

# Hepatitis B and C Virus Variants in Long-Term Immunosuppressed Renal Transplant Patients in Latvia

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## Key Words

Hepatitis B virus · Hepatitis C virus · Genotype · Subtypes · Variant · Mutation

## Abstract

The incidence of genome variants of hepatitis B and hepatitis C viruses among 38 long-term (2–15 years) immunosuppressed patients after renal transplantation and 10 patients undergoing dialysis was investigated. Twelve patients had only HBV infection, 9 had only HCV infection and 14 were co-infected. Regions corresponding to the HBV X/EnII/BCP, preC/C, preS/S and to the HCV core were sequenced for molecular characterization of the HBV and HCV genomes. Fifty-seven percent of HBV DNA isolates belonged to genotype D and 42% to genotype A, whereas 77% of HCV RNA isolates belonged to genotype 1b and only 17% to genotype 3a. One sample (6%) was of genotype 2c. Detailed analysis of the above-mentioned HBV genome regions revealed the presence of nucleotide point mutations, which, in some cases, resulted in amino acid substitutions. The clinical significance of such mutations is discussed.

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## Introduction

Renal transplantation is a common treatment modality for patients with end-stage renal disease. Liver disease or liver failure is common in renal transplant recipients and is a significant cause of death in 8–28% of long-term survivors [1]. The major cause of liver failure is chronic hepatitis B virus (HBV) and/or hepatitis C virus (HCV) infection. Most HBV- and HCV-positive kidney transplant recipients have become infected during dialysis before transplantation [2]. Infection can also be acquired through blood transfusions or transplantation of organs from infected donors. Before the introduction of vaccination against HBV, renal transplant recipients were often infected with both HBV and HCV. This co-infection is associated with a poor clinical outcome [3]. Immunosuppression after transplantation plays a significant role in the progression and outcome of the HBV and HCV infection in transplant recipients. Numerous studies support the concept that suppression of the cellular immune response against HBV infection may diminish acute hepatic injury, while ultimately worsening the clinical course of infection by preventing viral clearance [4].

In recent years, increasing attention has been focused on the impact of variant HBV and HCV strains on the

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clinical course of acute or chronic liver infection. Mutant HBV variants can exhibit enhanced virulence and increased levels of HBV replication, resistance to antiviral therapies (e.g., interferon- $\alpha$  or nucleoside analogues), facilitated cell attachment/penetration, or alteration of epitopes important for the host immune response. Mutations associated with increased virulence appear in the following HBV regions: gene X/Enhancer II (EnII)/BCP (basal core promoter), and genes preC/C and preS/S [5, 6]. Mutations diminishing the therapeutic response to treatment are preC and/or C mutations, which may modulate the response to interferon- $\alpha$  [5, 7]. Mutations in the immunodominant epitope 'a' of the S protein lead to immune escape and therefore reduce the efficiency of preventive HBV vaccination [5, 8]. Deletions and/or insertions in the preS region and especially in the C gene can be associated with a less favorable prognosis in immunosuppressed renal transplant patients [9].

HCV genome factors, such as genotype, viral RNA level, infection with more than one genotype, and quasispecies diversity, have not been found to affect patient survival after renal transplantation [10, 11].

The aim of our work was to identify and characterize variants of HBV and HCV genomes from patients after kidney transplantation in Latvia. We studied (1) HBV and HCV genotypes, (2) HBV subtypes, and (3) the

appearance of mutations and classification of nucleotide (nt) and amino acid (aa) substitutions in the HBV regulatory regions (EnII/BCP) and structural (preS/S and preC/C) and non-structural gene X.

## Materials and Methods

### Patients

A total of 38 consecutive patients receiving cadaveric renal transplants from January 1, 1990 to December 31, 2001 were admitted to the study and their medical histories retrospectively analysed. The mean age of the recipients was  $45.2 \pm 11.6$  years. Thirty patients had a single renal transplant and 8 had been transplanted twice (13 female and 25 male patients). Time to diagnosis of chronic HBV infection ranged from 2 to 10 years; 17 patients were anti-HCV positive. Ten patients dialyzed from 3 months to 12 years were also included in the study as a comparison group. The mean age of these patients was  $56.8 \pm 16.1$  years; 6 were female and 4 male, 7 of them were anti-HCV positive and the time to chronic infection ranged from 3 months to 12 years.

