Hepatitis B virus genotype G epidemiology and co-infection with genotype A in Canada

Carla Osiowy,1,2 Diane Gordon,1 Jamie Borlang,1 Elizabeth Giles1 and Jean-Pierre Villeneuve3

1Bloodborne Pathogens and Hepatitis, National Microbiology Laboratory, Winnipeg, MB, Canada
2Section of Hepatology, Department of Internal Medicine, University of Manitoba, Winnipeg, MB, Canada
3Hôpital Saint Luc du Centre Hospitalier, Universitaire de Montréal, Montréal, QC, Canada

Hepatitis B virus (HBV) genotype G (HBV/G) is an unusual variant, and little is known about its epidemiology and natural history, particularly the requirement for a co-infecting HBV genotype and their relationship during infection. This study investigated the quasispecies nature of co-infecting genotypes in 39 samples collected over a 6 year period from 13 HBV/G-infected patients. HBV/G infections were found to occur predominantly in males (92%) and were primarily associated with male homosexual sex (67%). All patients were infected with HBV/G and HBV/A, or a recombinant HBV/A/G strain. Co-infecting genotypic prevalence was often observed to fluctuate over time, with periods of HBV/G monoinfection in some patients. The average sequence divergence among Canadian HBV/G strains was 1.57 ± 0.62%. Thus, all HBV/G infections in Canada occur in the context of co-infection or recombination with HBV/A, and strains display increased sequence divergence compared with all known HBV/G sequences described to date.

Hepatitis B virus (HBV) genotype G (HBV/G) has only recently been discovered (Stuyver et al., 2000); it is characterized by a unique 36 bp insertion downstream of the core gene start codon and by mutations preventing expression of hepatitis B e-antigen (HBeAg) (Kato et al., 2002a). Epidemiological and clinical data regarding this genotype are limited, likely due to the low prevalence of HBV/G throughout the world (Tanaka & Mizokami, 2007). HBV/G infection has been frequently reported to be detected along with a co-infecting functional HBV strain, such as genotype A (Kato et al., 2002a) or genotype H (Sánchez et al., 2007); however, HBV/G monoinfection has also been described (Alvarado-Esquivel et al., 2006; Chudy et al., 2006; Pas et al., 2008). Recombination between HBV/G and the co-infecting genotype has been observed, but normally as a minority species within the HBV quasispecies population (Kato et al., 2002b). HBV/G was found to be a determinant of liver fibrosis in immunocompromised individuals (Lacombe et al., 2006) and chimeric mice carrying human hepatocytes (Sugiyama et al., 2007; Tanaka et al., 2008), thus suggesting a pathogenic characteristic.

In order to understand the prevalence of co-infection involving HBV/G and the relationship between the co-infecting strains better, HBV/G isolates from Canadian patients with acute or chronic HBV infection were investigated by clonal and phylogenetic analysis. Epidemiological and clinical associations of the HBV/G strains were also determined.

HBV/G-infected individuals were identified through the results of routine molecular diagnostic service at the National Microbiology Laboratory (NML) and through the Canadian enhanced hepatitis strain surveillance system (EHSSS). Clinical, demographic and risk factor data were obtained from the sending laboratory or physician. HBV DNA was extracted from clinical samples by silica gel filtration (QIAamp DNA Blood Mini kit; Qiagen) or by phenol/chloroform extraction methods to optimize sensitivity (Osiowy, 2002). Extracted DNA was amplified for genotyping, clonal and phylogenetic analysis using hepatitis B s-antigen (HBsAg)/polymerase coding region-specific primers: Spr1A (5’-GTCAGGAACAGTAA-GCCC-3’) and antisense (5’-GAAAGCCCTTGAATGG-GCC-3’). Where necessary, the following primers were used for nested PCR: sense (5’-GGTTGGACTTCTCTCAA-TTTCCTAGG-3’) and Spr2A (5’-ACTTTCCAATCAATAGCC-3’), using conditions described previously (Osiowy et al., 2006). Full-length genome amplification of certain samples was performed using a thermostable DNA polymerase blend (Expand High FidelityPlus System;
Roche) and primers P1 (5′-CCGGAAAGCTTATGCTC-TTCTTTTTCACCTCTGCTTATCATC-3′) and P2 (5′-CCGAGAGCTCATGCTCTTCCTAAAGTGGCATGGTGC-CTGTTG-3′), as described by Günther et al. (1995). The precore region of each co-infecting HBV/A strain was also amplified using the primers and methods described by Takahashi et al. (1995), to determine the presence of a mutation at nt 1896. The HBV viral load for samples from chronically infected patients was determined by the NML or the provincial public health laboratory from which the sample originated. Viral load was determined by using either the Digene Hybrid Capture System (Digene) in copies ml⁻¹, the Versant HBV bDNA Assay (Siemens Healthcare Diagnostics) in copies ml⁻¹ or the Cobas TaqMan Assay (Roche Molecular Systems) in IU ml⁻¹. The approximate conversion factor for copies ml⁻¹ to IU ml⁻¹ is 5 (Saldanha et al., 2001).

