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Molecular Characterization of Hepatitis B and Hepatitis C Viruses Circulating in Latvia

Academic Dissertation

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ABSTRACT

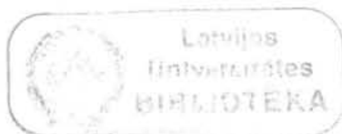
The aim of this study was to determine HCV and HBV genotypes distribution and to characterize HBV strain variants of Latvian patients. HBV-DNA and HCV-RNA were extracted from sera of different groups of Latvian patients; fragments of viral genomes were amplified and subjected to direct sequencing. HBV and HCV genotypes of were determinated by phylogenetic analysis of obtained sequences.

74 % of the tested HBV strains belonged to genotype D and only 26% - to genotype A. Both genotypes are distributed worldwide, but have different areas of predomination. Prevalence of genotype A is typical for North-West Europe, North America and Central Africa; genotype D is predominant in Mediterranean, Near and Middle East, South Asia.

About 88% of HCV isolates presented in Latvia belonged to genotype 1b. The genotype 3a was detected in about 9% of cases among Latvian patients infected with HCV. In a contrast with West-Europe countries, HCV genotypes 1a and 2 in Latvia are not common. The similar distribution of HCV genotypes was found in other former Soviet Union Republics and some of Asian countries. It is important, that predominant genotype 1b shows relatively poor response to treatment by traditional anti-viral drugs.

Comparison of obtained HBV sequences with published ones (GeneBank) showed relatively high genetic heterogeneity. We found number of classical mutations associated with natural history of infection. Mutations that affect structurally or functionally important regions of HBV genome but are not described in literature also were detected.

Obtained information could be useful to elaborate arrangements for limiting HBV and HCV transmission and prevention of the new outbreaks.



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INTRODUCTION

Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are the most common causes of liver disease worldwide. More than 300 million people worldwide are infected with HBV and about than 170 million are infected with HCV [1, 2]. They constitute a source of infection for the others and, more importantly, a population with high morbidity from chronic liver diseases including hepatocellular carcinoma (HCC). Hepatitis viruses are very topical public health problem in Latvia too, because of high potential to serious consequences of these infections. Thus, in 2004 214 cases of acute hepatitis B, 113 – of acute hepatitis C, 1134 – chronic hepatitis C and 427 of HBV surface antigen (HBsAg) carriers were registered in Latvia. The molecular characteristics of HBV and HCV are very important due to certain influence of virus genome characteristics on pathogenesis, treatment and outcome of disease.

REVIEW

1. HEPATITIS B VIRUS

1.1 Biology of HBV

HBV belongs to the genus *Orthohepadnavirus* of the *Hepadnairidae* family. Similar viruses were found in woodchucks (WHY), ground squirrels (GSHV), ducks (DHBV) and herons (HHBV). In serum of HBV- infected patients three forms of viral particles are found: (1) spherical, 42-nm diameter particles, which represent mature virions (Dane particles); (2) 20-nm diameter empty particles; (3) filaments of 20 nm in diameter and of varying length. Complete virions have an envelope consisting of virus encoded surface proteins and lipids derived from the host cell. Packed in the virion is a nucleocapsid 27 nm in diameter, which contains the viral genome and polymerase.

The HBV genome is a relaxed circular DNA of ~3,200 nucleotides and consists of a full-length of negative strand and a shorter positive strand [3]. The 5′ end of the negative strand is covalently linked to the viral reverse transcriptase, whereas the 5′ end of the positive strand bears an oligoribonucleotide. There are 4 partially overlapping open reading frames (ORF) encoding the envelope (preS/S), *core* (preC/C), polymerase, and X proteins (Fig. 1.). The preS/S open reading frame encodes the large (L), middle (M), and small (S) surface glycoproteins. The preC/C open reading frame is translated into a precore polypeptide, which is modified into a soluble protein, the hepatitis B e antigen (HBeAg) and the nucleocapsid protein, hepatitis B core antigen (HBcAg).

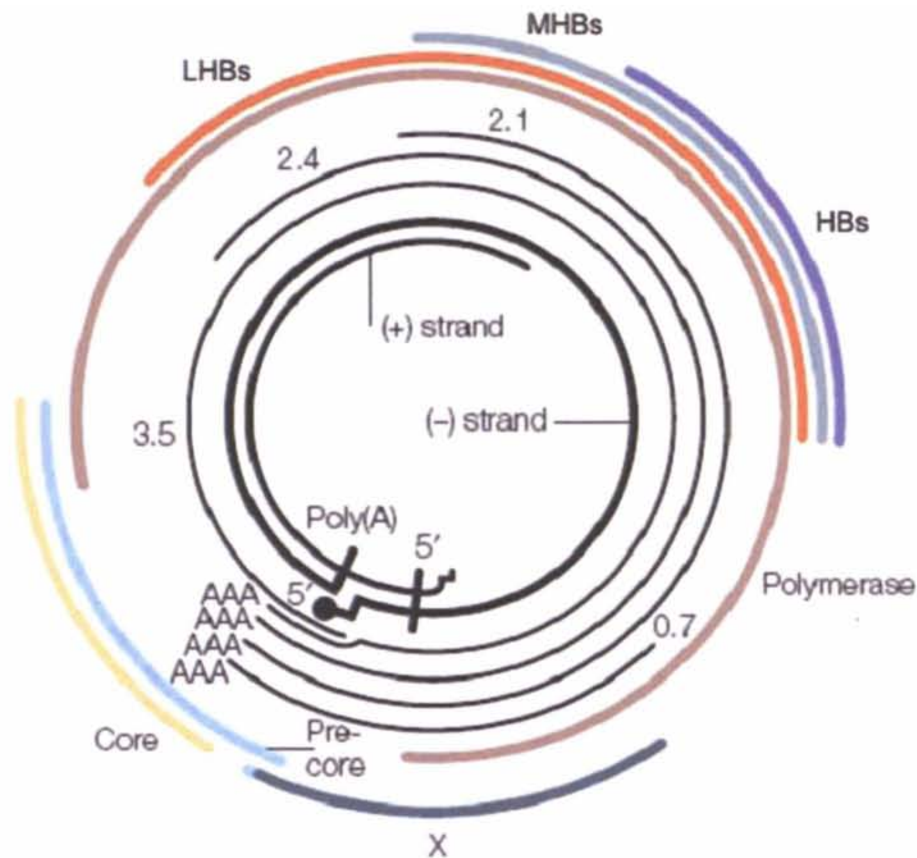

Figure 1. The genomic structure of hepatitis B virus The inner circles represent the full-length minus (–) strand (with the terminal protein attached to its 5′ end) and the incomplete plus (+) strand of the HBV genome. The thin black lines represent the 3.5, 2.4, 2.1 and 0.7 kilobase mRNA transcripts, which are all terminated near the poly(A) (polyadenylation) signal. The outermost colored lines indicate the translated HBV proteins: that is, large, middle and small HBV surface proteins, polymerase protein, X protein, and *core* proteins [4]

After virions enter hepatocytes, by an as-yet-unknown receptor, nucleocapsids transport their cargo, the genomic HBV DNA, to the nucleus, where the relaxed circular DNA is converted to covalently closed circular DNA (cccDNA). The cccDNA functions as the template for the transcription of four viral RNAs, which are exported to the cytoplasm and used as mRNAs for translation of the HBV proteins. The longest (pre-genomic) RNA also functions as the template for HBV replication, which occurs in nucleocapsids in the cytoplasm. Some of the HBV DNA and polymerase-containing capsids are then transported back to the nucleus, where they release the newly generated relaxed circular DNA to form additional cccDNA. Others are enveloped by budding into the endoplasmic reticulum and secreted after passing through the Golgi complex.

1.2. Genotypes and subtypes of HBV

Prior to the definition of the genotypes, HBV strains were distinguished by using subtype-specific antibodies against hepatitis B surface antigen HBsAg into nine serotypes or subtypes designated *ayw1*, *ayw2*, *ayw3*, *ayw4*, *ayr*, *adw2*, *adw4q–*, *adrq+*, and *adrq–* [5]. It was found that the prevalence of these serotypes varies in different parts of the world. In addition, antibody against the common determinant, "a", confers protection against all serotypes.

Advances in molecular biology techniques revealed significant diversities in sequences of HBV isolates, accounting for the allelic differences among the HBV serotypes. Based on an intergroup divergence of 8% or more in the complete nucleotide sequence, HBV can be classified into 8 genotypes A-H [6-9]. However, genotyping can be accomplished based on a partial sequence of the HBV genome such as the S gene.

With the identification of the molecular basis of the nine HBV serotypes, it seems in general possible to deduce the subtype from the primary structure of HBsAg (Tab.1). However, subtypes with new specificity combinations remain putative until confirmed by serological typing.

Table 1. Amino acid residues specifying determinants of HBsAg [10]

Position	Amino acid	Specificity
122	Lys	*d*
	Arg	*y*
127	Pro	*w*1*/*w*2
	Thr	*w*3
	Leu/Ile	*w*4
160	Lys	*w*
	Arg	*r*
* *w*1 reactivity also requires Arg 122, Phe 134 and/or Ala 159		

HBV genotypes have distinct geographical distribution (Fig. 2.). Genotypes A and D have global distribution, genotypes B and C are predominant in East and South East Asia, genotype E is predominant in West Africa, and the most divergent genotype F is found exclusively amongst indigenous peoples of Central and South America [11-14]. The geographical location HBV H is

restricted to Central and South America [15]. HBV genomes are of different lengths, depending on genotype. HBV genomes representing genotypes B, C, F and H are 3215 nucleotides in length. HBV genome genotype D is only 3182 nucleotides long. HBV genotypes E and G have a 3 nucleotide deletion in the same region of the polymerase. Genome of genotype A varies from the other genotypes by an insertion of six nucleotides in the C-terminal region of the *core* gene [16].

Figure. 2. Geographic distribution of hepatitis B virus genotypes [17]

1.3. Clinical differences between HBV genotypes

Although preliminary clinical studies indicate that there is an association between HBV genotype and natural history of infection and response to antiviral therapy [18-21], further evaluations on larger cohorts of patients are necessary to give a clearer picture of the subject.

It was suggested that HBV genotype C is associated with a more severe liver disease and with a lower rate of response to interferon therapy than genotype B infections [22, 23]. HBV genotype C carriers are more frequently positive for HBeAg [24]. Contrary, in other studies HBV genotype B has been associated with severe disease [25]. In India, patients infected with HBV genotype D had more severe disease, developed HCC and were younger than patients with genotype A [26]. A preliminary study from the Mayo Clinic with patients undergoing liver transplantation suggested that patients with genotype A had the lowest risk for HBV recurrence.

Patients with HBV genotype D appeared to have the highest risk for HBV recurrence and mortality after liver transplantation [27].

There are few reports of genotype correlations in fulminant hepatitis. However, a clear relationship between a particular genotype or co-infection of a particular genotype with HDV and fulminant hepatitis isn't observed until now [28].

Patients' responses to interferon α (IFN-α) therapy seem to be associated with specific HBV genotypes. Individuals infected with HBV representing genotypes C and D have a lower response rate to IFN-α compared to genotypes A and B [29, 30]. The better response rate of genotypes A and B individuals may correlate with the faster development of mutations in the basal *core* promoter (BCP) of genotype C and D compared with genotype A and B [31]. The emergence of resistance to antiviral therapy with lamivudine seems to be independent of the genotype after 1 year of therapy but may be related to the presence of HBeAg [32].

1.4. Mutations in HBV genome

The mutation rate and the origin of HBV are uncertain. As the virus contains a polymerase enzyme without proofreading activity, error frequencies on RNA or DNA copying are likely to be of the order measured for retroviruses and other RNA viruses. The presence of long deletions suggests that other mutagenic mechanisms not related to the reverse transcription operate. Candidates include slippage during mispairing between template and progeny DNA strand, the action of cellular topoisomerase, and gene splicing using alternative donor and acceptor site [33]. Mutation may also arise from recombination between co-infecting strains.

HBV seems to exhibit a mutation rate more than 10-fold higher than other DNA containing viruses. The rate for nucleotide substitutions vary depending on the stage of disease. The natural evolutionary rate for the HBV genome in chronic hepatitis B is approximately 1.4×10^{-5} to 3.2×10^{-5} substitutions/site/year [34], whereas in liver transplantation setting, it is almost 100-fold higher [35].

