ("j" for Japan) have been reported (27), and clinical differences between patients infected with HBV/Ba and HBV/Bj are being recognized (2, 28).

Similarly, a subtype of genotype A was reported and designated A' in sub-Saharan African countries (5). A large number

of HBV isolates from South Africa were found to cluster with this subtype A' by phylogenetic analyses of both the pre-S2/S region (5) and the complete genome (13, 16). This subtype has also been found in HBV isolates from Malawi (29). In African countries, genotype A isolates, most of which are subtype A', seem to be associated with low levels of HBV DNA in serum and a low prevalence of hepatitis B e antigen (HBeAg) in serum (14, 15). On the other hand, HBV carriers infected with isolates belonging to the remainder of genotype A, distributed widely in European countries and the United States, have a higher rate of sustained remission after seroconversion and a lower rate of death related to liver disease than other genotypes during long-term follow-up (8, 24, 33).

In this paper, we report a convenient assay with a subtype-specific PCR combined with a restriction fragment length polymorphism (RFLP) assay to distinguish these two subtypes of genotype A. Recently, a cross-sectional study in the United States showed a correlation of HBV genotypes not only with ethnicity but also with the place of birth of the carriers of the virus (6). By using the newly developed assay, we also examined the association between the subtypes of HBV/A and ethnicity in the United States. To avoid confusion, as previously proposed (30), the subgroup (subtype) A', which was proposed by Bowyer et al. (5), is renamed subtype HBV/Aa ("a" standing for African and Asian), the other subtype found in Euro-

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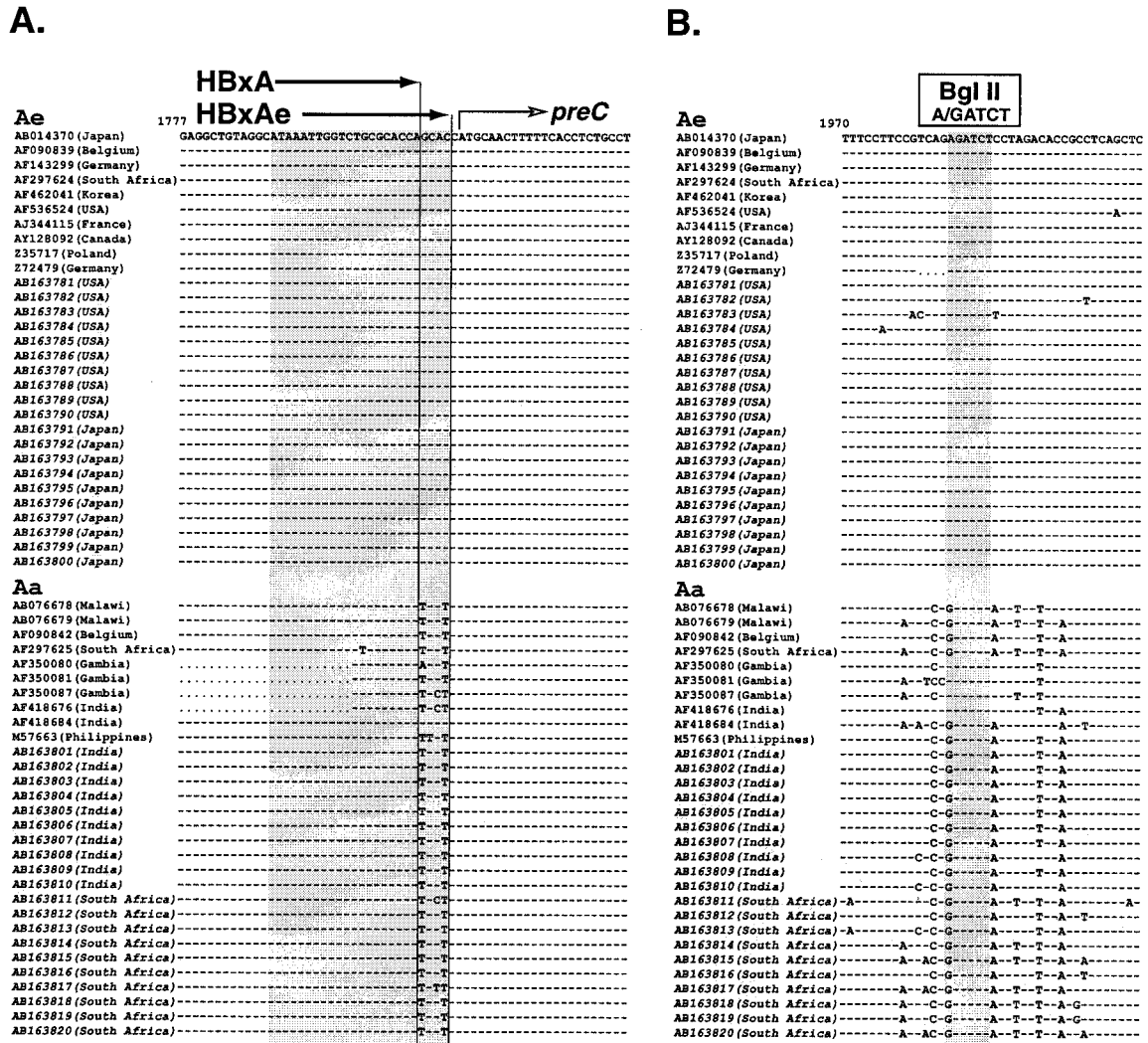