Amplicons were gel-purified prior to being sequenced or cloned using a TA-based ligation method (pDrive cloning vector; Qiagen). The directly amplified product and approximately 20–30 clonal inserts were sequenced to detect majority and minority genotype populations and to estimate their ratio. The genotype of each directly amplified and clonal sequence was initially determined by the NCBI HBV Genotyping Tool (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi) (Rozanov et al., 2004). In order to detect minority populations in directly amplified samples in a more sensitive manner to sequencing, reverse hybridization probe analysis using the line probe assay (LiPA) kit (INNO-LiPA HBV Genotyping; Innogenetics) was also performed on all directly amplified sequences, according to the manufacturer’s instructions.

Directly amplified sequences were assembled and analysed using DNA sequence analysis software (Lasergene software suite v7.1.0, DNASTAR). Sequence alignments were performed using CLUSTAL_X v1.8 (Thompson et al., 1997) and trimming of alignment ends to obtain the equivalent length sequences for each sample (411 bp; nt 261–671 of the HBsAg gene) was performed using BioEdit v5.0.9 (Hall, 1999). Sequence divergence was calculated based on the number of nucleotide changes per total number of nucleotides analysed (411 bp) in the alignment using MEGALIGN software (Lasergene v7.1.0). Pairwise genetic distances were estimated by Kimura two-parameter analysis and a phylogenetic tree was constructed by the neighbour-joining method with 1000 bootstrap replicates using MEGA software v4 (Tamura et al., 2007). Bootscan analysis software (SimPlot v3.5.1) was used to identify intergenotypic recombination (Lole et al., 1999). The full-length HBV genome sequences of patients 5 and 6 were queried against six GenBank reference sequences for each of genotypes A and G (potential parental) and six GenBank reference sequences for genotype D (outgroup control). One or two GenBank reference sequences for all other HBV genotypes were also included to assess informative sites for evidence of recombination with genotypes other than the suspected parents.

Thirteen patients infected with HBV/G were identified; three acutely infected patients were identified through the EHSSS, while 10 chronic HBV patients were identified through diagnostic HBV genotype testing. The HBV/G strains represented approximately 1.2% (3/244) of all acute cases and 2.3% (11/482) of all chronic HBV cases genotyped at the NML over a 6 year period, from 2001 to 2007. However, as initial detection of cases was by amplicon direct sequencing, samples with levels of HBV/G below the limit of sequence detection may have been missed. A total of 39 patient samples were collected during this period; 6 month consecutive samples were available for seven patients (33 of 39 samples).

HBV/G was observed in patients from three different provinces in Canada (Table 1). The majority of infections occurred in males (11/12; gender, age and risk factor was unavailable for one patient) having a median age of 46.5 years. The single female patient was 25-years-old and acutely infected. At the time of testing, seven of the 10 chronic patients were HBeAg-positive and eight patients were receiving adefovir therapy added to lamivudine therapy (all were genotypically and phenotypically resistant to lamivudine). Most HBV/G-infected chronic patients had significant viral load, which appeared to decrease over time in those patients on therapy (Table 1). Male homosexual sex (MSM) was the predominant risk factor associated with HBV/G infection (8/12; 67%).