Mutations are not limited to specific ORFs, and occur in all viral genes and regulatory elements. However, they tend to cluster into mutational patterns in particular, BCP, the pre-core region, the polymerase gene and the "a" determinant of the viral envelope [36].

Some authors discriminate between naturally occurring variants and selected variants (mutants). This distinction may be somewhat artificial and also used in other meanings. Moreover, the origin of the changes is often unknown and makes the definitions difficult to apply. The terms "mutant" and "variant" should be better used in a more general sense, i.e. to describe all genetically heterogeneous viruses, irrespective of the underlying causal mechanism [37]. In

terms of genetic variability, a distinction between HBV genotypes and mutants has to be made. The different genotypes of HBV are stable forms of the virus, which are the result of random changes selected over years of population pressure. Mutants arise in individuals under medically or naturally (chronic hepatitis B) induced immune pressure. They include vaccine and hepatitis B immune globulin therapy escape mutants. In liver transplant recipients, high titer antibody in a situation with low titer virus and a large number of susceptible cells (liver transplant) is an ideal breading ground for mutants. In chronic HBV infection, naturally occurring escape mutants may be selected by the anti-HBV response of the HBV carrier [38].

1.4.1. *Core* promoter (EII/CP) and PreC Mutants

The most characteristic feature of this group of HBV mutants is a decreased or abolished production of the preC protein and its secreted processing product HBeAg, either by influencing transcription or by nonsense mutations in the preC ORF. A recent detailed analysis showed that the HBe-negative mutants are more common than previously suspected, and their prevalence is increasing over time. These mutants are present worldwide, with marked variations in prevalence across different geographical regions. The median prevalence of the HBe-negative mutants among patients with chronic HBV infection drops from 33% in the Mediterranean to 15% in Asia Pacific and 14% in the USA and Northern Europe. The preC mutants are absolutely prevalent in the Mediterranean, while the *core* promoter mutants dominate in Asia [39].

1.4.1.1. *EII/CP Mutants*

Synthesis of both pregenomic and the 5'-terminally extended precore RNA is controlled by the EII/CP region; hence, mutations in this region can influence the ratio of the two transcript classes. The basal *core* promoter sequence (nt 1744–1804) is the most important of the numerous regulatory elements that have been mapped within the EII/CP region. The BCP mutations at nt 1762 (A→T) and 1764 (G→A) are most frequently observed in HBV-infected patients with chronic hepatitis, HCC and fulminant hepatitis, but are rare in asymptomatic HBe-positive carriers, in immunosuppressed patients and in carriers without HBV markers [40]. Additional mutations at nt 1653 (C→T) and 1753 (T→C/A/G) may have further association with progression of liver disease to cirrhosis in patients chronically infected with genotype C [41]. A mutation at nt 1752 (A→C) was reported also in conjunction with the 1762 and 1764 mutations and appeared to be associated with liver damage [42].

1.4.1.2. *PreC Mutants*

The frequent preC mutation at nt 1896 (G→A) leads to premature termination of the preC protein at codon 28 of the 29-amino acid (aa) preC region, and thus to elimination of the HBe protein [43]. This mutation also affects the RNA stem-loop structure of the encapsidation signal ε, which acts at the level of pregenomic RNA while preC RNA is not encapsidated [44]. The mutation is rarely found in genotype A (and some strains of genotype F), which contains a C at the pairing position 1858 (instead of T in other HBV genotypes), because the loss of a base pair destabilizes the ε stem-loop unless a stabilizing complementary mutation at nt 1858 (C→T) occurs. Simultaneously with the preC mutation at nt 1896, a 1899 (G→A) mutation, which also improves the stability of the stemloop, may be observed [45, 46]. It is present also in a high proportion of patients with fulminant hepatitis B. However, the implicated importance of the preC mutants in the prognosis of disease remains controversial [47-49]. Less common preC mutations are: start codon mutations at nt 1814 or 1815, a nonsense mutation at nt 1874, a missense mutation at nt 1862 and frameshift mutations [50]. Together, these data imply that preC and HBe are nonessential gene products, at least under certain conditions. However, the potential advantages for the virus of a decreased or abolished preC or HBe production will remain obscure as long as the normal functions of these proteins are not really clear.

1.4.2. *Core* mutants

Though also not finally settled, there is evidence suggesting that aggressive hepatitis B cases are connected with mutations others than those abolishing the HBeAg. First candidates for enhanced HBV virulence are mutations in the HBV core-derived proteins, most prominently in the central immunodominant region of the HBc protein. Such mutations frequently accompany preC mutations and may decrease HBV recognition by cytotoxic T cells. The appearance of the *core* mutants, in concert with the preC mutations, may significantly diminish the response to IFN-α therapy [51, 52].

1.4.2.1. *Substitutions within crucial HBc sites*

First experimental data supporting the idea that HBc mutants may alter the host immune response and modulate the clinical course of HBV infection appeared in 1991 [53]. Two generally accepted facts are that (1) mutations are clustered within immunologically relevant epitopes [54-56], and (2) mutant clones in patients with active liver disease outnumber the mutant clones in asymptomatic carriers [57, 58].

Severe liver damage in patients with chronic active hepatitis was attributed to changes clustering in three regions, i.e. codons 48–60, 84–101 and 147–155 [59]. Mutations within the region 84–

101, which overlaps the CTL epitope, accumulated during periods with frequent hepatitis exacerbation [60]. Numerous unique mutations were found in the region 48–60 in fulminant [61-63] and severe exacerbation hepatitis B patients [64-66]. In addition, mutation clustering regions at aa 21–34 [67] and 31–49 [68] were detected.

Although some unique mutations were attributed to fulminant rather than acute hepatitis B [69, 70], completely identical HBV strains were found to replicate in patients with acute and fulminant hepatitis B; hence, these mutants are, at least, not generally more pathogenic. Evolutionary studies in chronic hepatitis patients over a period of 6–11 years showed that the HBc mutations tended to increase with time and correlate with exacerbation of the disease [71], although interfamilial conservation of HBV *core* sequences for more than 20 years has been described [72]. Some other investigations, however, failed to detect diagnostically significant changes within the HBc protein, but found various numbers of missense mutations independent of the grade of liver disease [73-75].

The HBc protein can remain highly conserved for more than two decades during the immune tolerant phase of chronic HBV infection, whereas mutations appear typically after seroconversion to the anti-HBe-positive phase. This was observed in asymptomatic anti-HBe-positive carriers [76] but also in some cases of fulminant hepatic failure. Although the total viral load in such patients is drastically reduced, a small number of the newly appearing mutants may contribute to the fulminant outcome. Recent data suggest a sequential order in the mutational processes, i.e. first HBe clearance and seroconversion to anti-HBe, then emergence of the preC stop mutations preventing HBe generation, followed by *core* mutations in regions of B cell, T helper and CTL epitopes as well as in the arginine-rich C terminus, thus supporting the idea of immune escape [77, 78]. First direct evidence for the responsibility of the HBV *core* CTL epitope 18–27 mutations (aa changes S21N/A/V and V27A/I) was found in HLA-A2-positive patients with chronic hepatitis B [79]; however, another group of researchers failed to find significant mutations of this epitope in HLA-A2-positive patients with acute exacerbation during chronic infection [80]. Further, analyzing HBe to anti-HBe seroconverters, mutations were found mostly in the T helper epitope 50–69 and in the B cell epitope 74–83. Remarkably, the absence of serum anti-HBc was not a consequence of mutations within the HBc gene but rather of an aberrant immune reaction of the host [81].

Mutations I97L and P130I/T/S have been reported to be associated with exacerbation of chronic hepatitis and HCC [82, 83]. Interestingly, the I97L mutation is responsible for secretion of immature HBV cores, but this phenomenon can be offset by the P130T mutation [84]. Low secretion of HBV virions is caused by the naturally occurring P5T and L60V mutations [85].

1.4.2.2. *Deletions within the HBc Molecule*

According to recent data, the *core* internal deletions (CIDs) may be involved in the onset of hepatitis and the subsequent outcome of chronic infection [86]. Historically, the CIDs were found, first of all, in patients with long-standing HBe-positive infection [87-89], and in a familial cluster of chronic carriers with the absence of anti-HBc [90]. However, HBV variants with HBc deletions were also described as inhibiting HBV replication, and not persisting in preference to wild-type HBV under enhanced immune pressure, e.g. in patients with chronic active hepatitis B [91].

A special group of CID mutants is represented by the HBc variants with deletions in the central part of the molecule, which appear and accumulate in long-term immunosuppressed renal transplant recipients [92]. Moreover, persistence of these mutants seems to correlate with progressive liver disease [93]. Some of these variants, with relatively short deletions (for example, amino acid 86–93 and 77–93 deletions), retain their self-assembling capability, whereas mutants with longer deletions are unable to self-assemble [94].

Another group of the CID mutants is represented by a specific set of long in-frame internal HBc deletions (at aa 88–135 and 82–122), which were described as responsible for the appearance of defective interfering HBV particles [95]. Genomes carrying such CID variants possess some replication advantages in comparison to wild-type HBV genomes, but they are dependent on complementation by wild-type HBc protein. No detectable amounts of the CID proteins were found in infected cells. The interference phenomenon is not connected with the synthesis of deleted *core* proteins, since it was detected also in long out-of-frame deletions within the gene C [96].

Besides HBc deletions, insertions may also occur; for instance, an HBV variant with a 36-nt insertion in the preC region was described that forms an extended HBc protein with an extra 12 aa at the N-terminus [97]. Whether any pathogenic effects are associated with this variant has not been analyzed.

1.4.3. HBs Mutants

The a-determinant of the three surface proteins L, M and S is of special importance since it induces virus-neutralizing humoral responses and confers the protection raised by existing HBV vaccines. The *a* determinant is located within the major hydrophilic domain and consists of two loops that protrude from the outer surface of HBV virions and so-called 22-nm HBs particles. The tertiary structure of the *a* determinant is crucial since the vast majority of induced anti-HBs antibodies recognize conformational epitopes.

1.4.3.1. *Vaccine-induced mutations*

The most frequent vaccine-induced escape mutation is G145R [112], which is poorly detectable in traditional HBs diagnostic assays. The mutation was found in natural isolates, and immune pressure upon vaccination favours its selection. In countries with mass vaccination programmes, for example Taiwan [98], a substantial increase in HBs escape variants of up to 28% has been documented, concurrent with a 10-fold decrease in the HBs carrier rate in children [99]. Generally, escape mutations were found mainly in the aa 139–147 loop, or HBs4 region of the a determinant, predominantly at position 145 (G145R) [100].

1.4.3.2. *Mutations in chronic infection*

In contrast to the vaccine-induced escape mutations predominantly found in the aa 137–149 loop of the *a* determinant, the most frequent mutations in patients chronically infected with hepatitis B were detected within the aa 107–137 loop [101]. Mutations in the CTL epitope encompassing the 29–53 region, especially at aa positions 40 and 47, were found in the chronic patients [102], suggesting that they may contribute to chronicity.

1.4.4. PreS mutants

Mutations in the preS1 region may include long deletions of up to one half of the entire preS1 region [103], but the hepatocyte-binding site at aa 20–47 always remains conserved. The preS1 mutations destroy the integrity of the S promoter [104].

Deletions in the preS2 region have typical aspects of immune escape. Removing T and B cell epitopes [105], they may contribute to the generation of HCC [106]. Moreover, missense mutations at the start codon of the preS2 region that prevent the synthesis of the corresponding protein are frequently associated with fulminant hepatitis [107] and HCC [83].

In general, the preS mutations affect the ratio of the large, middle and small S proteins, and this could result in the accumulation and retention of these proteins in the endoplasmic reticulum. This may lead to a direct cytopathic effect in hepatocytes, and the appearance of the preS mutants might thus be linked to progression of liver disease [108].

1.4.5. Polymerase mutants

1.4.5.1. *Chronic infection*

Since the S gene completely overlaps with the P gene, preS and S mutations affect the structure of polymerase.