The immunosuppressive protocol is a triple-drug regimen of cyclosporin A, azathioprine or mycophenolate mofetil, and corticosteroids (methylprednisolone and prednisolone) in dosages according to local practice. Acute rejection episodes were initially treated with methylprednisolone (500 mg/day, i.v. for 3 days). Steroid-resistant acute rejection was treated with antithymocyte globulin. Nine of 38 patients experiencing one or more episodes of acute rejection received a higher cumulative dose of corticosteroids or polyclonal antibodies.

**Table 1.** Primers for PCR, RT-PCR, and sequencing of HBV and HCV genome fragments

Region of analysis	Primer	Sequence	Reference
<i>HBV</i>			
Pres/S	17p	5' TTATTTACATACTCTTTGGAAGGC 3'	17
2750–813 nt	8.15	5' AATGTATACCCAAAGACAG 3'	
Gene S (nested)	S1p	5' TTGTTGACAAGAATCCTCACAATACC 3'	17
215–710 nt	S2p	5' GCCCTACGAACCACTGAACAAATGG 3'	
preC/C	M3	5' CTGGGAGGAGTTGGGGGA 3'	16
1741–2516 nt	p19	5' AGGTACTGTAGAGGAATAAAGCCC 3'	17
geneC (nested)	C1	5' GTTACCTCACCATACTGCACTCAGGC 3'	17
2045–2387 nt	C2	5' GAGTCTTCTTCTAGGGGACCTGCCTCG 3'	
BCP/preC (nested)	M3	5' CTGGGAGGAGTTGGGGGA 3'	17
1741–2114 nt	2.364	5' CCCAGGTAGCTAGAGTCAT 3'	
Gene X	21p	5' CGTCCAGCCGACCACGGGGCGC 3'	17
1505–1825 nt	2-Sp	5' AAAAAGTTGCATGGTGCTGG 3'	15
<i>HCV</i>			
Core (nested)	1AS	5' ATGTACCCCATGAGGTCCGGC 3'	
476–725 nt	2S	5' TAGATTGGGTGTGCGCGCGA 3'	
	3S	5' CGCGCGACTAGGAAGACTTC 3'	
	4S	5' TGTGTGCGCGACGCGTAAA 3'	
	5AS	5' GCAYGTRAGGGTATCGATGACYT 3'	

### *Serological Tests*

Routine biochemical tests (ALT, AST, alkaline phosphatase, total bilirubin, platelets, leucocytes, and urea) were performed using standard clinical procedures. HBsAg was detected with an Enzygnost HBsAg 5.0 ELISA Kit (Dade Behring, Marburg, Germany). HBsAg-positive samples were tested for HBeAg and anti-HBe. HBeAg, anti-HBe, and anti-HBc were detected with ELISA kits (DiaSorin, Saluggia, Italy). Anti-HCV was detected with the Ortho HCV 3.0 ELISA Test System (Ortho Clinical Diagnostics, Raritan, N.J., USA).

### *Amplification of HBV Genome Fragments*

HBV DNA was extracted from 50 µl of serum with a commercially available DNA-RNA isolation kit based on phenol/chloroform extraction (LyteH, Moscow, Russia). Amplification of HBV genome fragments carrying the whole preS/S, preC/C regions and a fragment of the X gene, which included the EnII/BCP region, was performed by standard PCR with primers presented in table 1 [12–14]. When amplification of the whole fragments failed, we used nested PCR.

### *Amplification of a Fragment of the HCV Genome*

HCV RNA was extracted from 50 µl of serum with a commercial DNA/RNA extraction kit as above (LyteH). Amplification of the HCV core region was performed by nested RT-PCR. Primers are listed in table 1.

### *Sequencing of PCR Fragments*

PCR- and RT-PCR products were separated by electrophoresis on a 1 or 2% agarose gel in TBE (0.089 M Tris, 0.089 M boric acid and 0.002 M EDTA, pH 8.3) buffer. Bands of the appropriate size were excised from the agarose gel and the DNA fragments were purified with a DNA extraction kit (MBI Fermentas, Vilnius, Lithuania). Eluted DNA fragments were sequenced in both directions using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems, Foster City, Calif., USA) and electrophoregrams were obtained on an ABI Prism 377 sequencer (Applied Biosystems).

