Multiple genotypes and subtypes of hepatitis B and C viruses in Belarus: similarities with Russia and western European influences

C. M. Olinger\textsuperscript{1}, N. V. Lazouskaya\textsuperscript{2}, V. F. Eremin\textsuperscript{2} and C. P. Muller\textsuperscript{1}

\textsuperscript{1}Institute of Immunology, National Public Health Laboratory, Luxembourg, Luxembourg and
\textsuperscript{2}Institute for Epidemiology and Microbiology, Minsk, Belarus

ABSTRACT

The Republic of Belarus reports a seroprevalence of 4.8\% for hepatitis B virus (HBV) and 1.26\% for hepatitis C virus (HCV), but little is known about the molecular characteristics of the circulating viruses. This study analysed 69 HBV surface antigen (HBsAg)-positive and 113 anti-HCV-positive donors attending a national reference hospital in Minsk. Among the HCV patients, 70\% were co-infected with human immunodeficiency virus (HIV). Phylogenetic analysis of 12 complete genomes and 31 partial HBV sequences, as well as 78 core/E1 HCV sequences, revealed that multiple genotypes and subtypes of both viruses were circulating in Belarus. Of the HBV strains, 11.6\% were genotype A2 and 88.6\% were genotype D. The genotype D strains segregated into four recently described subtypes, with D2 being the most prevalent (58.1\%), followed by D3 (16.3\%), D1 (11.6\%) and D4 (2.3\%), but with inter-subtypic distances lower than the minimal 4\% distance proposed to define subtypes. The 78 HCV strains belonged to subtypes 1b (53.8\%), 3a (38.5\%), 1a (5.1\%), 4a (1.3\%) and 4d (1.3\%). Subtype 1b was less prevalent (45.1\% vs. 70.4\%) among HCV/HIV co-infected donors, while subtype 3a was more prevalent (29.6\% vs. 43.1\%). The relative prevalence of HBV and HCV genotypes in Belarus corresponded to the prevalence in Russia, although with a clear European influence that reflected the socio-political context of the country.

Keywords Belarus, epidemiology, genotypes, hepatitis viruses, molecular phylogeny, subtypes

Original Submission: 3 December 2007; Revised Submission: 9 January 2008; Accepted: 12 January 2008

Clin Microbiol Infect 2008; 14: 575–581

INTRODUCTION

With an estimated 387 million individuals worldwide who are chronically infected with hepatitis B virus (HBV), and a further 170 million who are infected with hepatitis C virus (HCV) [1,2], these infections pose major public health problems. There is increasing evidence that the risk of developing severe liver disease, and the varying response to antiviral treatment, is influenced by the virus genotypes and subtypes involved [3–5].

HBV has been classified phylogenetically into eight genotypes (A–H) with different geographical distributions [6,7]: genotype A is predominant in western Europe, North America, and South and East Africa [8,9]; genotypes B and C are prevalent in Asia [7,10]; genotype D has a worldwide distribution, with its highest prevalence in the Mediterranean region [8]; genotype E is found throughout sub-Saharan Africa [11–13]; genotype F is found in South and Central America [14]; genotype G is found in France, Georgia, the USA [15] and Germany [16]; and genotype H is found in the Amerindian populations of Central America [17].

HCV has at least six major genotypes (1–6), with more than 60 subtypes [18–20]. HCV subtypes 1a, 1b, 2a, 2b, 2c and 3a are responsible for >90\% of infections in North and South America, Europe and Japan. In the USA, subtypes 1a and 1b each account for c. 35\% of infections [21], while 73\% are caused by subtype 1b in Japan [22]. Subtype 3a is predominant among intravenous drug users in Europe and the USA [23], genotype 4 is prevalent in North Africa and the
MATERIALS AND METHODS

Clinical samples

In total, 157 serum samples were obtained during 2004–2005 from patients of known hepatitis B surface antigen (HBsAg) and anti-HCV status who were attending the Infectious Disease Hospital, Minsk, Belarus. The residents of Minsk account for c. 25% of the country’s population and are representative of the entire population. The Infectious Disease Hospital is the largest hospital in Belarus, and acts as a central referral hospital for patients from throughout the country. HBV and HCV were confirmed using the Murex HBsAg kit v.3 (Abbott Diagnostics, Louvain-la-Neuve, Belgium) and the Ortho HCV 3.0 ELISA system (Ortho-Diagnostics, Beere, Belgium). HBV DNA and HCV RNA were detected by RT-PCR and/or PCR (Table 1). Co-infection with human immunodeficiency virus (HIV) was diagnosed in 51% of all donors, including 8.7% of HBsAg carriers and 69.9% of anti-HCV-positive donors; 64.6% of the latter were positive for HCV RNA.