FIG. 1. Alignment of 40 HBV/A isolates sequenced in the present study with 20 reference isolates. (A) Alignment of sequences of HBV/A isolates within the subtype-specific region of the primer (nt 1777 to 1836). The positions of sense primers for the second-round PCR are shown by the arrows above alignments and are shaded in grey. (B) Alignment of sequences of HBV/A isolates within the core region containing a BglIII restriction site (nt 1970 to 2009). The position of the enzyme restriction site is shaded in grey. The isolates sequenced in the present study are shaded in light grey.

pean countries and the United States is HBV/Ae (“e” for European and U.S. type), and genotype A (HBV/A) includes both HBV/Ae and HBV/Aa.

MATERIALS AND METHODS

Serum samples. Forty HBV/A-positive serum samples were collected from carriers in four countries (10 each from Japan, the United States, India, and South Africa) and were used to examine the specificity and sensitivity of our HBV/A subtype-specific PCR combined with RFLP assay. These serum samples were stored at -80°C until assayed.

For the practical testing of this assay, 333 sera from HBsAg-positive paid donors in the United States recruited by ProMedDx Inc. (Plainville, Mass.) were used in this study. Of the 333 samples, 139 belonged to HBV/A. After hepatitis C virus- and/or human immunodeficiency virus-infected samples and samples whose ethnicity was unknown were omitted, 109 samples obtained from 38 African-American, 16 Asian, 24 Caucasian, and 31 Hispanic subjects were examined. All of them had not received current antiviral therapy, and their alanine aminotransferase (ALT) levels in serum were normal. For controls, 32 HBV/A isolates from South African black asymptomatic carriers of HBV with normal

ALT levels were used (age range, 18 to 44 years; 30 [94%] carriers were male). The study protocol was approved by ethics committees of the institutions, in accordance with the 1975 Declaration in Helsinki, and an informed consent was obtained from each subject.

Serological determination of HBeAg and HBV genotypes. HBeAg was detected by the chemiluminescent enzyme immunoassay (Ortho Clinical Diagnostics, Tokyo, Japan). The genotype of these samples was serologically confirmed as HBV/A by using commercial kits (HBV Genotypes EIA; Institute of Immunology) based on five monoclonal antibodies directed to the corresponding epitopes on the product of the pre-S2 region which are designated *b*, *m*, *k*, *s*, and *u* (31, 32).