In all cases PCR primers were used as sequencing primers. For sequencing of the complementary strand of the HBV preS region an additional primer p14 – 5' CTGTAACACGAGCAGGGGTCCTAG 3' was used [14]. The sequences were edited manually with the BioEdit Sequence Alignment Editor [15] and subsequently aligned in the FASTA format (<http://ngfnblast.gbf.de/docs/fasta.html>). The phylogenetic trees were constructed using the maximum-likelihood algorithm and the neighbour-joining method in the PHYLIP package [16].

## **Results and Discussion**

### *HBV and HCV Markers of Patients' Sera*

Controlling the spread of HBV and HCV infections in transplantation and dialysis units is one of the major advances in the treatment of patients with end-stage renal disease. For example, the frequency of HBsAg carriers on maintenance dialysis is low in developed countries, but the incidence rates of HBV infection among dialyzed patients in less developed countries remains very high [17].

We examined a group of patients with renal transplants and a control group from a dialysis unit in the largest Latvian hospital. Data of patients and testing sera for the presence of HBV and HCV markers are listed in table 2. Sera from 26/38 patients after kidney transplantation were HBsAg positive. Twenty-one were HBV DNA PCR positive, 18 were HBeAg positive and 3 were anti-HBe positive; the remaining 4 were only HBsAg and anti-HBe positive but had no HBV DNA. Sera from 2 patients were negative for all markers of HBV and HCV infections.

Sera from only 2 dialyzed patients were HBsAg positive and only 1 serum sample was HBV DNA positive. The 2 sera were negative for all other markers. This could be explained by definite improvements after introduction of preventive HBV vaccination in the dialysis unit since 1998.

Anti-HCV antibodies were detected in 12/38 and 7/10 sera from the transplantation and dialysis groups, respectively. HCV RNA was found in only 14/38 and 4/10 anti-HCV-positive sera. Sera from 10 patients contained both HBV DNA and HCV RNA.

### *Spread of HBV Genome Variants in Latvian Patients with Renal Transplants*

#### *HBV Genotypes and Subtypes*

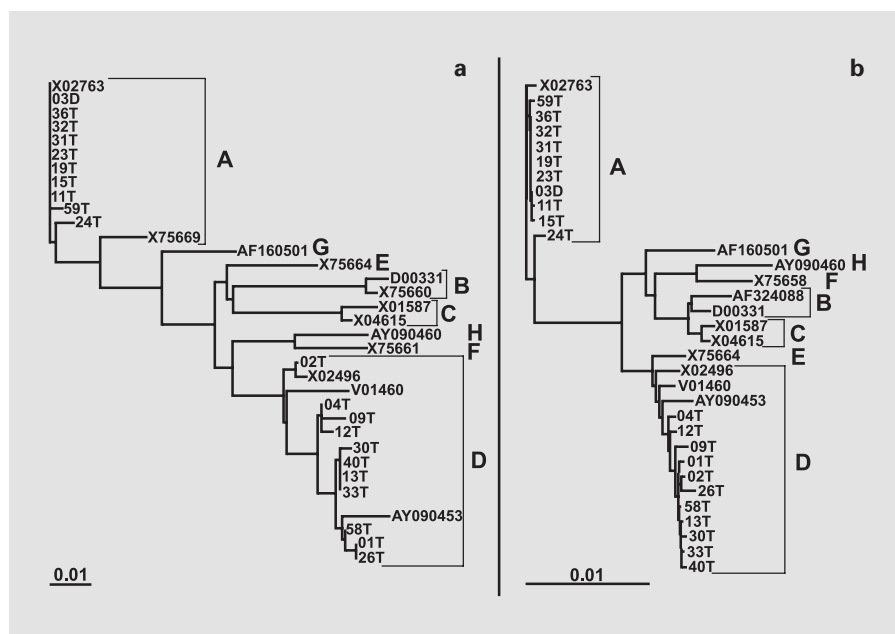
HBV regions X/EnII/BCP, preS/S, and preC/C were sequenced and analyzed in order to determine the HBV genotype/subtypes and to check for the presence of nucleotide and amino acid changes in comparison with prototype HBV sequences. Obtained sequences were submitted to GenBank (accession No. AY603427–AY603466). To identify the HBV genotype, the gene S nucleotide sequences were aligned to representative gene S sequences from HBV genotypes A–H [18–20], and the appropriate phylogenetic trees were constructed (fig. 1a). For comparison purposes, the phylogenetic trees were constructed for HBV gene C as well (fig. 1b). Genotype analysis showed that 12 samples of HBV DNA from transplanted patients belonged to genotype D and 9 to genotype A.