In order to determine whether a co-infecting HBV genotype was present in each patient infected with genotype G, LiPA and clonal analysis was performed for each patient sample. As shown in Fig. 1, all patients were found to be co-infected with genotypes G and A by either LiPA or clonal analysis or both. Often, LiPA exhibited greater sensitivity than clonal analysis, allowing both co-infecting genotypes to be detected. However, certain samples only demonstrated a single genotype by LiPA. This observation may be due to the co-infecting genotype falling below the level of LiPA sensitivity (for example, patients 9 and 11) or, in those samples having a dominant recombinant population (for example, patient 5), the HBsAg-coding region, hybridized by LiPA primers and probes, is HBV/G sequence specific. Genotype A and G populations were observed to fluctuate over time for several patients for whom consecutive samples were available. A complete switch in dominance of the genotype population was also observed with patient 11, from HBV/G to primarily HBV/A and back to primarily HBV/G, over the period of 42–72 months following the initiation of therapy (Fig. 1, see samples 11h, 11i and 11m). To explain the observation of HBeAg positivity in the HBV/G co-infected patients, the precore region of each co-infecting strain was sequenced. Wild-type sequence (G at nt 1896) was observed with all HBV/A co-infecting strains, except for the patient 4 genotype A sequence which was mutated (A at nt 1896).

HBV/G was frequently present as a minority species; however, for patients 9 and 11, putative HBV/G mono-
infection was observed at certain time points during antiviral therapy (at baseline, 6 and 18 months following initiation of therapy and at 6 and 18–42 months following initiation of therapy, respectively). At these time points, only HBV/G was observed by both LiPA analysis and following screening of an average of 30 clones (Fig. 1). This observation was likely due to the co-infecting genotype A falling below the level of detection sensitivity, as the patients clearly demonstrated HBV/A co-infection at an earlier time point. Full-length HBV genome sequence was determined for patients 5 and 6, who were observed to have a majority population of recombinants involving HBV/G with HBV/A (GenBank accession nos EU833889 and EU833890). Bootscan analysis determined that the breakpoints for both isolates were completely different (Supplementary Fig. S1, available in JGV Online).

Phylogenetic analysis was performed on all 39 directly amplified HBsAg sequences (GenBank accession nos EU833892–EU833930) to determine interrelatedness within Canadian HBV/G isolates and among HBV/G strains throughout the world. Canadian HBV/G strains all clustered within the HBV/G clade (Fig. 2), although for several patient time point samples, only the HBV/A sequence was directly amplified (10c–e and 11i). These sequences clustered with GenBank-derived HBV/A strains, although HBV/G was detectable in each sample by LiPA analysis but not by sequencing of the directly amplified product. Phylogenetic analysis of the HBsAg-coding region of co-infecting clonal sequences determined that all HBV/A co-infecting strains were subgenotype A2 (data not shown).

Within the HBsAg-coding region that was investigated (nt 261–671), the mean sequence divergence among the 35 HBV/G Canadian sequences was 1.57 ± 0.62 % (mean genetic distance 0.017 ± 0.003), while in comparison, the mean sequence divergence among all 33 GenBank HBV/G sequences, originating from Europe, North and South America and Asia, was 0.87 ± 0.51 % (mean genetic distance 0.007 ± 0.002), which lacked significance (calculated by Fisher’s exact test; P=0.3) due to the short region of the sequence that was analysed. A more accurate measure of genetic diversity would be derived by full-length genome analysis among the HBV/G sequences.

Despite the apparent low prevalence of HBV/G throughout the world, the viral genotype appears to circulate globally, as it has been reported in several European countries (De Maddalena et al., 2007; Jardi et al., 2008; Lacombe et al., 2006; Tran et al., 1991; Vieth et al., 2002), North and South America (Alvarado-Esquivel et al., 2006; Bottecchia et al., 2008; Kato et al., 2004; Osiowy & Giles., 2003; Sánchez et al., 2007), Nigeria (Oliger et al., 2006) and Asia (Shibayama et al., 2005; Suwannakarn et al., 2005; Toan et al., 2006). Canadian HBV/G was highly associated with

