Analysis of the entire nucleotide sequence of hepatitis B virus genotype B in the Philippines reveals a new subgenotype of genotype B

Futoshi Nagasaki, Hirofumi Niitsuma, Julieta G. Cervantes, Masanori Chiba, Shan Hong, Toshiaki Ojima, Yoshiyuki Ueno, Edgardo Bondoc, Koju Kobayashi, Motoyasu Ishii and Tooru Shimosegawa

The entire nucleotide sequences were determined for hepatitis B virus (HBV) genotype B (HBV/B) genomes extracted from five patients in the Philippines and designated GenBank AB219426, AB219427, AB219428, AB219429 and AB219430. The serotype of the first four isolates was ayw and that of GenBank AB219430 was adw. Divergences of entire sequences were 1–0–2 % between the first four isolates and 3–8–4–2 % between these four and GenBank AB219430. Phylogenetic-tree analysis revealed that, worldwide, HBV/B comprises five subgenotypes: B1, B2, B3, B4 and the new Philippines group, designated B5. Divergences of the entire genome sequences between four isolates in subgenotype B5 and isolates from other countries (subgenotypes) were 4–4–4–8 % with Vietnam (B4), 2–9–3–5 % with Indonesia (B3), 4–7–5–1 % with China (B2) and 5–4–6–0 % with Japan (B1). Similarly, GenBank AB219430 showed the lowest divergences: 3–4 % with the isolate from Indonesia (B3), 5–0 % with Vietnam (B4), 5–4 % with China (B2) and 6–1 % with Japan (B1). This is the first report of entire nucleotide sequences of HBV/B from the Philippines and the results show that these sequences belong to a new subgenotype, B5. The present study identified that HBV/B isolates throughout the world are divided genetically into five subgenotypes, the relationships between geographical distances and the genetic distances of HBV/B being well-correlated.

Hepatitis B virus (HBV) is an aetiological agent of liver cirrhosis and hepatocellular carcinoma and it remains a major health problem worldwide, especially in Asian countries (Lee, 1997). The subtypes of HBV have been determined serologically by the antigenic determinants of the HBV surface antigen (HBsAg), common determinant a and two mutually exclusive determinant pairs, dy and wr. As subtype, i.e. serotype, is mainly defined by a limited number of amino acids, one point mutation in the HBsAg gene could change the serotype in a patient (Okamoto et al., 1987). Therefore, serotyping has its limitations for the classification of HBV.

In 1988, Okamoto and colleagues proposed classifying HBV according to genotype, the genetic classification of HBV based on the comparison of entire genomes, and found an intergroup divergence of 8 % or more (Okamoto et al., 1988). As the classification of HBV by genotype is based on full genome sequences, it is more reliable.

Eight different genotypes of HBV, named A–H, have been determined to date (Okamoto et al., 1988; Norder et al., 1992; Stuyver et al., 2000; Arauz-Ruiz et al., 2002). The distribution of genotypes throughout the world has been confirmed, with genotype A being predominant in North
Africa and north-western Europe, genotype B in South-East Asia, genotype C in North-East Asia, genotype D in northern Europe and the Middle East, genotype E in Africa, genotype F in South America and genotype G in France, Germany, the USA and Mexico (Magnius & Norder, 1995; Lindh et al., 1997; Stuyver et al., 2000). Genotype H was recently reported to be detected in South and Central America (Arauz-Ruiz et al., 2002).

There have been some reports that clinical outcomes vary with HBV genotypes. For example, for genotypes B and C, which are characteristic of Asia, genotype B has been found to cause HBe seroconversion more frequently than genotype C, and those infected with HBV/B appear to have better prognoses (Sunitha et al., 2000).