Because the preS region overlaps the non-essential spacer domain of the polymerase, rather long deletions in the preS may remain indifferent with respect to the structure and function of the enzyme. By contrast, the catalytic reverse transcriptase domain coincides with the C-terminal part of the S protein, and changes therein could have drastic effects on polymerase activity. Indeed, this region of the S protein is not a mutational 'hot spot', and no polymerase mutations that could affect the susceptibility of HBV to antiviral nucleoside analogues were found in chronic patients [109]. From the other side, the *a* determinant overlaps the variable linker between the two conserved domains in the polymerase. Nevertheless, in isolates from individuals with anti-HBc reactivity only, an increased variability in the *a* determinant was found that consequently resulted in a similar hypervariable spot in the polymerase which might impair viral replication [110]. Interestingly, a missense mutation in the 5' region of the polymerase gene resulted in the inability to package pregenomic RNA into *core* particles [111].

1.4.5.2. *Antiviral chemotherapy*

Upon chemotherapy of hepatitis B with the nucleoside analogues lamivudine and famciclovir, the emergence of two classes of polymerase mutants is almost regularly observed, providing very strong evidence that they are selected by the presence of the drugs. First, the YMDD site in the C (catalytic) domain of the polymerase is subject to changes upon lamivudine therapy [112, 113], similar to the earlier described mutation M→V/I within the analogous site in HIV reverse transcriptase that is associated with lamivudine resistance. The variants M552V and M552I emerged in almost 50% of patients during lamivudine therapy [114], and their frequencies appear to rise even further upon prolonged therapy. Both mutations result in substitutions at aa 195 and 196 in the overlapping S gene. The YMDD mutants have lower replication efficiency than wild-type virus, and the latter displaces the mutant after cessation of lamivudine treatment. Lamivudine-resistant variants may have an additional L528M substitution in the B (template-binding) domain of the polymerase, often occurring together with the M552V mutation but rarely with the M552I mutation [115]. The additional L528M mutation restores the replication competence of the M552V mutant and increases resistance to various nucleoside analogues [116]. The L528M mutation has no effect on the aa sequence of the S gene. The M539V mutation is also able to confer resistance to lamivudine and other cytosine and thymidine

analogues [117]. More distant mutations, L430M and L428V/I, have been reported in genotype C patients in Japan [118]. These mutations were also associated with the basic M552V substitution.

The latter remains a central therapeutic problem, since the use of high doses of lamivudine does not prevent its emergence [119]. Interestingly, the HBV subtype may significantly influence the likelihood of the emergence of lamivudine resistance, possibly related to whether or not the aa sequence encoded by the overlapping S gene is affected [120].

The V555I [121] and V542I [122] mutations in the C domain confer resistance of famciclovir. Both mutations cause premature termination of the overlapping HBs proteins. Mild to moderate resistance was found in association with the V521L, P525L and L528M substitutions in the B domain. Although the M552I exchange was not associated with famciclovir resistance, no benefit was found in attempts to add famciclovir in patients with the M552I mutation after lamivudine failure [123]. Fortunately, lamivudine- and famciclovir-resistant viruses may remain sensitive to newer nucleoside analogues such as adefovir and lobucavir [124].

1.4.6. HBx Mutants

The importance of HBx mutants may appear in a new light after the recent discovery of HBx targeting of mitochondrial calcium regulation [125], although the HBx protein has been described as exhibiting numerous activities affecting intracellular transmission, gene transactivation, cell proliferation, DNA repair and apoptosis. Since the X gene overlaps the EII/CP region, mutations within the latter may influence the structure of the HBx protein.

For example, the mutation at nt 1653 (C→T) leads to the H94Y exchange in the immunodominant epitope of the HBx protein [41]. The EII/CP point mutations and deletion variants (for example, nt 1770–1777, 1752–1772) may produce truncated HBx protein variants. Such shortening of the HBx protein may have an effect on its transactivating and other properties. C-terminal insertions/deletions in the HBx protein have been described as connected with the appearance of HCC [83].

1.4.7. EI/XP Mutants

The EI/XP region (nt 959–1311) harbours binding sites for many regulatory cellular proteins, including hepatocyte-enriched nuclear factors, and is responsible for IFN stimulated responses [126]. The strongest decreasing effect on HBV replication was found for mutations which occur in the hepatocyte nuclear factor 3 and 4 binding sites of the EI *core* domain [127]. As previously described mutations, the EI mutations may also serve as an escape mechanism, switching viral replication from a high to a low level, as is frequently observed during chronic HBV infection.

2. HEPATITS C VIRUS

2.1. Biology of HCV

HCV is classified in the family *Flaviviridae*, although it differs in many details of its genome organization from the original (vector-borne) members of the family. HCV was the first virus discovered by molecular cloning without the direct use of biologic or biophysical methods. This was accomplished by extracting, copying into cDNA, and cloning all the nucleic acid from the plasma of a chimpanzee infected with non-A, non-B hepatitis [128].

HCV is an enveloped virus with a positive single-stranded RNA genome of approximately 9400 bp in length. The sequence contained a 5' untranslated region (5' UTR) of 341 bases containing an internal ribosome entry site, a long open reading frame coding for a polyprotein of 3,011 amino acids, and a 3' untranslated region (3' UTR) containing a poly(A) tail. The open reading frame length of each genotype is characteristically different [129].

The HCV polyprotein is cleaved co- and post-translationally by cellular and viral proteinases into ten different products, with the structural proteins located in the aminoterminal one-third and the nonstructural replicative proteins in the remainder [130, 131](Fig. 3.)

The structural components of the virion are the *core* protein and envelope glycoproteins, E1 and E2. These proteins are cleaved from the precursor by host-cell peptidases. The NS proteins, designated NS2 to NS5B, are involved in the further processing of the precursor polypeptide and virus replication. The structural proteins are separated from the non-structural (NS) proteins by a short peptide, P7. NS proteins are designated NS2 to NS5. NS4 and NS5 are each processed further into two subunits, A and B. NS2 is a component of the NS2–NS3 metalloprotease that autocatalyses cleavage of the NS2–NS3 junction; NS3 contributes to the NS2–NS3 protease and contains a separate serine protease that acts with co-factor NS4A to release the remaining NS proteins. The C-terminal region of NS3 contains an RNA helicase and nucleotide triphosphatase activity required for virus replication. NS5B is the RNA-dependent RNA polymerase. The functions of NS4B and NS5A in the virus life-cycle are unknown [133].

In common with other RNA-dependent polymerases, the HCV RNA polymerase (NS5B) does not have a proof-reading capability, and therefore generates considerable genetic diversity.

Despite a detailed knowledge of the molecular and structural biology of HCV, the pathogenesis of infection remains unclear. This is, at least in part, because there is no robust cell culture system for propagation of the virus or, despite recent advances, a widely accessible small animal model of HCV disease.

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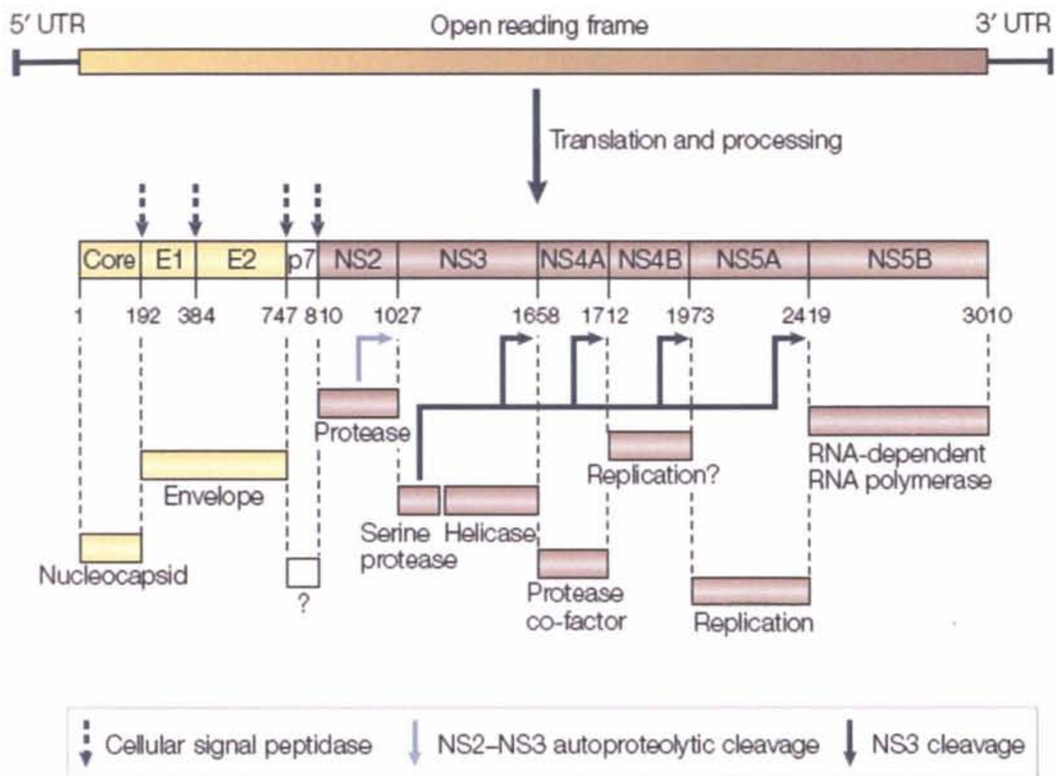


Figure. 3. The genomic structure of hepatitis C virus. A long open reading frame encodes a polyprotein of ~3,010 amino acids. The numbers below the polyprotein indicate the amino-acid positions of the cleavage sites for cellular and viral proteases. E – envelope protein; NS - non-structural protein; UTR - untranslated region. [132].

2.2. HCV genotypes.

After the complete HCV genome was determined by Choo et al. in 1991 [134], several HCV isolates from different parts of the world were obtained and sequenced [135-139]. Comparison of the published sequences of HCV has led to the identification of several distinct types that may differ from each other by as much as 33% over the whole viral genome [140]. Sequence variability is distributed equally throughout the viral genome, apart from the highly conserved 5' UTR and *core* regions and the hypervariable envelope (E) region [140-145].

As different investigators developed and used their own classification system for HCV strains, a confusing literature developed. However, at the 2nd International Conference of HCV and Related Viruses, a consensus nomenclature system was proposed to be used in future studies of HCV genotypes and subtypes [146]. According to this system, HCV is classified on the basis of

the similarity of nucleotide sequence into major genetic groups designated as genotypes. HCV genotypes are numbered (Arabic numerals) in the order of their discovery (Table 2). The more closely related HCV strains within some types are designated as subtypes, which are assigned lowercase letters (in alphabetic order) in the order of their discovery. The complex of genetic variants found within an individual isolate is termed the quasispecies. The quasispecies composition of HCV results from the accumulation of mutations during viral replication in the host [147].

Table 2. Classification systems for HCV genotypes [148]

Okamoto et al. [140]	Enomoto et al. [135]	Simmonds et al. [149]	Cha et al. [150]	Consensus [146]
I	PT	1a	I	1a
II	K1	1b	II	1b
				1c
III	K2a	2a	III	2a
IV	K2b	2b	III	2b
			III	2c
V		3	IV	3a
VI			IV	3b
		4		4a
			V	5a
				6a

The genomic sequences of different HCV isolates vary by as much as 35% [140]. The degrees of difference in nucleotide sequences among isolates vary from one genomic region to another. Sequence similarities between members of the different genotypes of a 222-bp segment of the NS5 region that we used in our laboratory range between 55 and 72%, whereas identities of subtypes range from 75 to 86% [146].

2.3. Geographical distribution of HCV genotypes

At least six major genotypes of HCV, each comprising multiple subtypes, have been identified worldwide [151]. Substantial regional differences appear to exist in the distribution of HCV genotypes (Fig. 4.). Although HCV genotypes 1, 2, and 3 appear to have a worldwide distribution, their relative prevalence varies from one geographic area to another.

HCV subtypes 1a and 1b are the most common genotypes in the United States [152]. These subtypes also are predominant in Europe [153-155]. In Japan, subtype 1b is responsible for up to 73% of cases of HCV infection [156]. Although HCV subtypes 2a and 2b are relatively common

in North America, Europe and Japan, subtype 2c is found commonly in northern Italy. HCV genotype 3a is particularly prevalent in intravenous drug abusers in Europe and in the United States [157]. HCV genotype 4 appears to be prevalent in North Africa and in the Middle East [158, 159], and genotypes 5 and 6 seem to be confined to South Africa and Hong Kong, respectively [160, 161]. HCV genotypes 7, 8, and 9 have been identified only in Vietnamese patients [162], and genotypes 10 and 11 were identified in patients from Indonesia [163]. There has been disagreement about the number of genotypes into which HCV isolates should be classified. Investigators have proposed that genotypes 7 through 11 should be regarded as variants of the same group and classified as a single genotype, type 6 [164-167].