Therefore, two predominant HBV genotypes were identified in two selected groups of patients. Fifty-seven percent of HBV-DNA-positive samples belonged to genotype D and 42% to genotype A. No other HBV genotypes have been found in Latvia so far. The phylogenetic tree of HBV isolates (fig. 1) shows that the representatives of genotype A are more conserved, whereas genotype D is definitely more divergent.

Sequences encoded by gene S of HBV DNA isolates were compared with aa sequences of 9 known HBV sub-

**Table 2.** HBV and HCV markers in the kidney transplant and dialysis patients' sera

Code	Age/sex	HBsAg	HBeAg	Anti-HBe	Anti-HBc	Anti-HCV	HBV PCR	HBV genotype	HBsAg subtype	HCV PCR	HCV genotype
1T	59/M	+	+	-	+	-	+	D	<i>adw3</i>	-	
2T	42/M	+	+	-	+	+	+	D	<i>ayw2</i>	+	1b
3T	65/M	+	-	+	+	-	-			-	
4T	40/M	+	+	-	+	+	+	D	<i>ayw3</i>	+	1b
5T	23/F	+	-	+	+	+	-			+	3a
6T	46/M	+	-	+	+	-	-			-	
9T	29/F	+	+	-	+	-	+	D	<i>ayw3</i>	-	
10T	53/M	+	-	+	+	-	-			-	
11T	50/M	+	+	-	+	-	+	A	<i>adw2</i>	-	
12T	44/M	+	+	-	+	+	+	D	<i>ayw3</i>	+	3a
13T	54/F	+	+	-	+	+	+	D	<i>ayw3</i>	+	1b
15T	54/F	+	+	-	+	+	+	A	<i>adw2</i>	-	
17T	39/F	-	-	-	-	-	-			-	
18T	69/M	-	-	-	-	-	-			-	
19T	36/F	+	+	-	+	-	+	A	<i>adw2</i>	-	
20T	49/F	-	-	-	-	+	-			+	1b
21T	51/F	-	-	-	-	+	-			-	
23T	41/M	+	+	-	+	-	+	A	<i>adw2</i>	+	1b
24T	32/M	+	-	+	+	-	+	A	<i>adw2</i>	-	
25T	67/M	-	-	+	-	+	-			+	1b
26T	51/M	+	-	+	+	-	+	D	<i>adw3</i>	-	
28T	43/M	+	-	+	+	-	-			+	1b
29T	33/M	+	-	+	+	-	+	D	<i>ayw3</i>	-	
30T	54/F	+	+	-	+	-	+	D	<i>ayw3</i>	+	1b
31T	42/M	+	+	-	+	+	+	A	<i>adw2</i>	-	
32T	56/M	+	+	-	+	-	+	A	<i>adw2</i>	-	
33T	46/M	+	+	-	+	-	+	D	<i>ayw3</i>	+	2c
34T	31/M	-	-	-	-	+	-			-	
35T	57/F	-	-	-	-	+	-			+	1b
36T	32/M	+	+	-	+	+	+	A	<i>adw2</i>	+	1b
37T	53/F	-	-	-	-	+	-			+	1b
39T	54/F	-	-	-	-	+	-			+	3a
40T	26/M	+	+	-	+	-	+	D	<i>ayw3</i>	-	
41T	30/M	-	-	-	-	+	-			-	
54T	48/M	-	-	-	+	-	-			-	
56T	72/M	-	-	-	+	+	-			+	1b
58T	49/F	+	+	-	+	+	+	D	<i>adw3</i>	+	1b
59T	40/M	+	+	-	-	+	+	A	<i>adw2</i>	+	1b
1D	74/F	-	-	-	-	+	-			-	
2D	63/M	+	+	-	+	+	-			-	
3D	67/F	+	+	-	+	+	+	A	<i>adw2</i>	-	
4D	65/F	-	-	-	+	+	-			+	1b
5D	37/M	-	-	-	-	+	-			+	1b
6D	59/F	-	-	-	+	+	-			+	1b
7D	31/F	-	-	-	-	-	-			-	
8D	68/M	-	-	-	-	-	-			-	
9D	27/F	-	-	-	+	-	-			+	1b
10D	63/F	-	-	-	-	+	-			+	1b



**Fig. 1.** Phylogenetic tree based on the 21 sequenced S (**a**) and C (**b**) genes. Database sequences (GenBank accession numbers are shown) representing genotypes A–H are included.

types [21]. It appeared that 8 HBV isolates were of HBsAg subtypes *ayw3*, one *ayw2*, and 3 *adw3* (genotype D), whereas 9 HBV isolates belonged to the *adw2* subtypes (genotype A). Among the dialyzed patients, HBV from the serum of patient 3D belonged to genotype A, subtype *adw2*.