Detection of HBV DNA and cloning. DNA was extracted from 100 µl of serum using a QIAamp DNA blood mini kit (QIAGEN Inc., Hilden, Germany). HBV DNA of X and precore/core genome sequences were amplified by a method reported previously (26). In brief, the first-round hemi-nested PCR was performed with sense primer HB7F (5'-GAG ACC ACC GTG AAC GCC CA-3' [nt 1611 to 1630]) and antisense primer HB8R-2 (5'-ATA GGG GCA TTG GTC T-3' [nt 2314 to 2299]; a modification of HB8R) for 5 min at 96°C, followed by denaturation for 1 min at 94°C, annealing for 1 min at 60°C, and extension for 1 min at 72°C (an additional 5 min in the last cycle), in a 96-well cycler (GeneAmp

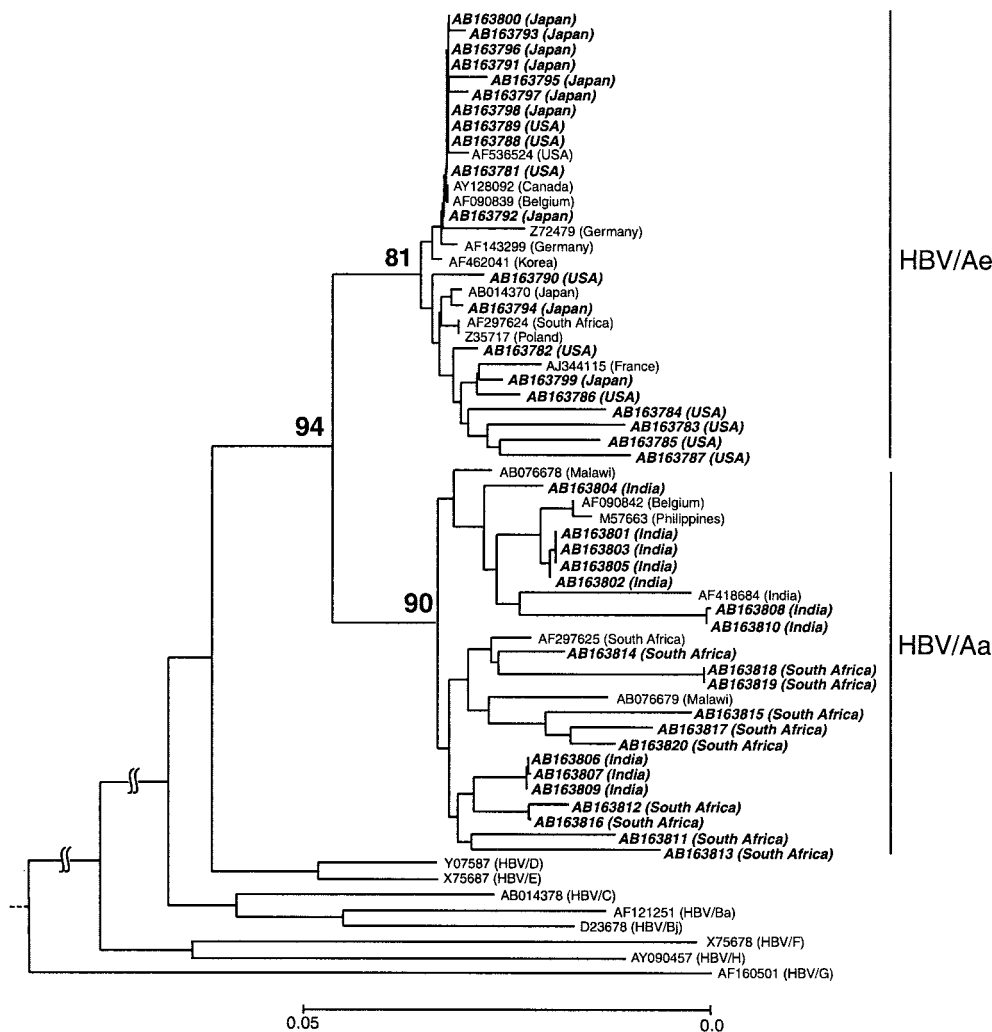


FIG. 2. A phylogenetic tree constructed using HBV X/precore/core sequences (nt 1631 to 2051). Isolates sequenced in the present study from four countries are shown in bold italic. Accession numbers: AB163781 to AB163790 (USA), AB163791 to AB163800 (Japan), AB163801 to AB163810 (India), and AB163811 to AB163820 (South Africa). Ten HBV/Ae and six HBV/Aa reference isolates obtained from the databases are labeled with corresponding accession numbers and countries of origin in parentheses. For the other genotypes (HBV/Ba, HBV/Bj, HBV/C, HBV/D, HBV/E, HBV/F, HBV/G, and HBV/H), reference isolates are also shown by accession numbers. The length of the horizontal bar indicates the number of nucleotide substitutions per site. The numbers on the nodes indicate the percentage of occurrences by bootstrap analysis.