The division of HBV/B into four subgenotypes, B1, B2, B3 and B4, based on the sequence divergence, has recently been reported (Norder et al., 2004). Ba (B2, B3 and B4) has recombined with genotype C and has a higher prevalence of HBe antigen, so it manifests a more severe clinical course than Bj (B1) (Sugauchi et al., 2002). Thus, HBV needs to be examined with regards not only to its genotype, but also to its subgenotypes.

The Philippines are located in South-East Asia, where HBV is hyperendemic. It is reported that about 10% of the population of the Philippines are HBV carriers, and that chronic HBV infection is a major health problem (Lingao et al., 1989), although to our knowledge, few data on HBV genotype distribution in the country are available. We obtained sera containing HBV isolates from carriers in the Philippines and investigated their features.

The serum samples were provided by St Luke’s Medical Center, Quezon City, the Philippines. Quezon City has a population of about 2 100 000 and is the biggest of the cities in the Metro Manila, the capital of the Republic of the Philippines. The patients were HBV carriers being followed at St Luke’s. All patients were negative for antibody to Hepatitis C virus and human immunodeficiency virus and were not intravenous drug abusers. The five patients were a 30-year-old male, a 48-year-old female, a 21-year-old male, a 60-year-old male and a 54-year-old male. They were all positive for HBe antigen and negative for anti-HBe. We explained the purpose of this study and collected serum samples with informed consent.

For amplification of HBV DNA by PCR, nucleic acids were extracted from 200 μl serum as described previously (Niitsuma et al., 1995). For analysis of the entire nucleotide sequence, we divided the entire HBV genome into six overlapping segments and amplified each segment. Extracted DNA was subjected to the first round of PCR with each set of primers. PCR was performed with TaKaRa Ex Taq for 35 cycles (consisting of denaturation for 1 min at 93 °C, annealing for 1 min at 55 °C and extension for 1 min at 74 °C), followed by an extension cycle at 74 °C for 8 min. The second round of PCR was carried out for 30 cycles consisting of the same protocol as those in the first round. The primers for the first and the second PCR rounds were as reported previously (Shan et al., 2002).

We used the standard numbering system in this report, the numbering of the bases commencing at the cleavage site for the restriction enzyme EcoRI in the preS2 region and the full length of 3215 bp being counted.

We used each set of sequencing primers for nucleotide sequences of HBV isolates shown previously (Shan et al., 2002). Direct sequencing of the PCR products was carried out by a fluorescence autosequencer (model 377; Perkin Elmer Applied Biosystems), using a BigDye Terminator Sequencing kit (Perkin Elmer Applied Biosystems) according to the manufacturer’s instructions. Six overlapping segments were joined and phylogenetic analysis of the sequences of HBV clones was performed by the neighbour-joining method with the aid of CLUSTAL W (http://www.ddbj.nig.ac.jp/search/clustalw-j.html).

We compared the clones determined in our present study with eight reported HBV clones and confirmed their genotype to be B by phylogenetic analysis. The names of clones and the genotypes of their HBV sequences used in the

Table 1. Percentage divergences of entire nucleotide sequences among HBV/B isolates from five subgenotypes