Amplification and cloning

Nucleic acid extraction and complete HBV genome amplification were performed as described previously [13]. For amplification of the core/E1 region of HCV, a semi-nested PCR was performed in a 25-μL reaction containing 0.5 μL cDNA, 2.5 mM MgCl₂, 200 nM dNTPs, 50 nM each primer (fw290utr(+), 5’-TGGCTCTGATAGGGTGCTTGCGAG, pos. 290–311; 1321e1(-), 5’-ACCAGTTCATCATCATATCCCATG, pos. 1321–1320), and 1 U Platinum Taq DNA polymerase with 1 x PCR buffer. PCRs comprised 95°C for 5 min, followed by 40 cycles of 95°C for 1 min, 63°C for 1 min and 72°C for 1 min. Nested PCRs were performed using the same conditions with 5 μL of the first-round product diluted 1:100, but with a different forward primer (fw480c(+), 5’-CCCGCCGACTAGGAAGACTTC, pos. 480–499; rv1321e1), 0.10 μM each primer, 2 μM MgCl₂ and an annealing temperature of 62°C. The product of the first round S fragment PCR was cloned using the pCR4-TOPO kit (Invitrogen, Hilden, Germany) according to the manufacturer’s protocol.

**Table 1.** Comparison of PCR results with the results of ELISAs for hepatitis B surface antigen (HBsAg) and anti-hepatitis C virus (HCV) antibodies

<table>
<thead>
<tr>
<th>HBV DNA PCR</th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>HBsAg</td>
<td>Positive</td>
<td>Negative</td>
<td>Total</td>
</tr>
<tr>
<td></td>
<td>42 (26.7%)</td>
<td>27 (17.2%)</td>
<td>69 (43.9%)</td>
</tr>
<tr>
<td>ELISA</td>
<td>Negative</td>
<td>1 (0.6%)</td>
<td>88 (56.1%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>114 (72.6%)</td>
<td>157</td>
</tr>
<tr>
<td>Total</td>
<td>43 (27.4%)</td>
<td>114 (72.6%)</td>
<td>157</td>
</tr>
<tr>
<td>HCV RNA RT-PCR</td>
<td>Anti-HCV</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Total</td>
</tr>
<tr>
<td></td>
<td>78 (49.7%)</td>
<td>44 (28%)</td>
<td>122 (72%)</td>
</tr>
<tr>
<td>Total</td>
<td>78 (49.7%)</td>
<td>79 (50.3%)</td>
<td>157</td>
</tr>
</tbody>
</table>

Sequencing and phylogenetic analysis

The nested and M13 PCR products were purified and sequenced as described previously [13]. Phylogenetic analysis was performed using MEGA4 [28] with the neighbour-joining and Kimura two-parameter method. HCV sequences included the core to E1 region with a total length of c. 844 bp (pos. 480–1323, according to HCV H77). For each HBV strain, phylogenetic analysis was performed for the combined preS and S fragment sequences (pos. 56–2423, according to X75664), as well as the full-length genome, if available. All sequences were submitted to EMBL/Genbank/DDBJ under accession numbers EU414031–EU414184.