9600; Perkin-Elmer Cetus, Norwalk, Calif). The second-round PCR was performed with the sense primer HB7F and antisense primer HB7R-2 (5'-CCT GAG TGC TGT ATG GTG AGG-3' [nt 2072 to 2052]; a modification of HB7R) under the same conditions as the first-round PCR. The amplicons were analyzed by electrophoresis on 3% agarose gels, stained with ethidium bromide, and observed under UV light.

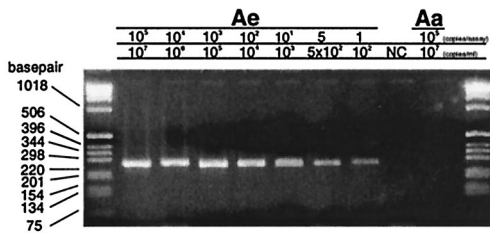
To confirm the specificity of the amplification, PCR products were directly sequenced with Prism Big Dye (Applied Biosystems, Foster City, Calif.) in the ABI 3100 DNA automated sequencer. For positive controls of HBV/Aa and HBV/Ae isolates, the second-round PCR products were cloned into TOPO TA cloning vector (Invitrogen Corp., Carlsbad, Calif.). Plasmids were amplified in *Escherichia coli* DH5 α cells (TaKaRa Shuzo Co. Ltd., Tokyo, Japan) and purified by the QIAGEN procedure.

Molecular evolutionary analyses. A phylogenetic tree was constructed to distinguish the HBV/A subtypes. An alignment of 421 nt (nt 1631 to 2051) of the 40 isolates amplified in this study, together with 24 sequences of HBV/A to HBV/H isolates obtained from DDBJ, EMBL, and GenBank DNA databases, was analyzed. By using the computer program Hepatitis Virus Database Server (<http://s2as02.genes.nig.ac.jp/>), the number of nucleotide substitutions per site and the genetic distances between the isolates were estimated by the six-parameter method (11), and a neighbor-joining tree was constructed based on these values

(23). To confirm the reliability of the phylogenetic tree, bootstrap resampling tests were performed 1,000 times (10).

HBV/A subtype-specific PCR combined with RFLP assay. A novel HBV/A subtyping assay, based on HBV/A subtype-specific PCR combined with a RFLP assay, was developed and applied to HBV/A isolates only, after the genotype had been determined serologically using HBV Genotypes EIA. The first-round hemi-nested PCR was performed with the sense primer HB7F and the antisense primer HB7R-2 for 5 min at 96°C, consisting of denaturation for 1 min at 94°C, annealing for 1 min at 60°C, and extension for 1 min at 72°C (additional 5 min in the last cycle), in a 96-well cycler. The second-round PCR consisted of one of two sets of primers: set I, HBV/A confirmation PCR using the sense primer HBx A (5'-ATA AAT TGG TCT GCG CAC CA-3' [nt 1789 to 1808]) within conserved X regions (Fig. 1A) and the antisense primer HB7R-2 (for 5 min at 96°C, consisting of denaturation for 45 second at 94°C, annealing for 45 second at 62°C, and extension for 45 second at 72°C [additional 5 min in the last cycle]); and set II, HBV/Ae-specific PCR using the sense primer HBx Ae (5'-ATT GGT CTG CGC ACC AGG AC-3' [nt 1793 to 1812]) with the specific sequences for HBV/Ae at the 3' end (Fig. 1A) and the antisense primer HB7R-2 under the same conditions as the second-round PCR in set I. In order to distinguish some unusual isolates and take advantage of another subtype-specific restriction site (Fig. 1B), the PCR assay was combined with a RFLP assay using BglII. Restriction