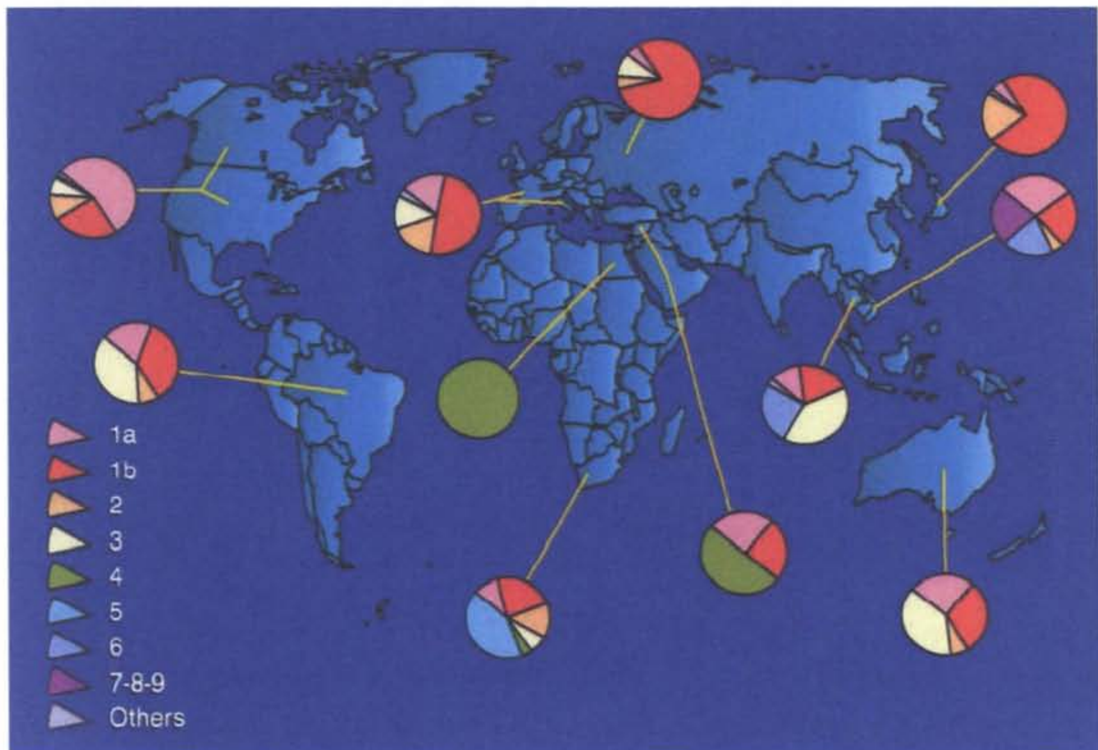


Figure 4. Worldwide geographic distribution of HCV genotypes. "Others" indicate unclassified sequences [148].

The geographic distribution and diversity of HCV genotypes may provide clues about the historical origin of HCV [168]. The presence of numerous subtypes of each HCV genotype in some regions of the world, such as Africa and Southeast Asia, may suggest that HCV has been endemic for a long time. Conversely, the limited diversity of subtypes observed in the United States and Europe could be related to the recent introduction of these viruses from areas of endemic infection.

2.4. Clinical impact of HCV genetic diversity

The major features of HCV structure, replication, transmission and ability to establish persistent infection are shared between all known variants. Indeed, viewed purely as a survival machine, the widespread distribution of genotypes 1–6 in human populations indicates that that each is equally successful in maintaining infections in human populations. Despite this obvious evidence for phenotypic similarity, there is growing evidence for genotype-specific differences in persistence and interactions with innate cell defences and the immune system that have important repercussions for current and probable future therapy.

The clearest difference between genotypes is in their susceptibility to treatment with IFN monotherapy or IFN/ribavirin (RBV) combination therapy. Typically, only 10–20 and 40–50% of individuals infected chronically with HCV genotype 1 on monotherapy and combination therapy, respectively, exhibit complete and permanent clearance of virus infection. This long-term response rate is much lower than the rates of 50 and 70–80% that are observed on treatment of HCV genotype 2 or 3 infections [169, 170]. This difference has proved to be highly significant in patient management and has led to the use of higher doses and longer durations of treatment for type 1 (and type 4) infections, in order to achieve acceptable efficacy. In numerous multivariate analyses, genotype-specific differences in treatment response have been shown to be independent of host variables, such as stage of disease progression, age, duration of infection, sex and HIV and other virus co-infections. It is similarly independent of virus-specific factors, such as pre-treatment viral load, although this also correlates independently (inversely) with response[171].

The most promising evidence for a relationship between virus sequence and persistence/treatment resistance was demonstrated in the region of NS5A that interacts with the dsRNA-dependent protein kinase (PKR). Long before its function was known, it was observed that there was a clustering of amino acid changes in NS5A during IFN treatment. An association was also found between treatment response and possession of the so-called 'prototype' NS5A sequence in the region where mutations occurred [172]. Prototype 'IFN-sensitivity determining region' (ISDR) sequences were also associated with higher circulating virus loads in untreated patients [173]. As the ISDR colocalizes with the part of NS5A that interacts with PKR, it was suggested that PKR evasion was a key determinant in the persistence of HCV and, potentially, other aspects of virus–host interaction.[149]

Since the original study, several groups have sought to reproduce the findings of a dependence on ISDR sequence of treatment response in other patient cohorts. Despite highly variable results between studies, a recent metaanalysis of all the available data has demonstrated a clear

correlation between the prototype ISDR sequence and treatment resistance and, as a corollary, a large number of diverse amino acid changes in non-responders [174]. It has also been shown that the same differential response exists in HCV genotype 2a and 2b infections [175]. In trying to unravel the mechanism of this interaction, it remains curious that whilst the 'prototype' ISDR sequence of NS5A is found specifically in individuals who resist IFN therapy, there is no evident selection for this sequence in viruses with non- 'prototype' sequences that are treatment-sensitive.

In contrast to the clear-cut differences between genotypes in their response to antiviral therapy, the data on the differences in natural history and pathogenicity between HCV genotypes is match more controversial.

Longitudinal studies, where the course of HCV disease over time in individuals with known times of infection is monitored prospectively, are few in number and frequently limited to patients who are infected with a single genotype. For example, natural history studies of the Irish and East German anti-D cohorts considered individuals who were infected only with genotype 1b [176, 177]. Similarly, a prospective study in the USA of individuals who were exposed to HCV by blood transfusion in the 1970s was limited to predominantly genotype 1a or 1b infections [178]. However, more genotype diversity is found in several European cohorts in which an early diagnosis of infection was possible through specific risk factors, such as haemophilia, or in community-based case-control studies. In these studies, genotype 1 appeared invariably to be more likely to establish persistence and, in carriers, to be associated with more severe liver disease, compared with genotypes 2 and 3 [179-182].

Surprisingly, and in contrast to the probable greater long-term pathogenicity of genotype 1, infections with genotype 3 are associated with a higher incidence of steatosis [183, 184], which is thought to result from direct cytopathic damage to hepatocytes from a block in lipoprotein secretion [185]. As with the many other manifestations of biological differences between genotypes (including the vexed question of whether genotype 1 is more likely to cause HCC [186]), the availability of an in vitro system for investigating differences in the replication of different genotypes would be of considerable value in dissecting out the differences in virus–host cell interactions that underlie these clinical observations.

AIM OF THE STUDY

The focus of this study was molecular monitoring of HBV and HCV genomes occurring in Latvia and characterization of mutated HBV genomes. Specifically this study set out to address the following issues:

1. creating a representative collection of sera from HBV and HCV infected patients ;
2. PCR-amplification, sequencing and phylogenetic analysis of HBV and HCV genome fragments obtained from different cohorts of Latvian patients;
3. determination of genotypes and subtypes of HBV distributed in Latvia;
4. determination of genotypes of HCV distributed in Latvia
5. finding of definite mutations at the nucleotide and amino acid levels in the HBV regulatory regions (EnII/BCP), as well as in structural (preS/S and preC/C) and non-structural (X) genes in the obtained HBV isolates.

DESCRIPTION OF THE METHODS

1. Serological tests

HBsAg was detected with an Enzygnost HBsAg 5.0 ELISA Kit (Dade Behring, Marburg, Germany). 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).

2. 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 3. When amplification of the whole fragments failed, we used nested PCR with inner primers [187]

3. 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 mentioned above (Lyteh). Amplification of the HCV *core* region was performed by nested RT-PCR. Primers used for RT and PCR are listed in Table 3.

4. 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 [188].

Table3. Primers for PCR, RT-PCR, and sequencing of HBV and HCV genome fragments

Region of analysis		Primer	Sequence	Reference
HBV				
pres/S	2750-813 nt	17p	5' TTATTTACATACTCTTTGGAAGGC 3'	[189]
		8.15	5' AATGTATACCCAAAGACAG 3'	
gene S (nested)	215-710 nt	S1p	5' TTGTTGACAAGAATCCTCACAATACC 3'	[190]
		S2p	5' GCCCTACGAACCACTGAACAAATGG 3'	
preC/C	1741-2516 nt	M3	5' CTGGGAGGAGTTGGGGGA 3'	[191]
		p19	5' AGGTACTGTAGAGGAATAAAGCCC 3'	[192]
geneC (nested)	2045-2387nt	C1"	5' GTTCACCTCACCATACTGCACTCAGGC 3'	[193]
		C2'	5' GAGTTCTTCTTCTAGGGGACCTGCCTCG 3'	
BCP/preC (nested)	1741-2114 nt	M3	5' CTGGGAGGAGTTGGGGGA 3'	[194]
		2.364	5' CCCAGGTAGCTAGAGTCAT 3'	[187]
gene X	1505-1825 nt	21p	5' CGTTCCAGCCGACCACGGGGCGC 3'	[195]
		2-Sp	5' AAAAAGTTGCATGGTGCTGG 3'	[196]
HCV				
core (nested)	476-725 nt	1AS	5' ATGTACCCCATGAGGTCGGC 3'	[187]
		2S	5' TAGATTGGGTGTGCGCGCGA 3'	
		3S	5' CGCGCGACTAGGAAGACTTC 3'	
		4S	5' TGTGTGCGCGACGCGTAAA 3'	
		5AS	5' GCAYGTRAGGGTATCGATGACYT 3'	

5. Phylogenetic analysis

The sequences were edited manually by the BioEdit Sequence Alignment Editor [197] and subsequently aligned in the FASTA format (http://ngfnblast.gbf.de/docs/fasta.html). The phylogenetic trees were constructed using DNA-distance algorithm and the neighbour-joining method in the PHYLIP package [198].

RESULTS AND DISCUSSION

1. Serological analysis of collected sera

The representative collection of sera of Latvian patients with hepatitis B and C has been created. It includes sera from three groups of patients :(1) Kidney Transplantation centre (48 samples), (2) Paediatric oncology ward (46 samples), and (3) Latvian Infectology center (89 samples of patients with chronic hepatitis B and C). Collected sera were tested for presence of HCV- and HBV-associated serological markers: HBsAg, a-HBc, HBeAg, a-HBe, as well as for the presence of HBV DNA and HCV RNA. Results of testing are shown in the Tables 4-7.