Our longitudinal data on the distribution of HBV genomes [22] showed clear evidence that the subtype *ayw2* (GenBank accession No. X02496) is most common in Latvia. However, the present study of renal transplant patients reveals a predominance of *ayw3* (GenBank accession No. V01460) and *adw3* (GenBank accession AY090453) subtypes of genotype D, whereas subtype *adw2* (GenBank accession No. X02763) is fully predominant in genotype A.

Detailed analysis of sequenced HBV genes revealed the presence of silent and missense mutations (fig. 2).

#### Gene X/EnII/BCP

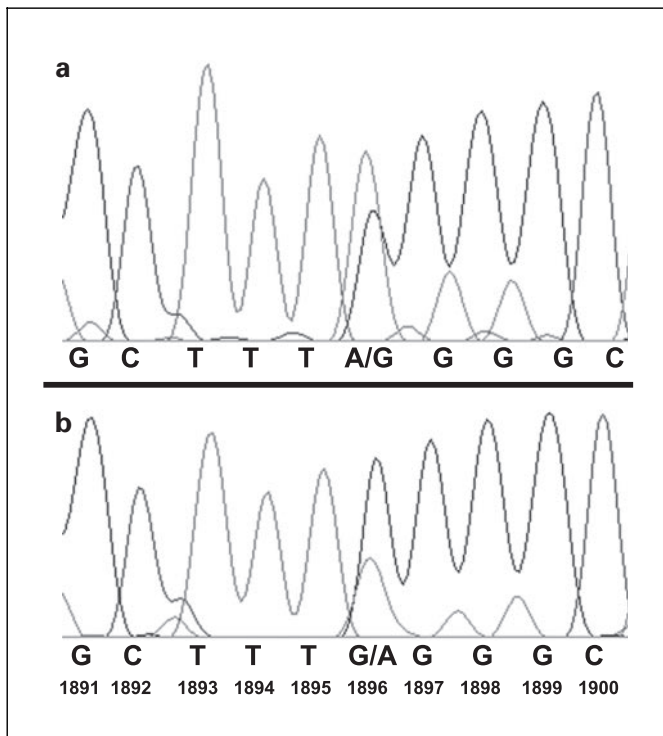
A set of mutations affecting the amino acid sequence of the X protein was been found (fig. 2). However, the functional role of these mutations may be explained by their influence on the EnII and BCP regions, which overlap with gene X. For example the BCP mutation G1752A/C appears to be associated with liver damage [23]. A double mutation, G1753A and T1754C, is frequently found in anti-HBe-positive patients, and is sometimes accompanied by the preC stop codon mutation

A1896 [24]. Moreover, although the HBV isolate from this patient (12T) carries no mutation at position 1896 and possesses an HBe-positive phenotype, it contains a double mutation in the central part of the BCP: A1762T and G1764A. These mutations are most frequently observed in HBV-infected patients with chronic hepatitis, hepatocellular carcinoma, and fulminant hepatitis, but are regarded as rare in immunosuppressed patients [25]. Although these mutations are not directly associated with HBeAg negativity, they could suppress HBeAg titres in HBeAg-positive patients. These changes, considered ‘hot spot mutations’, are found in patients with hepatocellular carcinoma carrying an *ayw* subtype, genotype D [26]. The HBV DNA isolate from patient 12T was also an *ayw* subtype of genotype D, but patient 12T has got chronic hepatitis B and C and thus far, no signs of hepatocellular carcinoma. In total, 6 HBV isolates from transplanted patients contained mutations in the BCP region, and 4 isolates contained nucleotide substitutions. These can be attributed to EnII mutations.

#### PreC/C Gene

Severe liver damage in chronic HBV infection may be related to the emergence of a preC stop-codon mutation, and clustering of missense mutations in the immunological epitopes at amino acids 18–27, 48–69, 74–83, 84–101, at position 130 and at the carboxy-terminal processing site of the C protein (aa 147–155) [27–30]. This may con-





**Fig. 3.** Simultaneous presence of preC stop-codon mutant and wild-type DNA in the sera of patients 30T (a) and 40T (b). Results of automatic sequencing are shown.

mutants ranging from 14 to 33% in different areas have been observed in European and Asian countries, however [36].