### Table 1. Demographic, clinical and risk factor association of Canadian HBV/G

Patient numbers in bold refer to those patients for which consecutive 6-month samples were available. NA, Data not available.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Province</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Disease state*</th>
<th>Viral load†</th>
<th>HBeAg‡</th>
<th>Risk factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>British Columbia</td>
<td>NA</td>
<td>NA</td>
<td>Acute</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Nova Scotia</td>
<td>F</td>
<td>25</td>
<td>Acute</td>
<td>NA</td>
<td>NA</td>
<td>Born in endemic country (Vietnam)</td>
</tr>
<tr>
<td>3</td>
<td>British Columbia</td>
<td>M</td>
<td>47</td>
<td>Acute</td>
<td>&lt;1.4 x 10^5</td>
<td>NA</td>
<td>MSM, injection drug user</td>
</tr>
<tr>
<td>4</td>
<td>Quebec</td>
<td>M</td>
<td>56</td>
<td>Chronic (ADV + LAM)</td>
<td>7 x 10^-4–9.4 x 10^5</td>
<td>NA</td>
<td>None reported</td>
</tr>
<tr>
<td>5</td>
<td>Quebec</td>
<td>M</td>
<td>40</td>
<td>Chronic (ADV + LAM)</td>
<td>9.9 x 10^-2–4 x 10^-4</td>
<td>NA</td>
<td>MSM</td>
</tr>
<tr>
<td>6</td>
<td>Quebec</td>
<td>M</td>
<td>46</td>
<td>Chronic (ADV + LAM)</td>
<td>1.2 x 10^-6–4.5 x 10^-6</td>
<td>NA</td>
<td>MSM</td>
</tr>
<tr>
<td>7</td>
<td>Quebec</td>
<td>M</td>
<td>48</td>
<td>Chronic (ADV + LAM)</td>
<td>3.9 x 10^-5–5.8 x 10^-5</td>
<td>NA</td>
<td>MSM</td>
</tr>
<tr>
<td>8</td>
<td>Quebec</td>
<td>M</td>
<td>64</td>
<td>Chronic (ADV + LAM)</td>
<td>1.4 x 10^-8–2.8 x 10^-8</td>
<td>NA</td>
<td>Unprotected sex (male homosexual)</td>
</tr>
<tr>
<td>9</td>
<td>Quebec</td>
<td>M</td>
<td>70</td>
<td>Chronic (ADV + LAM)</td>
<td>&gt;1.7 x 10^-1–1.4 x 10^-1</td>
<td>NA</td>
<td>Born in endemic country (Cambodia)</td>
</tr>
<tr>
<td>10</td>
<td>Quebec</td>
<td>M</td>
<td>37</td>
<td>Chronic (ADV + LAM)</td>
<td>1.7 x 10^-2–&lt;1.4 x 10^-2</td>
<td>NA</td>
<td>MSM</td>
</tr>
<tr>
<td>11</td>
<td>Quebec</td>
<td>M</td>
<td>52</td>
<td>Chronic (ADV + LAM)</td>
<td>8.9 x 10^-3</td>
<td>NA</td>
<td>MSM</td>
</tr>
<tr>
<td>12</td>
<td>Nova Scotia</td>
<td>M</td>
<td>42</td>
<td>Chronic</td>
<td>3.6 x 10^-4</td>
<td>NA</td>
<td>MSM</td>
</tr>
<tr>
<td>13</td>
<td>Nova Scotia</td>
<td>M</td>
<td>33</td>
<td>Chronic</td>
<td>3.6 x 10^-4</td>
<td>NA</td>
<td>MSM</td>
</tr>
</tbody>
</table>

* Patients on treatment with adefovir (ADV) and lamivudine (LAM) are indicated.
† Range of viral load for consecutive samples from first consecutive sample (baseline) to last time point sample.
‡ Positive and negative HBeAg results are indicated by + and −, respectively.
§ Copies ml⁻¹. Viral load determined by Digene Hybrid Capture System (Digene).
∥ Copies ml⁻¹. Viral load of last time point sample determined by Versant HBV DNA Assay (bDNA; Siemens Healthcare Diagnostics). Initial time point sample determined by Digene Hybrid Capture.
¶ IU ml⁻¹. Viral load determined by Roche Cobas TaqMan Assay (Roche Molecular Systems).
MSM as a risk factor for infection, corresponding to previous studies (Bottecchia et al., 2008; Shibayama et al., 2005; Sánchez et al., 2007). When information is available regarding HBV/G co-infection, the co-infecting genotype is normally that which is highly prevalent within the geographical region, i.e. genotype A in Europe and North America, genotype H in Mexico and genotype C in Vietnam, thus suggesting that HBV/G is capable of co-infecting with any other HBV genotype. Results of the present study show that Canadian HBV/G infection occurs exclusively in the context of HBV/A co-infection, although genotypic dominance and the genotype population ratio appear to fluctuate over time. It is important to note that the patients, for whom longitudinal samples were available, were all being treated with adefovir and lamivudine. Antiviral therapy may have influenced the quasispecies population to initiate a switch in genotypic dominance. This has been suggested following the selection of specific strains during treatment of HBV/A/D co-infected patients (Hannoun et al., 2002; Jardi et al., 2008).