Table 4. Analysis of sera from patients with chronic HBV infection from Latvian Infectology center

Code	HBsAg	Anti-HBc	HBe	Anti-HBe	Anti-HCV	HBV PCR
LIC2-2857	+	-	-	+	NT	+
LIC3-577	+	+	+	-	-	+
LIC3-987	+	+	-	+	-	+
LIC4-1725	+	+	NT	NT	NT	+
LIC4-1835	+	+	+	-	-	+
LIC4-2015	+	+	+	-	-	+
LIC4-2307	+	+	+	-	-	+
LIC4-2684	+	NT	NT	NT	NT	+
LIC4-2688	+	+	NT	NT	NT	+
LIC4-2971	+	NT	NT	NT	NT	+
LIC4-3445	+	+	-	+	-	+
LIC4-775	+	+	-	+	+	+
LIC4-778	+	+	-	+	-	+
LIC5-2628	+	-	NT	NT	NT	+
LIC5-2878	+	+	-	+	-	+
LIC5-556	+	+	NT	NT	-	+
LIC6-217	+	+	+	-	-	+
LIC6-220	+	+	+	-	-	+
LIC6-444A	+	+	-	+	-	+
LIC7-1010	+	+	+	-	-	+
LIC7-1117A	+	+	+	-	-	+
LIC7-1275	+	+	-	-	+	+
LIC7-1442	+	+	+	-	-	+
LIC7-1884	+	+	+	-	-	+
LIC7-283	+	+	+	-	-	+

Code	HBsAg	Anti-HBc	HBe	Anti-HBe	Anti-HCV	HBV PCR
LIC7-624	+	+	-	+	-	+
LIC7-640	+	+	-	-	-	+
LIC8-2186	+	+	-	+	-	+
LIC8-3662	+	+	-	+	-	+
LIC9	+	**NT**	**NT**	**NT**	**NT**	+
LIC9-2772	+	+	-	-	-	+
LIC9-296	+	+	+	-	-	+
LIC9-3113	+	+	+	-	-	+
LIC9-3163	+	+	+	-	-	+
LIC9-4070	+	+	+	-	-	+
LIC9-599	+	+	+	-		+
LIC10-1108	+	+	+	-	-	+
LIC10-1612	+	+	-	+	-	+
LIC10-2085	+	+	+	-	-	+
LIC10-2400	+	+	+	-	-	+
LIC10-2425	+	+	+	-	-	+
LIC10-2596	+	+	+	-	-	+
LIC10-486	+	+	-	+	+	+
LIC10-737	+	+	+	-	**NT**	+
LIC11-1941	+	+	-	+	-	+
LIC11-2343A	+	+	+	-	-	+
LIC11-2514	+	+	-	**NT**	+	+
LIC11-2759	+	+	+	-	-	+
LIC11-3028	+	-	+	-	-	+
LIC11-884	+	-	+	-	-	+
LIC11-913	+	-	+	-	-	+
LIC11-965	+	+	-	+	-	+
LIC12-82	+	+	+	-	-	+
LIC12-958	+	+	-	+	-	+

Table 5. Analysis of sera from patients with chronic HCV infection from Latvian Infectology center.

Code	Anti-HCV	HCV RT-PCR
LIC0233	+	+
LIC0257	+	+
LIC0258	+	+
LIC0280	+	+
LIC0283	+	+
LIC0287	+	+
LIC0296	+	+
LIC0311	+	+
LIC0315	+	+
LIC0321	+	+
LIC0346	+	+
LIC0358	+	+
LIC0375	+	+
LIC0428	+	+
LIC0448	+	+
LIC0493	+	+
LIC0639	+	+
LIC0941	+	+
LIC1108	+	+
LIC1159	+	+
LIC1171	+	+
LIC1221	+	+

Table 6. Analysis sera from patients from Kidney Transplantation centre renal transplant and dialysis patients (T – after transplantation, D – ongoing dialysis)

Code	HBsAg	Anti-HBc	HBeAg	Anti-HBe	Anti-HCV	HBV PCR	HCV RT-PCR
1T	+	+	+	-	-	+	-
2T	+	+	+	-	+	+	+
3T	+	+	-	+	-	-	-
4T	+	+	+	-	+	+	+
5T	+	+	-	+	+	-	+
6T	+	+	-	+	-	-	-
9T	+	+	+	-	-	+	-
10T	+	+	-	+	-	-	-
11T	+	+	+	-	-	+	-
12T	+	+	+	-	+	+	+
13T	+	+	+	-	+	+	+
15T	+	+	+	-	+	+	-
17T	-	-	-	-	-	-	-
18T	-	-	-	-	-	-	-
19T	+	+	+	-	-	+	-
20T	-	-	-	-	+	-	+
21T	-	-	-	-	+	-	-
23T	+	+	+	-	-	+	+
24T	+	+	-	+	-	+	-
25T	-	-	-	+	+	-	+
26T	+	+	-	+	-	+	-
28T	+	+	-	+	-	-	+
29T	+	+	-	+	-	+	-
30T	+	+	+	-	-	+	+
31T	+	+	+	-	+	+	-
32T	+	+	+	-	-	+	-
33T	+	+	+	-	-	+	+
34T	-	-	-	-	+	-	-
35T	-	-	-	-	+	-	+
36T	+	+	+	-	+	+	+
37T	-	-	-	-	+	-	+
39T	-	-	-	-	+	-	+
40T	+	+	+	-	-	+	-
41T	-	-	-	-	+	-	-
54T	-	+	-	-	-	-	-
56T	-	+	-	-	+	-	+
58T	+	+	+	-	+	+	+
59T	+	-	+	-	+	+	+
1D	-	-	-	-	+	-	-
2D	+	+	+	-	+	-	-
3D	+	+	+	-	+	+	-
4D	-	+	-	-	+	-	+
5D	-	-	-	-	+	-	+
6D	-	+	-	-	+	-	+
7D	-	-	-	-	-	-	-
8D	-	-	-	-	-	-	-
9D	-	+	-	-	-	-	+
10D	-	-	-	-	+	-	+

Table 7. Serological analysis sera from paediatric oncology ward's patients

Code	HBsAg	a-HBc	HBeAg	a-HBe	HBV PCR	HCV RT- PCR
01B	+	+	+	NT	+	+
02B	-	+	-	NT	-	+
03B	+	+	+	NT	+	-
04B	-	+	-	NT	-	+
05B	-	+	-	NT	-	-
06B	+	+	+	NT	+	+
07B	+	+	+	NT	+	-
08B	+	-	+	NT	+	-
09B	+	-	+	NT	-	+
10B	+	+	-	+	-	+
11B	+	+	+	NT	-	+
12B	+	-	-	NT	-	+
13B	+	-	-	NT	-	+
14B	+	-	-	NT	-	+
15B	+	+	-	+	-	-
16B	+	+	+	NT	+	-
17B	-	-	NT	NT	-	+
18B	+	+	+	NT	+	-
19B	-	+	-	NT	-	+
20B	+	-	-	-	-	+
21B	-	+	NT	NT	-	-
22B	+	+	+	NT	+	-
23B	+	+	-	NT	-	+
24B	+	+	+	NT	+	+
25B	-	+	-	NT	-	+
26B	+	+	+	NT	+	-
27B	+	+	-	NT	-	+
28B	+	+	+	NT	+	-
29B	+	-	NT	NT	-	+
30B	+	+	-	-	-	-
31B	+	+	+	NT	+	-
32B	+	+	-	NT	-	+
33B	+	-	-	NT	-	+
34B	+	-	-	NT	-	-
35B	+	+	-	NT	-	-
36B	+	-	+	NT	+	+
37B	+	+	-	NT	-	+
38B	+	+	NT	NT	-	-
39B	+	+	+	NT	+	-
40B	+	-	NT	NT	+	+
41B	+	+	+	NT	+	+
42B	+	+	+	NT	+	-
43B	NT	+	NT	NT	-	+
44B	NT	+	NT	NT	-	+
45B	+	+	+	NT	+	-
46B	NT	NT	-	-	-	-

2. Distribution of HBV genotypes in Latvia

HBV regions X/EnII/BCP, preS/S, and preC/C were sequenced and analyzed in order to determine the HBV genotype/subtype and to check the presence of nucleotide and amino acid changes in comparison with prototype HBV sequences. To identify the HBV genotype, the gene S nucleotide sequences were aligned with representative gene S sequences from HBV genotypes A-H obtained from GeneBank database [199-201], and the appropriate phylogenetic trees were constructed (Fig.5). For comparison purposes, the phylogenetic trees were constructed also for the HBV gene C (Fig. 6). The results of the genotype analysis are shown in the Table 8.

Two predominant HBV genotypes were identified in the selected groups of patients. 73.56% of HBV DNA positive samples belonged to the genotype D and 26.44% to the genotype A. No other HBV genotypes have been found in Latvia so far. The phylogenetic trees of HBV isolates (Fig.1, 2) showed that the representatives of the A genotype are more conserved, whereas the D genotype is definitely more divergent.

The groups of patients showed strongly pronounced difference in ratio of distributed HBV genotypes. This phenomenon could be explained by different transmission pattern of HBV infection in different groups of patients. Thus, most of patients from the paediatric oncology ward were infected in the outbreak [202]. Most of HBV sequences obtained from patients of this group indicate close evolutionary relationship and therefore possible transmission of the virus between patients. There wasn't found distinct connection between HBV infected renal transplant patients, but according to results of phylogenetic analysis a number of HBV sequences obtained from these persons belonged to genotype A are very similar. Finally, the distribution of HBV genotypes in patients with chronic hepatitis is closest to general tendency.

The subtype of HBV was deduced from the primary structure of HBsAg according to Magnius and Norder, 1995 (Table 1.). In the case of one patient (LIC9) it was impossible to detect subtype because of mutations affecting subtype specifying determinants. The results of subtype analysis are summarized in the Table 8. HBV isolates belonged to genotype D represent the HBsAg subtypes *ayw3*, *ayw2*, and *adw3*, whereas HBV isolates belonged to genotype A represent the HBsAg subtype *adw2*. Three isolates belonged to putative subtype *adw3* [203] were found only in renal transplant patients.

Longitudinal data on distribution of HBV genomes in Latvia [204], showed clear evidence that the subtype ayw2 (GenBank accession X02496) is most common in Latvia. However, present study reveals predominance of the ayw3 (GenBank accession V01460) subtype of the D genotype, whereas the adw2 subtype (GenBank accession X02763) is fully predominant in the A genotype.

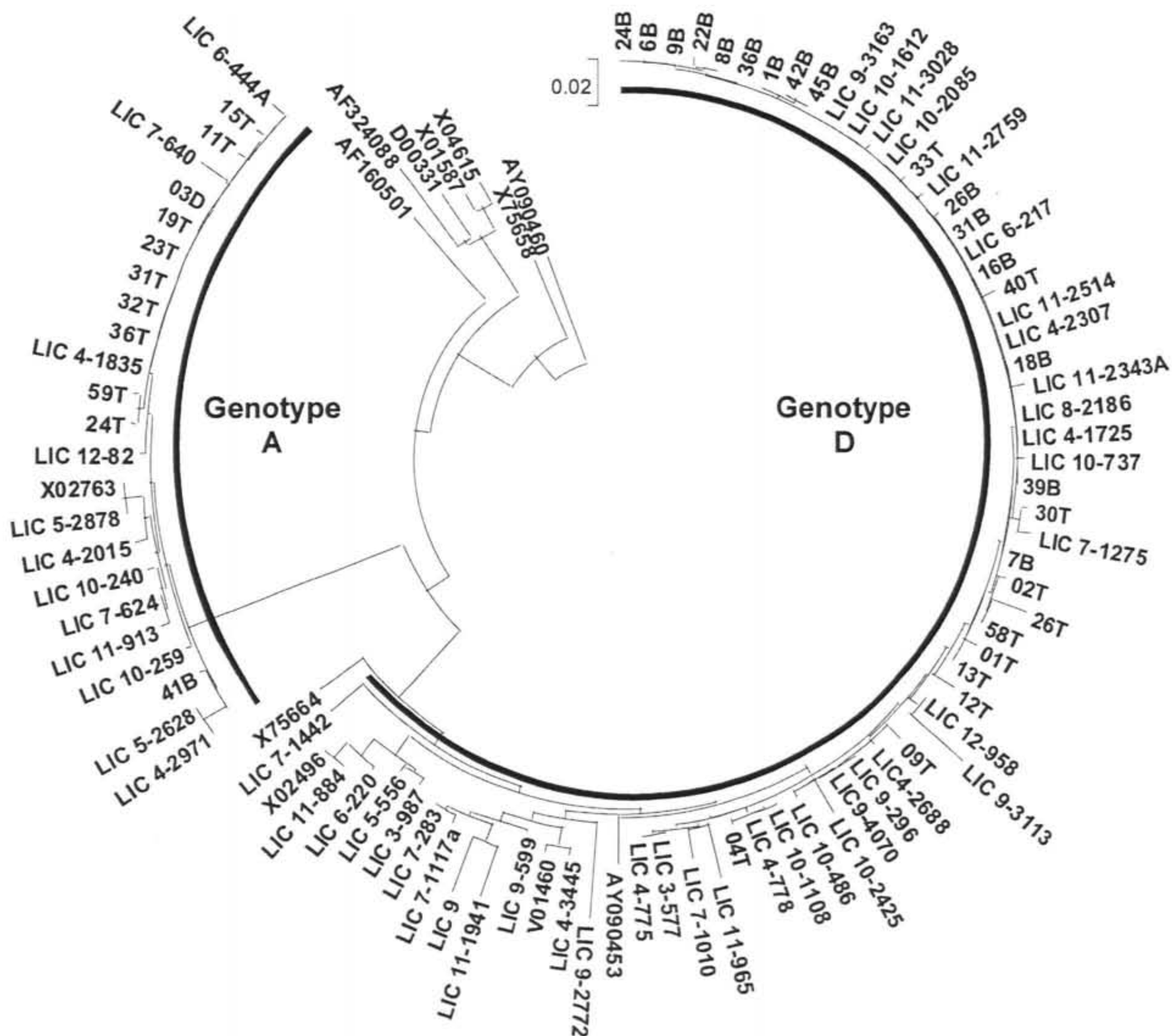


Figure 5. Phylogenetic tree based on the HBV S gene sequences. Sequences from patients with chronic hepatitis have the prefix LIC, sequences from patients after kidney transplantation have the prefix T, sequences from patients ongoing dialysis have the prefix D and sequences from patients from the paediatric oncology ward have the prefix B.