A. HBxAe



B. HBxA

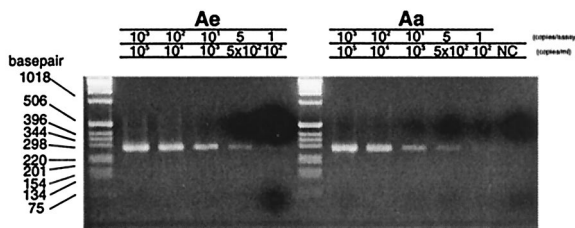


FIG. 3. Sensitivity of subtype-specific PCR assay. Serial 10-fold dilutions containing known numbers of HBV copies of HBV/Ae and HBV/Aa were tested using HBxAe-specific primer (A) and HBxA confirmation primer (B). Molecular weight markers are on the right and/or left lanes.

tion digestions were carried out using 5 μ l of the 282-bp amplicon from set II, with 10 U of restriction enzyme BglII (New England BioLabs, Beverly, Mass.) for 3 h. The digested PCR products were electrophoresed on 3% agarose gels and stained with ethidium bromide. The RFLP pattern was examined under UV light; HBV/Ae amplicons were digested into two fragments (221 plus 61 bp), whereas HBV/Aa amplicons were not restricted.

Statistical analyses. Statistical differences were evaluated by Mann-Whitney U test, Fisher's exact probability test, and chi-square test with Yates' correction, where appropriate, with use of STATA software version 8.0 (StataCorp. LP, College Station, Tex.). Differences were considered significant for *P* values less than 0.05.

Nucleotide sequence accession numbers. Isolates sequenced in this study were submitted to the GenBank/DDBJ/EMBL/ databases under the following accession numbers: AB163781 to AB163790 (USA), AB163791 to AB163800 (Japan), AB163801 to AB163810 (India), and AB163811 to AB163820 (South Africa).

RESULTS

Molecular evolutionary analyses of HBV/A. Based on the alignment of 40 isolates from four countries and 24 reference sequences including HBV/A to HBV/H sequences from the DDBJ, EMBL, and GenBank DNA databases, a phylogenetic tree was constructed for the HBV X/precure/core region (Fig. 2). All 20 HBV/A isolates from the United States and Japan were confirmed to be HBV/Ae, and all 20 isolates from India and South Africa were classified into HBV/Aa with high bootstrap values.

Alignment of HBV/A isolates. Figure 1 shows the alignment of 60 HBV/A sequences (56 sequences from the sequences used for phylogenetic analysis above and 4 additional short sequences from Gambia or India), within the subtype-specific region of the primer (Fig. 1A) and within the core region corresponding to the BglII RFLP site (Fig. 1B). Almost all

HBV/Ae isolates had G at nt 1809 and C at nt 1812 (G1809/C1812 pattern), whereas HBV/Aa isolates had T1809/T1812. Taking advantage of these two single nucleotide polymorphisms, two sense primers were designed to distinguish HBV/Ae from HBV/Aa, and two sets of hemi-nested PCRs were developed. Examining available sequences from the databases, we noted exceptions. A few HBV/Aa isolates had G1809/C1812, and therefore may initially be classified as HBV/Ae. In order to distinguish these unusual isolates, a RFLP assay using BglII was developed in a different region of the HBV genome that contains a single nucleotide polymorphism distinguishing HBV/Aa and HBV/Ae. Because the restriction enzyme, BglII, recognizes the nucleotide sequence A/GATCT (nt 1984 to 1989) (Fig. 1B), HBV/Ae isolates were restricted to 221-plus-61-bp fragments and HBV/Aa isolates were not (282-bp fragment). Furthermore, some atypical isolates of HBV/Aa with A1984 were found in the databases; however, these would be excluded initially by the PCR step because of the nucleotide pattern (T1809 or A1809/T1812) (Fig. 1A).

Sensitivity of HBV/A subtype-specific PCR combined with RFLP assay. The sensitivity of the newly developed PCR assay to distinguish HBV/Ae from HBV/Aa was evaluated on serial 10-fold dilutions containing known copy numbers of both HBV/Ae and HBV/Aa clones (Fig. 3). As shown in Fig. 3A, the subtype-specific PCR with sense primer HBxAe could detect 10² copies per ml of the isolates which have G1809/C1812, and HBV/Aa isolates with T1809/T1812 were not amplified. On the other hand, the PCR with primer HBxA could detect both HBV/Ae and HBV/Aa, and the detection limits were 10² copies per ml (Fig. 3B).