<table>
<thead>
<tr>
<th>HBV/B subgenotype</th>
<th>GenBank accession no.</th>
<th>Country of origin</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. B5</td>
<td>AB219426</td>
<td>Philippines</td>
<td>–</td>
<td>1.5</td>
<td>1.5</td>
<td>1.0</td>
<td>3.8</td>
<td>4.5</td>
<td>3.0</td>
<td>4.9</td>
<td>5.6</td>
</tr>
<tr>
<td>2. B5</td>
<td>AB219427</td>
<td>Philippines</td>
<td>–</td>
<td>2.0</td>
<td>1.7</td>
<td>4.2</td>
<td>4.7</td>
<td>3.5</td>
<td>5.1</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td>3. B5</td>
<td>AB219428</td>
<td>Philippines</td>
<td>–</td>
<td>1.7</td>
<td>3.8</td>
<td>4.4</td>
<td>2.9</td>
<td>4.7</td>
<td>5.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. B5</td>
<td>AB219429</td>
<td>Philippines</td>
<td>–</td>
<td>4.0</td>
<td>4.8</td>
<td>3.3</td>
<td>5.0</td>
<td>5.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. B3</td>
<td>AB219430</td>
<td>Philippines</td>
<td>–</td>
<td>5.0</td>
<td>3.4</td>
<td>5.4</td>
<td>6.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. B4</td>
<td>AB031267</td>
<td>Vietnam</td>
<td>–</td>
<td>4.2</td>
<td>4.0</td>
<td>5.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>7. B3</td>
<td>AB033554</td>
<td>Indonesia</td>
<td>–</td>
<td>4.8</td>
<td>5.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8. B2</td>
<td>AF282917</td>
<td>China</td>
<td>–</td>
<td>4.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9. B1</td>
<td>D00329</td>
<td>Japan</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
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Fig. 1. Phylogenetic tree of entire nucleotide sequences constructed by the neighbour-joining method using the present five isolates, HBV/B isolates and other HBV genotype (A, C, D, E, F, G and H) isolates retrieved from DDBJ/GenBank. Bootstrap values are indicated for each group.
analysis were as follows: GenBank AB014370 (genotype A), X97850 (genotype B), X75665 (genotype C), J02203 (genotype D), X75664 (genotype E), X75663 (genotype F), AF160501 (genotype G) and AY090457 (genotype H).

We used four representative clones from four subgenotypes of HBV/B, i.e. B1–B4, in addition to the present HBV/B clones from the Philippines for the study of the divergences in the entire sequences among the subgenotypes of HBV/B in Asian countries. The GenBank accession numbers of HBV sequences and countries from which they were derived were as follows: D00329 (Japan, B1); AF282917 (China, B2); AB033554 (Indonesia, B3); and AB031267 (Vietnam, B4).

We compared the precore and core sequences of our five clones from the Philippines and four previous isolates from subgenotypes B1–B4 with HBV/C and HBV/B1 by following the method of Sugauchi et al. (2002, 2003). The GenBank accession numbers and the countries from which they were derived are as follows: HBV/C, AB014378 (Japan) and D23684 (Japan); HBV/B1, AB073845 (Japan) and AB073855 (Japan).

Entire nucleotide sequences of five HBV/B isolates from the Philippines were determined. They were registered with DDBJ/GenBank under the accession numbers AB219426, AB219427, AB219428, AB219429 and AB219430.

Serotypes were determined from aa 122 and 160 in the HBs gene. GenBank AB219430 was found to be of serotype adw and AB219426, AB219427, AB219428 and AB2194294 were of serotype ayw.

We constructed a phylogenetic tree by using the entire nucleotide sequences determined from the five isolates, other HBV/B isolates retrieved from DDBJ/GenBank and additional representative genotypes (A, C, D, E, F, G and H). The HBV isolates are illustrated in Fig. 1, the HBV/B isolates detected worldwide being divided into five groups: subgenotypes B1, B2, B3, B4 and the Philippines group. We suggest the latter to be a new HBV/B subgenotype, B5. Among the present five isolates, four isolates belonged to subgenotype B5 and the other was subgenotype B3.

Divergences in the entire genome sequences of the present five isolates with HBV/B subgenotype isolates registered with GenBank are shown in Table 1. The four isolates in subgenotype B5 showed 1–0 to 2–0 % divergence from each other. Among the sequences registered with GenBank, AB033554 (subgenotype B3, serotype adw) from Indonesia showed the lowest divergences with the four isolates, 2–9–3–5 %. These four isolates showed divergences of 3–8–4–2 % with GenBank AB219430 and, similarly, 4–4–4–8 % with Vietnam (B4), 4–7–5–1 % with China (B2) and 5–4–6–0 % with Japan (B1).