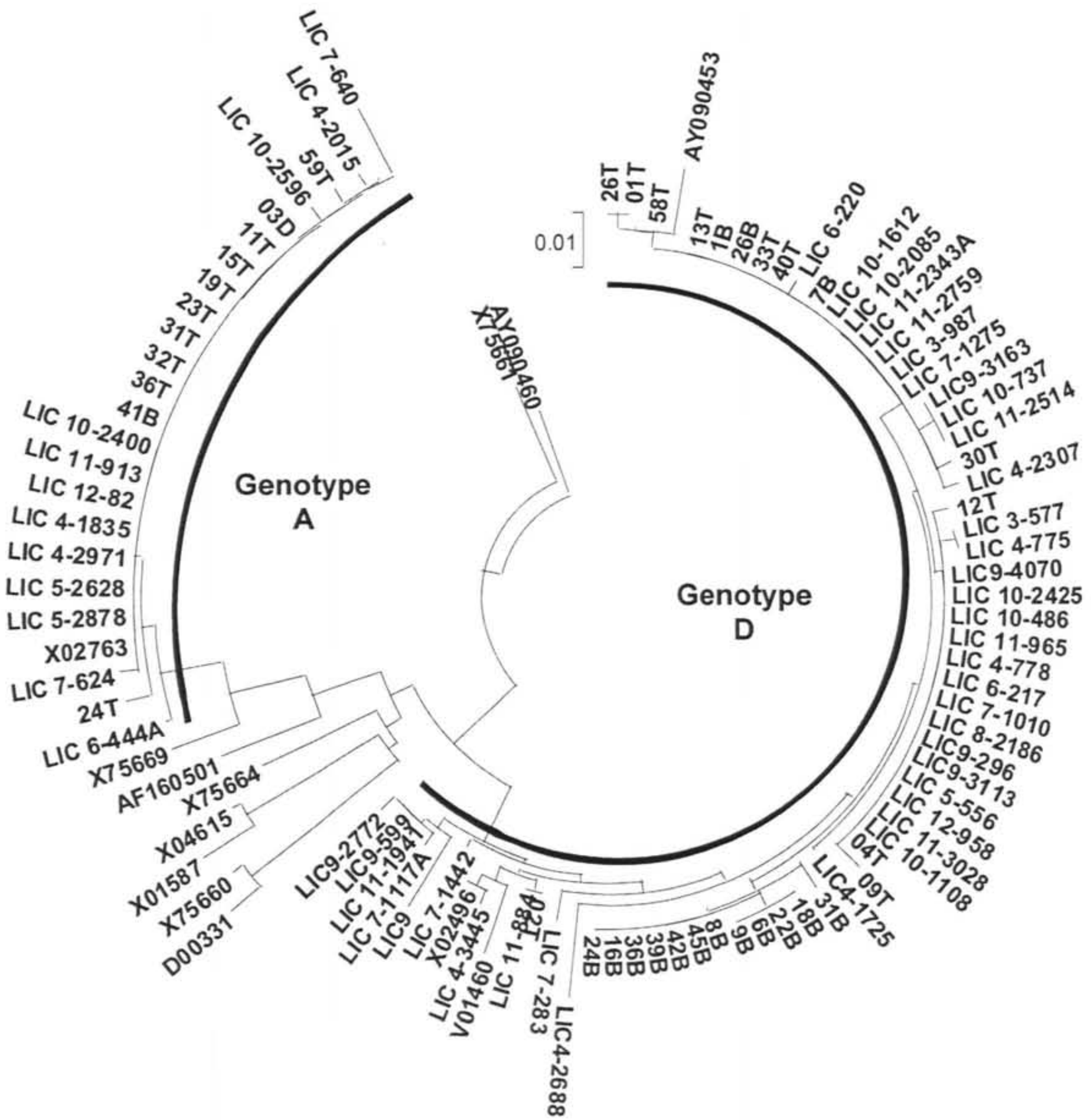


Figure 6. Phylogenetic tree based on the HBV C gene sequences.

Table 8. Distribution of HBV genotypes and subtypes among different groups of patients.

	HBV genotype		HBsAg subtype			
	A	D	*adw2*	*ayw2*	*ayw3*	*adw3*
Kidney transplantation, hemodialysis	10 (43.48%)	13 (56.52%)	10 (43.48%)	3 (13.04%)	7 (30.43%)	3 (13.04%)
Paediatric oncology ward	1 (7.69%)	12 (92.31%)	1 (7.69%)	9 (69.23%)	3 (23.08%)	0
Chronic hepatitis	12 (23.53%)	39 (76.47%)	12 (24.00%)	8 (16.00%)	30 (60.00%)	0
Total	23 (26.44%)	64 (73.56%)	23 (26.74%)	20 (23.26%)	40 (46.51%)	3 (3.49%)

3. HBV mutants

3.1. Gene X/EnII/BCP

A set of mutations affecting the amino acid sequence of the X protein has been found (Fig 7-9). The mutation at nt 1652 (A→T) found in two HBV isolates from patients with chronic hepatitis leads to the His94Tyr exchange in the immunodominant epitope of the HBx protein [41]. The functional role of these mutations may be explained also by their influence on the EnII and BCP regions, which overlap with the gene X.

The double mutation in the central part of the BCP - A1762T and G1764A – was found in five HBV isolates: one from renal transplant patient and four from patients with chronic hepatitis. 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 [205]. Although these mutations are not directly associated with HBeAg negativity, they could suppress HBeAg titers in HBeAg-positive patients. These changes, considered "hot spot mutations", are found in patients with hepatocellular carcinoma carrying an *ayw* subtype, the D genotype [206]. In our case, three of five HBV isolates belong to the genotype D, so, these patients may be at increased risk of development of liver cancer. In one case HBV isolate has mutation G1764A but nucleotide in position 1762 is changed not to "classical" T but to G. Interesting also is that 8 from 13 HBV isolates obtained from patients from paediatric oncology ward have a single mutation G1764A but had no changes in position 1762.

The BCP mutation A1752C was reported also in conjunction with the 1762 and 1764 mutations and appeared to be associated with liver damage [207]. This mutation was found in four HBV isolates - two from renal transplant patients and two from patients with chronic hepatitis, but in all cases it was only unique mutation in the BCP region.

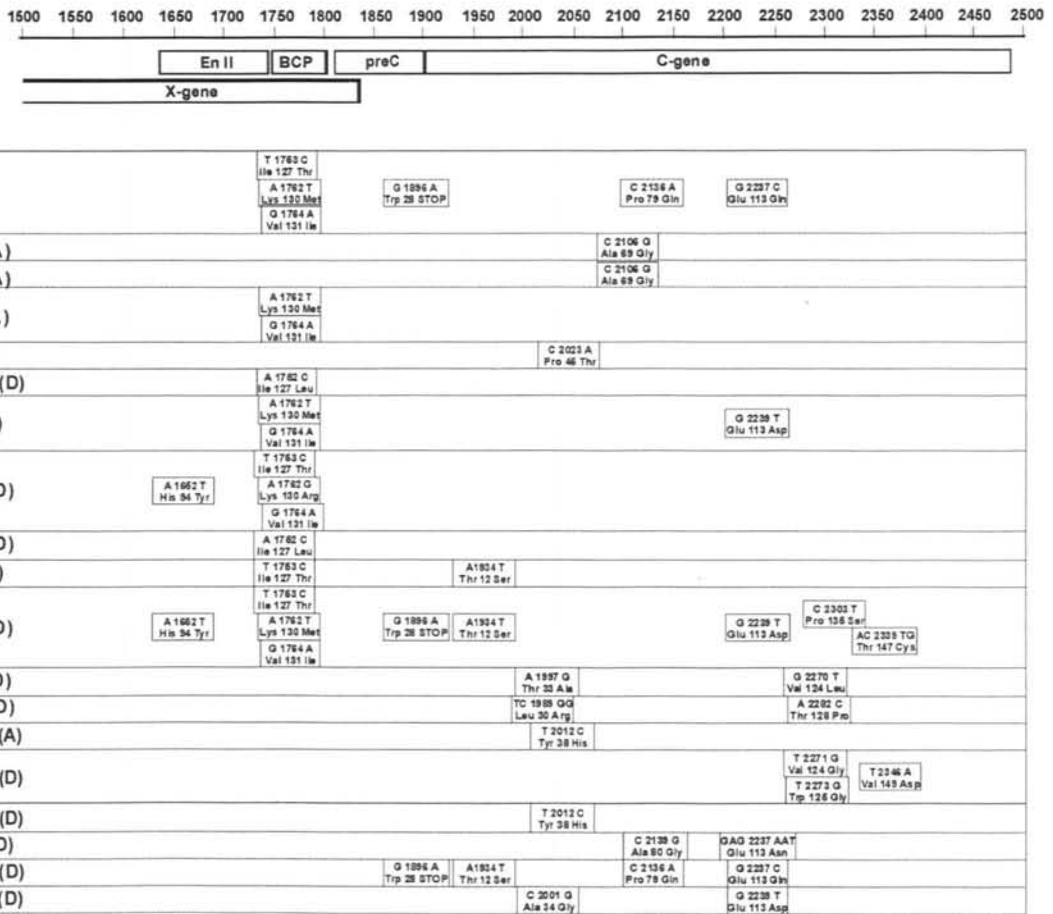
The mutation T1753C was found in four HBV isolates form from patients with chronic hepatitis and two isolates from renal transplant patients. This mutation has association with progression of liver disease to cirrhosis in patients chronically infected with genotype C [41], but in our case all isolates belong to genotype D.

In contrast to other patients groups HBV isolates from renal transplant patients contain also number of mutations in EII/BCP region that are not described in literature: C1665A, T1678C, A1729T, A1761C, C1773A. On the other hand, there only one G1764A mutation in EII/BCP region was found in isolates from patients from paediatric oncology ward.

3.2. Gene C

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) [208-211]. This may contribute to the adaptive mechanisms decreasing the production and secretion of HBeAg and viral persistence.

The preC stop codon mutation G1896A was detected in six HBV isolates: three from patients with chronic hepatitis, two from renal transplant patients and one from patient from paediatric oncology ward. This mutation is common in HBeAg-negative HBV phenotype with aborted HBeAg expression and is known to cause chronic hepatitis [212], although G1896A mutations occurred in 15% [213] and 38% [214] of cases in HBeAg-positive patients. In our patient cohort, we also found HBeAg in three sera with the preC stop codon mutation, two from renal transplant patients and one from chronic hepatitis patient. The presence of HBeAg in the sera could be explained by the appearance of wild-type HBV DNA along with the preC stop codon mutant, which is clearly evident from the sequencing data (Fig. 10).