Strategy for the subtyping assay of HBV/A based on HBV/A subtype-specific PCR combined with RFLP assay. Figure 4A shows the final strategy of the subtyping assay of HBV/A based on subtype-specific PCR followed by a RFLP assay. The hemi-nested PCR assay consisted of two sets of second-round PCR, with either sense primer HBxAe (named the HBV/Ae-specific primer) or sense primer HBxA (named the HBV/A confirmation primer), combined with a RFLP assay using BglII, and allowed HBV/Aa to be clearly distinguished from HBV/Ae. In brief, when HBV/A isolates were detected by using both sets of PCR primers and the amplicons were not restricted by the RFLP step (Fig. 4B), the isolates were classified as HBV/Aa. On the other hand, when HBV/A isolates were detected by both sets of PCR primers and the amplicons restricted by BglII, the isolates belonged to HBV/Ae. The isolates positive for the PCR with primer HBxA only and not primer HBxAe were also recognized as HBV/Aa (Fig. 4B).

Application of the HBV/A subtyping assay to U.S. samples. In order to confirm the practical usefulness of this assay, HBV isolates from sera of 109 paid donors from the United States, which had been shown to be HBV/A by enzyme-linked immunosorbent assay, were examined using the subtyping assay. Of the 109 samples obtained from 38 African-American, 16 Asian, 24 Caucasian, and 31 Hispanic subjects, 79 were classified as HBV/Ae and 30 as HBV/Aa. There were no differences between the ethnic groups in the age, gender, and ALT levels in serum of the donors (Table 1). Interestingly, the prevalence of HBeAg in the Asian group tended to be lower than that of the other groups. By examining the ratio of HBV/A subtypes among isolates from each ethnic group with our new assay,

A

Hemi-nested PCR (subtype-specific PCR)

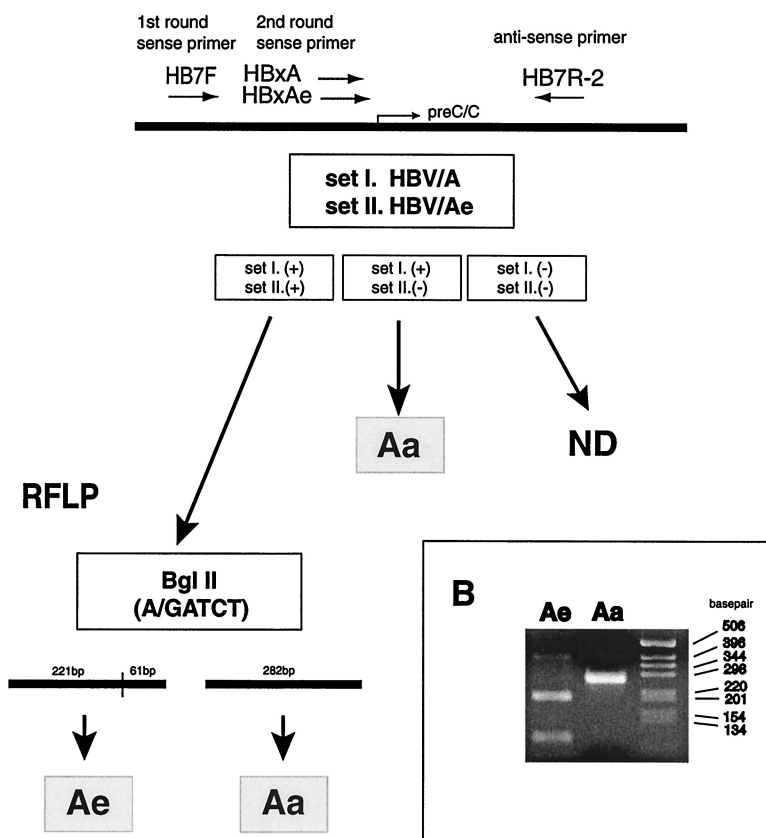


FIG. 4. (A) The strategy of novel subtyping assay of HBV/A based on subtype-specific PCR combined with RFLP assay with BglIII. (B) Ethidium bromide-stained 3% agarose gel showing the restriction patterns obtained by BglII digestion. HBV/Ae isolates were restricted to a 221-plus-61-bp fragment and HBV/Aa isolates were not (282-bp fragment). ND, not determined.