RESULTS AND DISCUSSION

HBV

Overall, 44 (28%) patients were positive for HBsAg only, 88 (56%) were positive for anti-HCV antibodies only, and 25 (15.9%) were positive for both serological markers. For HBV, <10% were HIV-positive. Similar values have been reported in other countries [29]. Thirty-seven (84.1%) of the 44 HBsAg-positive samples, one (1.2%) of 88 anti-HCV-positive samples and five (20%) of 25 double-positive samples (20%) were positive according to at least one of the four nested PCRs covering the preS, S, X and C genes of HBV (Table 1) and were adequate for genotyping. One patient was HBsAg-negative but HBV DNA-positive. Complete genomic sequences were obtained for 12 strains. Phylogenetic analysis, based on the preS or the complete genome sequences, revealed that 38 (88.4%) patients were infected by genotype D and five (11.6%) by subtype A2. Despite the co-circulation of multiple genotypes in Belarus, cloning experiments revealed no evidence of mixed infections or recombinations. Subtypes of genotype D have been recognised only recently in phylogenetic studies [1]. The strains from Belarus segregated into four subtypes (Table 2): D1 (n = 5), D2 (n = 25), D3 (n = 7) and D4 (n = 1) (Fig. 1).
was no significant difference in genotype or subtype distribution between HIV-positive and HIV-negative patients. Three complete genome sequences were obtained for each of the identified subtypes except D4, for which the fragments from nucleotides 198–2418 were missing.

Analysis of sequences available from Genbank/DDBJ/EMBL revealed that countries north (Estonia, Latvia) [30] and south (Hungary, Serbia) [31,32] of Belarus had a similar distribution of genotypes A and D, with the prevalence of genotype D ranging from 71% to 82%, and that of genotype A ranging from 18% to 28%. In Estonia, Latvia and Hungary, subtype D2 was the most prevalent (50.8–66.6%), followed by subtypes D3 and D1. Further south in Serbia, subtype D3 was dominant (43.2%), followed by subtype D2 (32.9%). To the west of Belarus, e.g., in Poland [33] and the Czech Republic [34], genotype A becomes dominant over genotype D (86.5% vs. 13.5%, and 73% vs. 27%, respectively).

No sequence data were available from the latter countries. In contrast, genotype D is predominant in Russia [35], accounting for >90% of isolates, consisting mainly of subtype D2 (80.6%), followed by subtype D3 (12.9%) and subtype D1 (3.2%). Thus, as in the Baltic States, the genotype distribution found in Belarus resembles that found in Russia, although not without clear influences from western Europe. No HBV genotype data were available for Lithuania, Romania, Ukraine and Bulgaria.

Since genotype D subtypes have, to date, been described only rarely [1,36,37], the Belorussian strains were considered in the context of the proposed criteria for the definition of new subtypes. Sequence analysis of complete genomes revealed an average intra-subtypic genetic distance of 1.5% within the genotype D subtypes (subtype D4 excluded), while the maximal inter-subtypic genetic distance (subtype D4 excluded) was 2.9%, with subtype D3 being the most divergent from the other two subtypes (2.7% from subtype D1 and 2.9% from subtype D2). The average distance between subtypes D1 and D2 was 2.2% (Table 3). The inter-subtypic distances of all available strains were actually lower than the minimal 4% distance proposed to define subtypes [1], but phylogenetic reconstructions nevertheless allowed an unambiguous separation, with bootstrap values of 100% at the nodes separating the different subtypes on the phylogenetic tree calculated using complete genome sequences (Fig. 1b).

Subtypes D1 and D2 are phylogenetically close and are thought to have evolved in Europe and western Russia [1]. Subtype D3 probably originated in Russia and spread to Japan [1,38]. Subtype D4 is found predominantly in Australia and Papua New Guinea, and is also the subtype that is genetically most distant from the other genotype D subtypes. BLAST searches of complete genomes revealed that the closest relatives with known origins were: subtype D1, AB222711 from Uzbekistan; subtype D2, Z35716 from Poland; subtype D3, DQ111987 from Mongolia; and subtype A2, AB116079 from Japan. A BLAST search, based on the S gene of subtype D4, returned AB033559 from Papua New Guinea.
HCV

Almost 70% of HCV patients were co-infected with HIV, which is similar to the percentages reported in other countries [39]. In 60 of the 88 patients who were positive only for anti-HCV, and in 18 of the 25 patients who were positive for HBsAg and anti-HCV (Table 2), HCV RNA was detected and sequenced in the core/E1 region (Tables 1 and 2). Phylogenetically, the 78 strains belonged to HCV subtypes 1b (53.8%), 3a (38.5%), 1a (5.1%), 4a (1.3%) and 4d (1.3%) (Table 2; Fig. 2). A previous study in 1997 [40] reported that subtype 1b was the most prevalent subtype (76%), followed by subtype 3a (19%) and single cases of subtypes 1a and 2a, which sug-