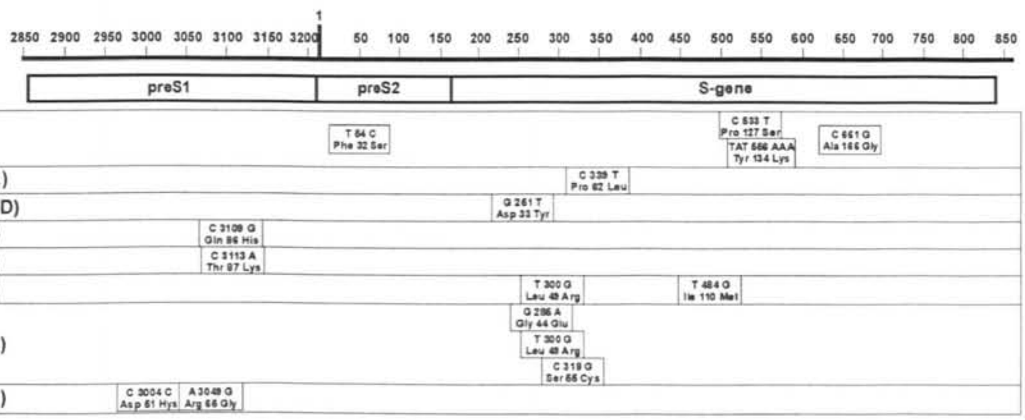


Figure 7. Nucleotide sequences of HBV DNAs isolated from sera of patients with chronic hepatitis, numeration is given according to adw2 (X02763). Boxes show changes of nt (upper line) and aa (lower line) against the corresponding subtype sequence.

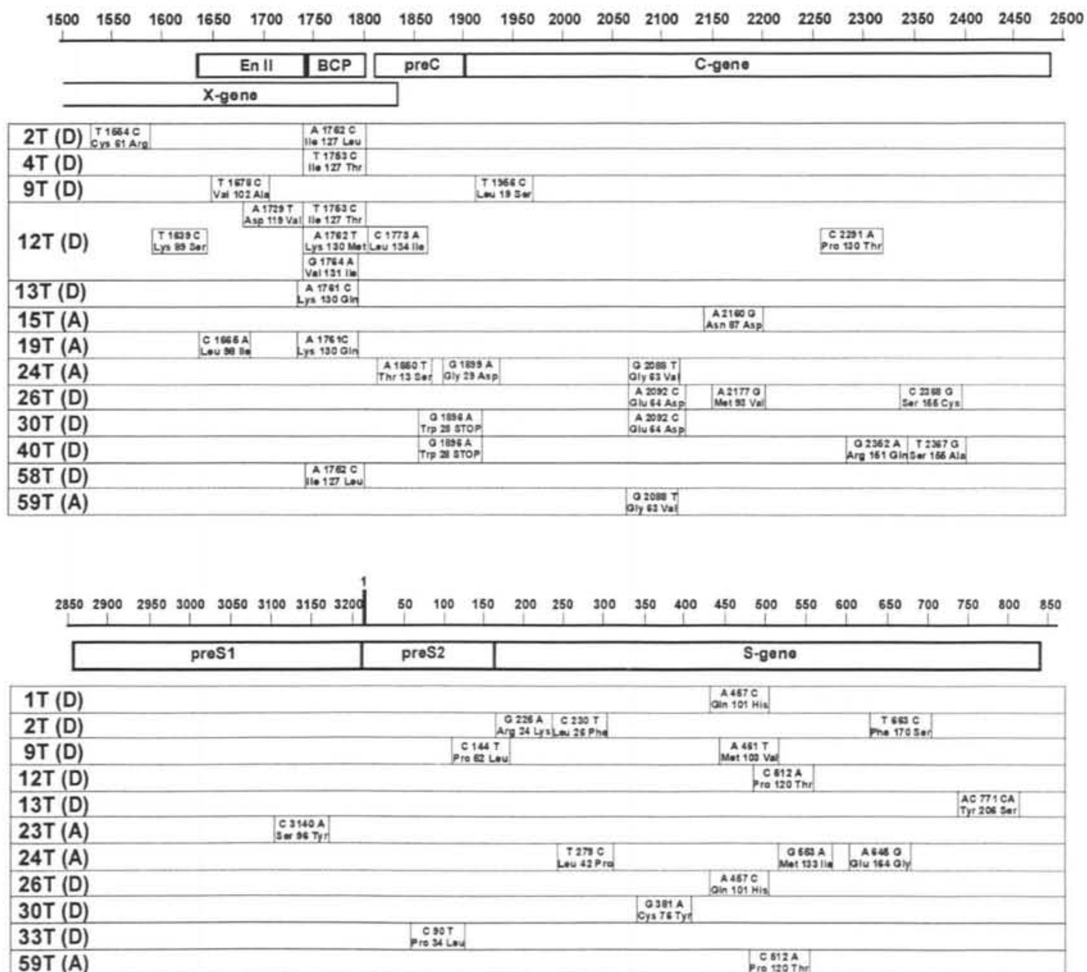
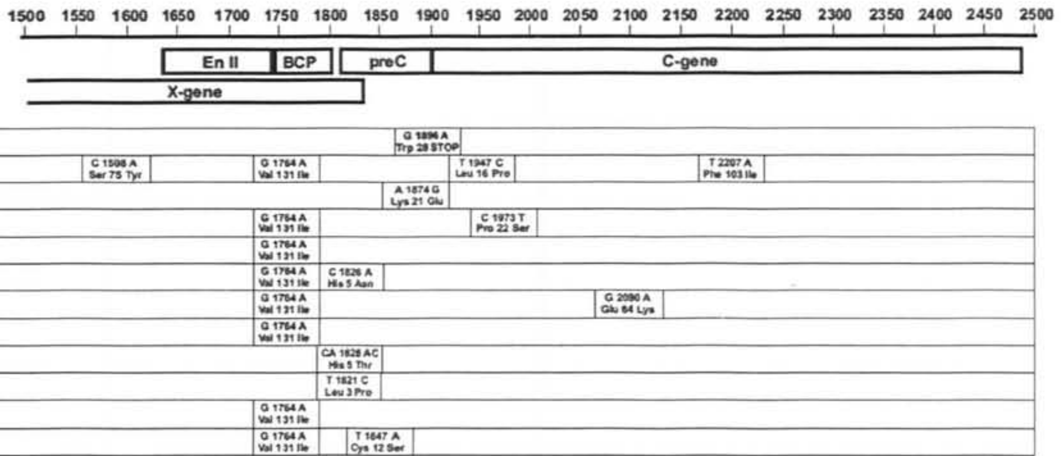


Figure 8. Nucleotide sequences of HBV DNAs isolated from sera of renal transplant patients, numeration is given according to adw2 (X02763). Boxes show changes of nt (upper line) and aa (lower line) against the corresponding subtype sequence.



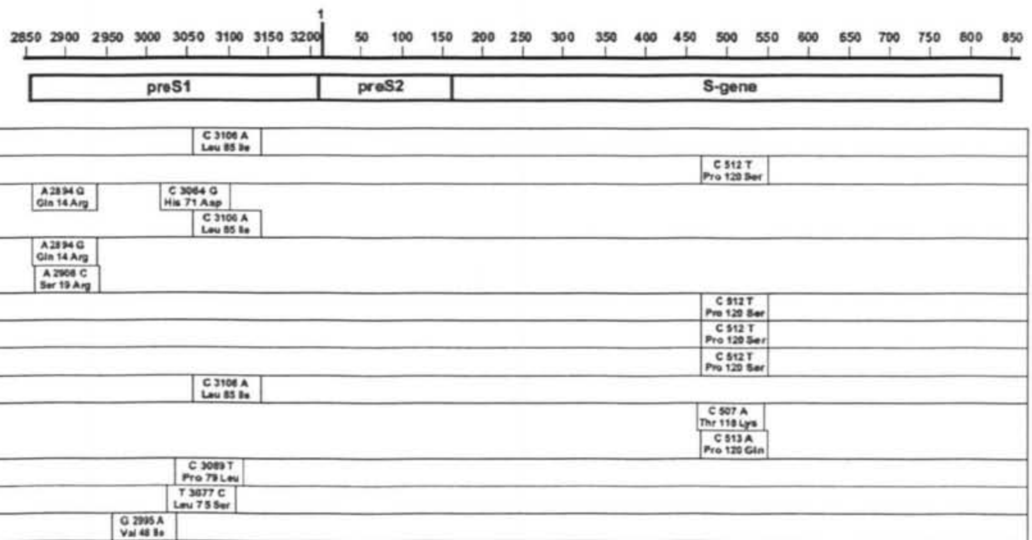


Figure 9. Nucleotide sequences of HBV DNAs isolated from sera of patients of paediatric oncology ward, numeration is given according to adw2 (X02763). Boxes show changes of nt (upper line) and aa (lower line) against the corresponding subtype sequence

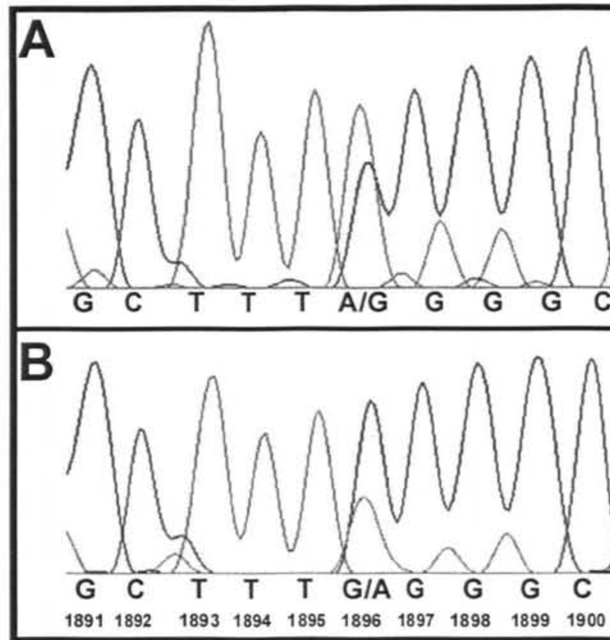


Figure 10. Simultaneous presence of preC stop-codon mutant and wildtype DNA in the sera of patients 30T (a) and 40T (b). Results of automatic sequencing are shown.

Most often preC stop codon mutation was detected in isolates from renal transplant patients (9%) next are patients from paediatric oncology ward (8%) and the last are patients with chronic hepatitis (6%). According to literature data, this mutation is rarely found in genotype A and relatively often occurs at immunosupressed patients. In our case all HBV isolates with preC stop codon mutation belonged to genotype D.

We found number of substitutions in the previously described CTL epitopes of C protein: Leu19Ser, Pro22Ser, Gly63Val, Glu64Asp, Ala69Glu, Pro79Gln, Ala80Gly, Asn87Asp, Met93Val. Most of them were found in HBV isolates from renal transplant patients. Contrary, HBV isolates from patients from paediatric oncology ward demonstrate a relative conservatism in a *core* gene region. HBV isolates from patients with chronic hepatitis are more unsteady in this region, but most of mutations do not affect sufficient regions of *core* protein and could be explained by the natural variability of HBV.

We found one Pro130Thr substitution, which was detected exclusively in patients with chronic hepatitis with or without HBeAg. In our case it was found in HBV isolate from renal transplant patient. 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 [215]. This mutation appears as a result of immune selection and is associated with exacerbation of chronic hepatitis and hepatocellular carcinoma.

The carboxy-terminal processing site of the C protein was found to be mutated in two HBV isolates from renal transplant patients and two isolates from patients with chronic hepatic: Thr147Cys, Val149Asp, Arg151Gln, Ser155Cys, Ser155Ala.
In contrast to earlier observations [216], we did not find any HBV *core* deletion variants. This could be explained by the milder liver disease in our patient cohort.