84.2% of African-American, 83.3% of Caucasian, and 74.2% of Hispanic isolates were classified as HBV/Ae, whereas 75% of the Asian isolates were HBV/Aa. This indicates that the prevalence of HBV/Aa was significantly higher in Asian isolates than in the other groups (Fig. 5A). As a control study, the subtyping assay was applied to 32 HBV/A sera from South Africa, where subtype Aa is known to predominate (5, 13, 16).

A total of 96.9% of HBV isolates belonged to HBV/Aa (Fig. 5B), which was different than the prevalence of HBV/Aa among African-American donors from the United States. Moreover, when the HBeAg positivity of subjects younger than 40 was evaluated, we found that the proportion of carriers infected with HBV/Aa that were positive was lower than with those infected with HBV/Ae (data not shown).

TABLE 1. Clinical backgrounds of blood donors from the United States^a

Ethnicity (n)	Mean age, (yr)	Male sex (%)	ALT median (IU/liter)	ALT range (IU/liter)	HBeAg positive (%)
African-American (38)	34.2 ± 11.4	19 (50.0)	15	6–22	6/33 ^b (18.2)
Asian (16)	39.3 ± 17.1	12 (75.0)	22	19–40	2/13 ^b (15.4)
Caucasian (24)	40.3 ± 16.3	15 (62.5)	14	6–40	6/24 (25.0)
Hispanic (31)	38.7 ± 14.4	19 (61.2)	18	4–38	5/31 (16.1)

^a P values were not significant for any data.

^b Five samples in the African-American group and three samples in the Asian group were not available.

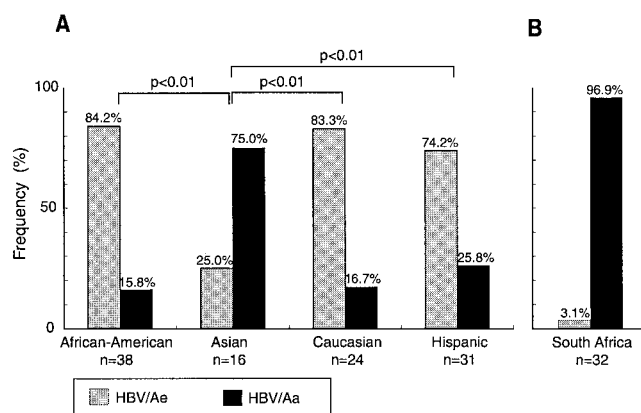


FIG. 5. The prevalence of HBV/Ae and HBV/Aa among different races from 109 U.S. paid donors (A) and 32 black African carriers (B) determined using the HBV/A subtyping assay. The vertical axis indicates percent frequency of HBV/A subtypes. The prevalence was statistically significant between African-Americans and Asians, between Caucasians and Asians, and between Hispanics and Asians.

DISCUSSION

In this study, a subtype-specific PCR combined with a RFLP assay was developed. For HBV/A isolates tested in this study and data extrapolated from sequences in the database, the specificity of this new assay in the X/precure/core overlapping region was 100% and could clearly distinguish HBV/Ae from HBV/Aa.

In previous studies, when HBV isolates showing intergenotypic recombination were excluded, subtype A' (HBV/Aa) was clearly distinguished by phylogenetic analyses from the remaining genotype A (HBV/Ae) in each of the open reading frames except for the small S region (29, 30). Examining all available full HBV sequences from the databases, nucleotide sequences that could distinguish HBV/Aa from HBV/Ae were found in the X/precure/core region. Notably, T1809/T1812 is a nucleotide pattern very specific for HBV/Aa, and almost all HBV/Ae isolates and isolates belonging to the other genotypes have G1809/C1812. Thus, the region of the genome that includes nt 1809 and 1812 was suitable for the subtyping of HBV/A.