On the other hand, similar examination showed that GenBank AB219430 has the lowest divergence, i.e. 3–4 %, with AB033554 (B3). Furthermore, GenBank AB219430 showed 5–0 % divergence from isolates from Vietnam (B4), 5–4 % with China (B2) and 6–1 % with Japan (B1).

In addition, we constructed a phylogenetic tree by using the partial nucleotide sequences (369 bp in the HBs region) determined from 30 HBV/B isolates from the Philippines (Fig. 2). As for the four HBV/B subgenotypes, they were not as obvious in this tree as in that constructed from entire nucleotide sequences. To the contrary, the 17 isolates constructed an obvious subgroup with the serotype ayw, with one exception, which is characteristic for B5.

Similar to the study reported by Sugauchi et al. (2002), a comparison of nucleotide similarities from positions 1814 to 2452 in the precore and core gene of the present five isolates from the Philippines and representative HBV/B isolates from the other four subgenotypes, B1–B4, with isolates from HBV/C and B1 is shown in Supplementary Table S1 in JGV Online. The divergences confirmed that HBV/B in the Philippines had recombed with HBV/C in the precore and core gene, like in B2, B3 and B4. In this study, we determined the entire nucleotide sequences of five HBV/B isolates from the Philippines, where data on HBV have been scant.

We showed that HBV/B isolates from all over the world, including the Philippines, can be divided into five subgroups in the phylogenetic tree based on entire nucleotide sequences, whose geographical distances and genetic distances are well-correlated. There were long genetic distances among the subgroups, i.e. subgenotypes. In general, subgenotypes vary by > 4–0 % at the nucleotide level over the entire genome, as described by Norder et al. (2004) and Kramvis et al. (2005). In our study, the divergence of the entire-genome nucleotide sequences between GenBank AB219427 (B5) and D00329 (B1) was 6–0 %, that with AF282917 (B2) was 5–1 %, that with AB033554 (B3) was 3–5 % and that with AB031267 (B4) was 4–7 %.

We suggest that the subgroup composed of four HBV/B isolates from the Philippines is a new subgenotype, B5, for the following three reasons. First, a significant bootstrap value was confirmed on the phylogenetic-tree analysis. Second, the divergences between B5 and the other four subgenotypes, B1, B2, B3 and B4, were nearly 4 %. Third, as for the serotypes, B3 was adw and B5 was ayw. We confirmed an additional 25 HBV clones in the Philippines to be genotype B from the sequence analysis of 369 bp (nt 278–646) in the HBs region. Phylogenetic-tree analysis of partial nucleotide sequences also showed the subgenotype B5. Seventeen clones among the 30 isolates, including the current four isolates, belonged to B5. Their serotypes were ayw with one exception (adw). This may be derived from just one point mutation in the HBsAg region. HBV infection is known to show clinical differences among genotypes. Therefore, these five HBV/B subgenotypes might show clinical differences, as previously reported for B1 (Bj) and B2 (Ba) (Orito et al., 2001).

This is the first report of the entire nucleotide sequences of HBV/B from the Philippines, where there have been scant data on HBV. It shows that they belong to a novel HBV/B.
Fig. 2. Phylogenetic tree of partial nucleotide sequences (nt 278–646 in the HBs region) constructed by the neighbour-joining method using the present 30 isolates, HBV/B isolates and other HBV genotype (A, C, D, E, F, G and H) isolates retrieved from DDBJ/GenBank. The 25 isolates are described as PHL-number-serotype. Isolates classified in different subgroups on the basis of phylogenetic analysis by entire and partial nucleotide sequences are marked with an asterisk.

New HBV subgenotype B5 in the Philippines
subgenotype, B5. Further studies, including other genotypes and their clinical relevance, should be conducted.

References


Sugauchi, F., Orito, E., Ichida, T. & 10 other authors (2003). Epidemiologic and virologic characteristics of hepatitis B virus genotype B having the recombination with genotype C. *Gastroenterology* 124, 925–932.