Clinical monoinfection with HBV/G has been suggested following LiPA analysis of an acutely infected blood donor and the corresponding platelet recipients (Chudy et al., 2006), and by LiPA analysis of Mexican (Alvarado-Esquivel et al., 2006) and European (Pas et al., 2008) chronic carriers. HBV/G has also been shown to be capable of replication in the absence of a co-infecting genotype following in vitro transfection of Huh7 cells (Li et al., 2007) or uPA/SCID mouse infection (Sugiyama et al., 2007; Tanaka et al., 2008), albeit at close to undetectable levels. However, results of the present study suggest that the observation of monoinfection by LiPA or clonal analysis is due to the co-infecting genotype falling below the level of detection sensitivity. In both cases of apparent monoinfection, the patient was determined to be co-infected at an earlier and later time point during infection. This observation, together with the findings from uPA/SCID mouse studies, suggests that chronic infection with HBV/G can only occur in the context of co-infection with another HBV genotype, and thus would be transmitted as such.
Fig. 2. Phylogenetic analysis of the HBsAg coding region (411 bp; nt 261–671) of each patient isolate. Thirty-nine patient sequences were aligned with representative sequences of all eight HBV genotypes available from GenBank, including 33 HBV/G sequences (shown as the GenBank accession number followed by the country of origin for each strain). Longitudinal, consecutive patient samples are numbered as described for Fig. 1. Bootstrap confidence values of 60% or greater are shown. Bar, Branch length for a pairwise distance equal to 0.005.
Recombination between the co-infecting genotypes was frequently observed among the genotypic population of each patient, and HBV/G/A was present as the dominant strain in two patients. Recombinant genomes demonstrated variable breakpoints within the polymerase, preS1 and HBsAg coding regions, as well as within the X gene. Several of these regions have been described as putative ‘hot spot’ sites of recombination (Simmonds & Midgley, 2005; Yang et al., 2006), although a breakpoint within the X coding region has not yet been described.

HBV genotype G has been described as being genetically homologous due to the observed low nucleotide divergence among globally distributed isolates (Lindh, 2005; Tanaka et al., 2008). However, the Canadian HBV/G HBsAg-coding sequences (411 bp) examined in this study were found to have a mean genetic distance over twofold greater than HBV/G sequences described to date (0.017 versus 0.007). Increased sequence divergence may be the result of antiviral therapy, as an increased mutation rate within the HBsAg/polymerase overlap region is known to occur during treatment with lamivudine (Conzelmann et al., 2005). This selection pressure likely contributes to increased spontaneous mutation as well as quasispecies selection of more fit minor strains. However, phylogenetic analysis that included only the first available sample from each of the 13 patients (data not shown) still resulted in a mean intragroup distance of 0.015. Therefore, global intragenotypic HBV/G homology, at least within the HBsAg-coding region, may be closer to 96.8–98.9%. This remains a very high sequence identity among such disparate strains, with respect to geography and co-infecting genotypes, and thus raises the interesting question as to the mechanisms controlling HBV/G evolution.

Acknowledgements

The investigators are grateful to Dr Todd Hatchette, Director, Virology and Immunology, Queen Elizabeth II Health Sciences Centre, Halifax, Nova Scotia, and to Jessica Ip Chan, Vancouver Coastal Health, Vancouver, British Columbia, for providing patient information.

References


Osiowy, C., Villeneuve, J., Heathcote, J., Giles, E. & Borlang, J. (2006). Detection of rtN236T and rtA181T/T mutations associated with resistance to adefovir dipivoxil in samples from patients with...