We found the following substitutions in the previously described CTL epitopes of C protein [6]: 18–27 – Leu19→Ser (once), 63–71 – Gly63→Val and Glu64→Asp (both twice), 87–97, or 88–100 – Asn87→Asp and Met93→Val (both once). The CTL epitope 87–97 may play an important role in the pathogenesis of HBV infection [37]. For this reason, it is intriguing to discover the Asn87→Asp substitution. To date, this has never been described.

We found one Pro130→Thr substitution, which was detected exclusively in patients with chronic hepatitis with or without HBeAg [38]. The 130-aa belongs to both T helper and B cell epitopes and is regarded by some authors as one of the most important immunogenic sites in the HBc mutation. This appears as a result of immune selection and is associated with exacerbation of chronic hepatitis [30] and hepatocellular carcinoma [39].

The 155-aa was found to be mutated in 2 patients: Ser155→Cys (patient 26T) and Ser155→Ala (40T). The Ser155→Cys is new, but the Ser155→Ala is a very rare

substitution. The last mutation is accompanied with an Arg151→Gln substitution, which can be regarded as common. Similarly, the Ile116→Leu substitution was found in 3 HBV isolates in the absence of other mutations.

In contrast to earlier observations [9], we did not find any HBV core deletion variants. This could be explained by the milder liver disease in our patient cohort.

#### PreS/S Gene

The immunologically important HBs1 to HBs5 stretches of protein S cover 99–169, two structural loops of which 107–137 and 139–147 may be regarded as immunodominant epitope ‘a’. Mutations in epitope ‘a’ play a crucial role in the formation of a vaccine escape phenomenon. They appear mainly within the 139- to 147-aa loop, and the Gly145→Arg substitution is the most frequent vaccine escape mutation thus far characterized [40]. In our patients, we found no mutations in the 139- to 147-loop and only 3 mutations in the 107- to 137-loop. The Pro120→Thr was described earlier [41]. Substitutions of Pro120 to Glu [42], or to Ser [43] have been previously found to be significant. The Met133→Ile substitution is a new mutation as well. Most of the mutations found in patients living in Latvia after kidney transplantations are located within the CTL and T helper epitopes of protein S: 19–28, 41–49, 97–106, and 187–216 (Arg24→Lys, Leu26→Phe, Leu42→Pro, Gln101→His (twice), Met103→Val, Tyr206→Ser). It has been shown that chronic HBV carriers and patients with hepatocellular carcinoma frequently have mutations encompassing residues 29–53 [44, 45], but we found only a single Leu42→Pro mutation in this region. Typical deletions within the preS1 and preS2 regions [46] were not found. Interestingly, the preS region is extremely conservative in comparison with other regions of the HBV isolates [47]. We also found only 3 mutations: 1 in the preS1 (Ser96→Tyr) and 2 in the preS2 (Pro34→Leu and Pro52→Leu).

#### Spread of HCV Genomes in Latvian Renal Transplant Patients

HCV infection is present in 2–50% of renal transplant recipients and haemodialyzed patients [48]. In contrast to HBV infection, absence of preventive HCV vaccination results in infection of 70% of patients before or during dialysis. In renal transplant patients, we found anti-HCV in 26 samples of sera (51%), but HCV RNA was present in 24 samples (47%).

Comparison of sequencing data of HCV RNA from positive samples with published representative HCV se-

quences belonging to genotypes 1a, 1b, 2c, 3a (GenBank accession No. AF009606, AJ132997, L38322, and D14305, respectively) allowed us to establish the HCV genotypes of our isolates. Seventy-eight percent of HCV-RNA-positive samples belonged to genotype 1b, 16.67% to genotype 3a, and 1 sample (5.55%) to genotype 2c. Therefore, the distribution of HCV genotypes reflects the distribution in Western and Eastern Europe and North America.

Among dialyzed patients, all 5 HCV-RNA-positive samples belonged to genotype 1b.

In conclusion, a long-term follow-up study of selected renal transplant patients will provide us with additional

data on the significance of discovered mutations and their impact on the pathogenesis of viral-induced liver disease.

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