Table 3. Distances (%) within (shown in bold) and among the different genotypes and subtypes of hepatitis B virus present in Belarus

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<th>D1</th>
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<th>D3</th>
<th>D4</th>
</tr>
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<tbody>
<tr>
<td>A2</td>
<td>0.9 (1.04)</td>
<td>1.3 (1.41)</td>
<td>1.7 (1.98)</td>
<td>NA (NA)</td>
<td></td>
</tr>
<tr>
<td>D1</td>
<td>10.5 (17.52)</td>
<td>2.2 (2.78)</td>
<td>2.9 (3.75)</td>
<td>NA (5.77)</td>
<td></td>
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<tr>
<td>D2</td>
<td>10.6 (17.52)</td>
<td>2.7 (3.18)</td>
<td>1.5 (1.48)</td>
<td>NA (5.14)</td>
<td></td>
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<tr>
<td>D3</td>
<td>10.5 (17.33)</td>
<td>2.7 (3.18)</td>
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<td>NA (5.77)</td>
<td></td>
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<tr>
<td>D4</td>
<td>NA (17.68)</td>
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gests that the prevalence of subtype 3a has increased and that fewer subtype 1b strains are now circulating. Subtypes 1b and 3a are also dominant over other subtypes in Russia, Estonia and western Europe [41]. While both subtypes have a similar prevalence (45%) in Russia, the relative prevalence of subtype 1b over subtype 3a is three-fold greater in Estonia (71% vs. 24%; [30]) and 6.5-fold higher in western Europe (58% vs. 9%). In Russia and Estonia, the prevalence of all other subtypes is <2% and <1%, respectively, while subtype 1a accounts for 20% of strains in Europe. Thus, with a 1.5-fold dominance of subtype 1b over subtype 3a, and a prevalence of 5.1%, the subtype pattern of HCV in Belarus is similar to that in Russia, although not without a clear western European influence. Subtypes 1a, 4a and 4d were found only in HIV-positive patients, while the prevalences of subtypes 1b and 3a were 70.4% and 29.6%, respectively, in HIV-negative patients, and 45.1% and 43.1%, respectively, in HIV-positive patients (Table 2). Similar studies, e.g., in Spain [42], have also revealed that subtypes 1a and 3a are more prevalent among HIV-positive donors, indicating that separate transmission networks exist for different HCV subtypes, e.g., among intravenous drug users [43].

In the HCV strains found in Belarus, the average distances at the nucleotide level were 5.7–7.1% within subtypes, and 22.4–33.8% among subtypes, with an average distance among all Belorussian strains of 21.8%. The diversity within subtype 3a in Belarus was 1.7-fold less than the diversity of all worldwide strains belonging to the same subtype, while for subtype 1b it was 1.2-fold less. Despite the higher prevalence of subtype 1b, this may indicate that subtype 3a has been circulating longer in Belarus, or that it has been introduced on multiple occasions from various sources and/or countries. BLAST searches of the different HCV strains did not reveal a closer relationship of Belorussian subtypes with other strains from the same geographical region, but indicated similarities for both subtypes 1b and 3a with strains found worldwide. For subtype 1b, this could be explained by the worldwide use of contaminated blood products, e.g., in anti-D immunoglobulin in 1977 [44]. However, for subtype 3a, circumstances similar to those in Egypt may have caused its spread [45]. At the protein level, the HCV strains did not reveal any amino-acid substitutions that were specific to strains or subtypes found in Belarus, thereby confirming that multiple introductions from abroad had probably occurred.

In conclusion, HBV genotype D strains in Belarus form phylogenetic clusters (D1–D4) that are compatible with the four subtypes proposed recently, although the inter-subtypic distances may be lower than required. The relative prevalence of genotypes of both HBV and HCV in Belarus reflect the frequencies found in Russia, although with clear European influences, possibly explained by the socio-political context of the country. Surprisingly, the virus variants do not seem to be related to those from neighbouring countries.

ACKNOWLEDGEMENTS

Support by the Ministry of Foreign Affairs of the Grand-Duchy of Luxembourg and the Centre de Recherche Public – Santé is gratefully acknowledged. C. Olinger was supported by a BFR fellowship of the Ministry of Research. The authors declare that they have no conflicts of interest in relation to this work.

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