In early studies, the individuals infected with HBV in Africa were shown to very frequently seroconvert from HBeAg positivity to negativity in childhood or early adulthood (4, 7), compared with those in the other countries where HBV is endemic (9, 17, 19). Furthermore, HBV-related virus markers were frequently found in young patients with hepatocellular carcinoma in South Africa (12). The unique subtype of HBV/A' (HBV/Aa) was first reported by Bowyer et al. in 1997 (5) and is known to be the major genotype of HBV in South Africa (16). Taken together, these observations allude to HBV/Aa isolates in Africa being different than HBV/Ae isolates in western countries. Therefore, it is useful to distinguish the HBV/Aa with possible different clinical characteristics from the HBV/Ae on the basis of the unique sequences in the X/precure/core region.

Of the 40 original isolates from four countries where HBV is endemic (Japan, the United States, India, and South Africa) and the 28 reference isolates from the database, just one un-

usual exceptional isolate was found (AF418684 in Fig. 1A). This isolate clustered with subtype HBV/Aa following a phylogenetic analysis but was found to have the characteristics of HBV/Ae according to the subtype-specific PCR assay. When a total of 132 available HBV/Aa sequences deposited in the databases and from our unpublished data (India and South Africa) were investigated in order to determine the frequency of isolates that were exceptions, 14 isolates (10.6%) from South Africa and India (five isolates from South Africa [accession numbers AF297621, AF297622, AF297623, U87742, and U87746] and nine isolates from India [accession numbers AF418684, AF418685, AF418686, AF418687, AF418688, AF418689, AF418690, AF418691, and AF418692]) had the G1809/C1812 pattern, and therefore could not be distinguished from HBV/Ae isolates by using the subtype-specific PCR step. However, because these unusual isolates had G instead of A at position 1984, they were not restricted by BglII and could therefore be correctly classified as HBV/Aa. On the other hand, 22 HBV/Aa isolates (16.7%) from Gambia and India (19 isolates from Gambia [accession numbers AF350080 to AF350098] and 3 isolates from India [accession numbers AF418676, AF418677, and AF418678]) had A1984 at the RFLP site that would allow the amplicons to be digested by BglII, a characteristic of HBV/Ae, but because they had T1809 or A1809/T1812, they would not be amplified by the HBV/Ae-specific primers and therefore would not be classified as HBV/Ae.

The application of this assay for HBV/A isolates from sera obtained from paid donors from the United States and HBV carriers from South Africa showed that the majority of HBV isolates from African-Americans, Caucasians, and Hispanics in the United States were HBV/Ae, whereas isolates from Asians living in the United States and black South Africans were mainly HBV/Aa (Fig. 5). Although almost all HBV/A isolates from black Africans were classified as HBV/Aa, which was consistent with previous reports (5, 13, 16), 84.2% of African-Americans had HBV/Ae. This difference between the subtypes in African-Americans and the Africans may be an indication that the infection may be a consequence of the interaction of African-Americans with other races in the United States as opposed to the introduction of HBV from Africa during the slavery period in the 19th century. However, further studies with a larger number of African-Americans and more detailed sequencing analysis would be necessary to support this assumption. On the other hand, the high prevalence of HBV/Aa in the Asian community in the United States may be a reflection of the high rate of migration from Asia to the United States in recent years.

Infection with HBV/Aa is associated with low prevalence of HBeAg in serum and early seroconversion (14, 15). Recently Ahn and coworkers showed that the T1809/T1812 mutations interfere with the translation of HBeAg in vitro and may be responsible for the early loss of HBeAg seen in southern African black carriers of HBV (1). Because of the low frequency of HBeAg positivity among the paid donors, however, we did not find a significant difference between the prevalence of HBeAg in carriers infected with the two different subtypes of genotype A. A large scale case-control study would be necessary in order to investigate this further.

In conclusion, we have described a novel HBV/A subtyping

assay that can differentiate between subtypes Aa and Ae. The specificity and sensitivity of the HBV/A subtype-specific PCR combined with a RFLP assay were confirmed, and the usefulness of this assay in a practical context was demonstrated. This assay will contribute to further clinical or epidemiological studies that will allow us to clarify the differences between the two subtypes Aa and Ae.

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