3.3. PreS/S Gene

Analysing the S gene sequences of obtained HBV isolates we found number of mutations affecting the *a*-determinant region. Mutations in the 107-137 aa loop, associated with chronic form of HBV infection were found in 11 HBV isolates. The most often these mutations were found in HBV isolates from patients of paediatric oncology ward. Four of this isolates (6B, 9B, 18B, 22B) belonged to genotype D had an identical aa substitution Pro120Ser. The phylogenetic analysis demonstrated a high similarity of sequences corresponding to isolates 6B, 9B and 22B, and so it is possible that it was the same HBV isolate. Possible, these patients infected each other or were infected from one source.
The mutations affecting the subtype specifying determinants (Pro127Ser and Tyr134Lys) were found in the isolate from patient with chronic hepatitis LIC9. Unfortunately we have no data about serological subtyping of this isolate.
Most of the mutations found in HBV isolates from renal transplant patients were located within the CTL and T helper epitopes of protein S: aa 19–28, 41–49, 97–106, and 187–216 [217]. Mutations in aa 29–53 region contributing to chronicity were found in five HBV isolates from patients with chronic HBV and in one isolate from renal transplant patient, but weren't found in any isolate from paediatric oncology ward.
Typical deletions within the preS1 and preS2 regions were not found. Interestingly, the preS region was relative conservative in comparison with other regions of the HBV isolates from renal transplant patients and patients with chronic hepatitis but it was highly heterogeneous in the HBV isolates from paediatric oncology ward.

4. Distribution of HCV genotypes in Latvia

HCV *core* region was sequenced and analysed in order to determine the HCV genotype. Phylogenetic analysis of HCV *core* region nucleotide sequences showed that 57 isolates belonged to genotype 1b, six isolates to genotype 3a, one isolate to genotype 1a and one isolate to genotype 2c (Fig. 11.). Distribution of HCV genotypes in isolates from different patient groups are shown in Table 9.

Obtained results showed a strong domination of genotype 1b in all groups of patients: 87% in case of chronic HCV infection, 78% in case of posttransplantation, 100% in case of dialysis and 95% of case of patients from the paediatric oncology ward. Not all of analysed groups are equally representative. As it was shown earlier [202], most of patients from the paediatric oncology ward were infected in the outbreak. According to phylogenetic analysis 13 from 19 patients were infected with the same HCV isolate belonged to genotype 1b. On the other hand, the rate of evolution to chronicity after acute exposure to HCV was 92% in patients exposed to HCV genotype 1b infection, compared to 33% - 50% in patients exposed to other genotypes [148]. Distribution of HCV genotypes among patients ongoing dialysis and after kidney transplantation is similar to the distribution among non–renal patients in the same country [218]. Taking all this obstacles into consideration, about 85% of HCV isolates presented in Latvia, belonged to genotype 1b. It is known, that genotype 1b was seen more often among patients, acquired HCV through blood transfusion of unscreened blood products and medical procedures and showed relatively poor response to treatment by traditional anti-viral drugs [219].

The genotype 3a was detected in about 9% of cases among Latvian patients infected with HCV. According to published data, this genotype showed a good response to interferon therapy and is the most common among intravenous drug users [220], but in our case no one of patients infected with HCV genotype 3a was registered as drug user.

In a contrast with Western Europe countries, HCV genotypes 1a and 2 are not widespread in Latvia. We think, that the main transmission pattern of HCV in Latvia is 1b genotype infection that most likely spreading through blood transfusion and medical procedures and it is similar to other former Soviet Union Republics and some of Asian countries [221-223].

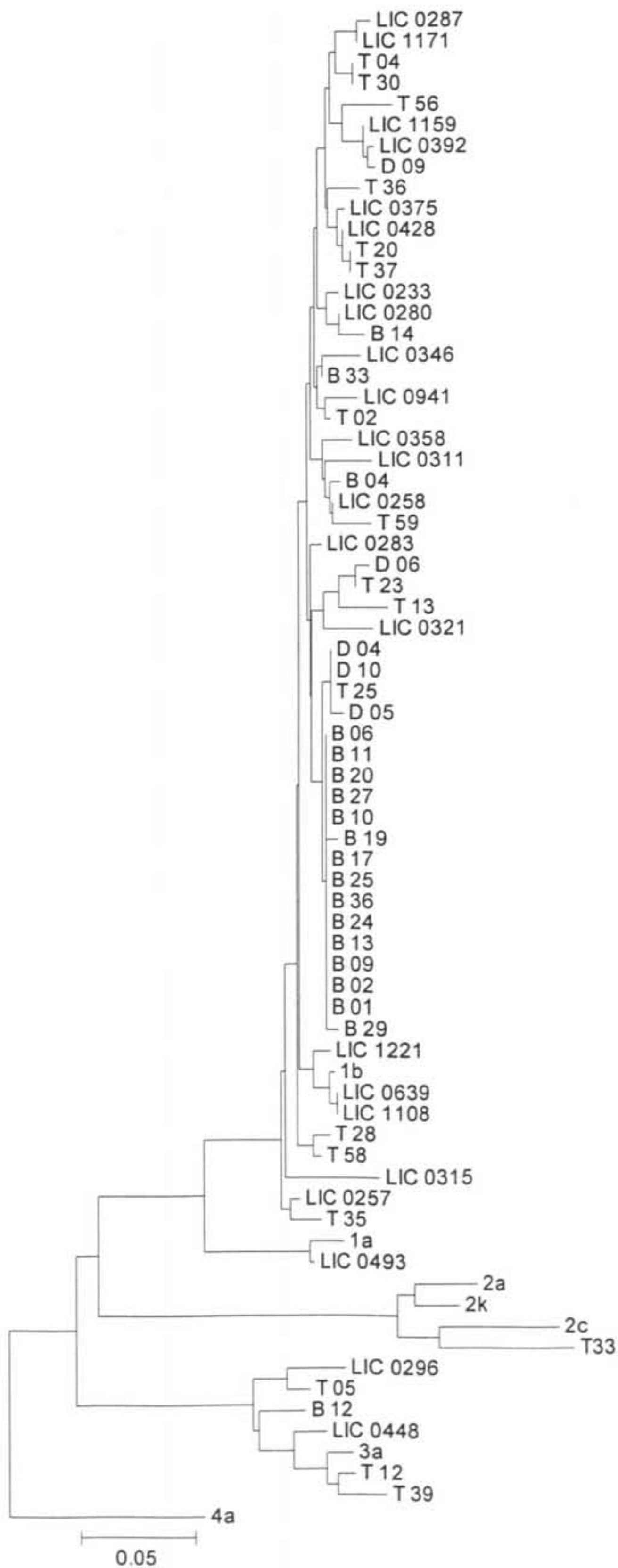


Figure 7. Phylogenetic tree of sequenced fragments of HCV *core* region. Reference sequences representing published genotypes 1a, 1b, 2a, 2c, 2k 3a and 4a are included.

Table 9. Distribution of HCV genotypes among different groups of patients

Groups of patients	HCV genotypes			
	1b	1a	2c	3a
Patients with chronic HCV infection	20 (86.95%)	1 (4.35%)	0	2 (8.70%)
Patients after kidney transplantation	14 (77.78%)	0	1 (5.56%)	3 (16.66%)
Patients ongoing dialysis	5 (100%)	0	0	0
Patients from the paediatric oncology ward	18 (94.74%)	0	0	1 (5.26%)
Total	57 (87.69%)	1 (1.54%)	1 (1.54%)	6 (9.23%)

CONCLUSIONS

1. The representative collection of sera of Latvian hepatitis B and C patients has been created. It includes sera from three groups of patients:(1) Kidney Transplantation centre (48 samples), (2) Paediatric oncology ward (46 samples), and (3) Latvian Infectology center (74 samples of patients with chronic hepatitis).
2. Phylogenetic trees were created on the basis of obtained nucleotide sequences of HBV genes S and C and HCV *core* region.
3. Two predominant HBV genotypes were identified in the selected groups of patients: 73.56% of HBV DNA positive samples belonged to the genotype D and 26.44% belonged to the genotype A. HBV isolates belonging to the genotype D were represented by the HBsAg subtypes *ayw*3, *ayw*2, and *adw*3, whereas HBV isolates belonging to the genotype A were represented by the HBsAg subtype *adw*2.
4. 87.69% of analyzed HCV isolates belonged to the genotype 1b, 9.23% belonged to the genotype 3a, 1.54% belonged to the genotype 1a, and 1.54% belonged to the genotype 2c.
5. Mutations affecting the HBV X/EnII/BCP, preC/C and preS/S regions were detected in the analyzed HBV isolates. The most significant of them were:

5.1 <u>X/EnII/BCP region</u> - His94Tyr, the double mutation A1762T and G1764A, A1752C, T1753C;

5.2 <u>preC</u>- Thr13Ser, preC stop codon mutation G1896A, Gly29Asp;

5.3 <u>*core*</u> - Leu19Ser, Pro22Ser, Gly63Val, Glu64Asp, Ala69Glu, Pro79Gln, Ala80Gly, Asn87Asp, Met93Val, Pro130Thr, Thr147Cys, Val149Asp, Arg151Gln , Ser155Cys, Ser155Ala;

5.4 <u>gene S</u> - Leu26Phe, Asp33Tyr, Leu42Pro, Gly44Glu, Leu49Arg, Ser55Cys, Thr118Lys, Pro120Thr, Pro127Ser, Met133Ile, Tyr134Lys.

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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-α 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-α [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 ± 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 ± 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
HBV			
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′ GTTCACCTCACCATACTGCACTCAGGC 3′	17
2045–2387 nt	C2	5′ GAGTTCTTCTTCTAGGGGACCTGCCTCG 3′	
BCP/preC (nested)	M3	5′ CTGGGAGGAGTTGGGGGA 3′	17
1741–2114 nt	2.364	5′ CCCAGGTAGCTAGAGTCAT 3′	
Gene X	21p	5′ CGTTCCAGCCGACCACGGGGCGC 3′	17
1505–1825 nt	2-Sp	5′ AAAAAGTTGCATGGTGCTGG 3′	15
HCV			
Core (nested)	1AS	5′ ATGTACCCCATGAGGTCGGC 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′ CTGTAACACGAGCAGGGGTCCT-AG 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-

BOIS:C85:ZINTV769XA.96 FF: ZUF9 E1: 48X3

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	*adw3*	–	
2T	42/M	+	+	–	+	+	+	D	*ayw2*	+	1b
3T	65/M	+	–	+	+	–	–			–	
4T	40/M	+	+	–	+	+	+	D	*ayw3*	+	1b
5T	23/F	+	–	+	+	+	–			+	3a
6T	46/M	+	–	+	+	–	–			–	
9T	29/F	+	+	–	+	–	+	D	*ayw3*	–	
10T	53/M	+	–	+	+	–	–			–	
11T	50/M	+	+	–	+	–	+	A	*adw2*	–	
12T	44/M	+	+	–	+	+	+	D	*ayw3*	+	3a
13T	54/F	+	+	–	+	+	+	D	*ayw3*	+	1b
15T	54/F	+	+	–	+	+	+	A	*adw2*	–	
17T	39/F	–	–	–	–	–	–			–	
18T	69/M	–	–	–	–	–	–			–	
19T	36/F	+	+	–	+	–	+	A	*adw2*	–	
20T	49/F	–	–	–	–	+	–			+	1b
21T	51/F	–	–	–	–	+	–			–	
23T	41/M	+	+	–	+	–	+	A	*adw2*	+	1b
24T	32/M	+	–	+	+	–	+	A	*adw2*	–	
25T	67/M	–	–	+	–	+	–			+	1b
26T	51/M	+	–	+	+	–	+	D	*adw3*	–	
28T	43/M	+	–	+	+	–	–			+	1b
29T	33/M	+	–	+	+	–	+	D	*ayw3*	–	
30T	54/F	+	+	–	+	–	+	D	*ayw3*	+	1b
31T	42/M	+	+	–	+	+	+	A	*adw2*	–	
32T	56/M	+	+	–	+	–	+	A	*adw2*	–	
33T	46/M	+	+	–	+	–	+	D	*ayw3*	+	2c
34T	31/M	–	–	–	–	+	–			–	
35T	57/F	–	–	–	–	+	–			+	1b
36T	32/M	+	+	–	+	+	+	A	*adw2*	+	1b
37T	53/F	–	–	–	–	+	–			+	1b
39T	54/F	–	–	–	–	+	–			+	3a
40T	26/M	+	+	–	+	–	+	D	*ayw3*	–	
41T	30/M	–	–	–	–	+	–			–	
54T	48/M	–	–	–	+	–	–			–	
56T	72/M	–	–	–	+	+	–			+	1b
58T	49/F	+	+	–	+	+	+	D	*adw3*	+	1b
59T	40/M	+	+	–	–	+	+	A	*adw2*	+	1b
1D	74/F	–	–	–	–	+	–			–	
2D	63/M	+	+	–	+	+	–			–	
3D	67/F	+	+	–	+	+	+	A	*adw2*	–	
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

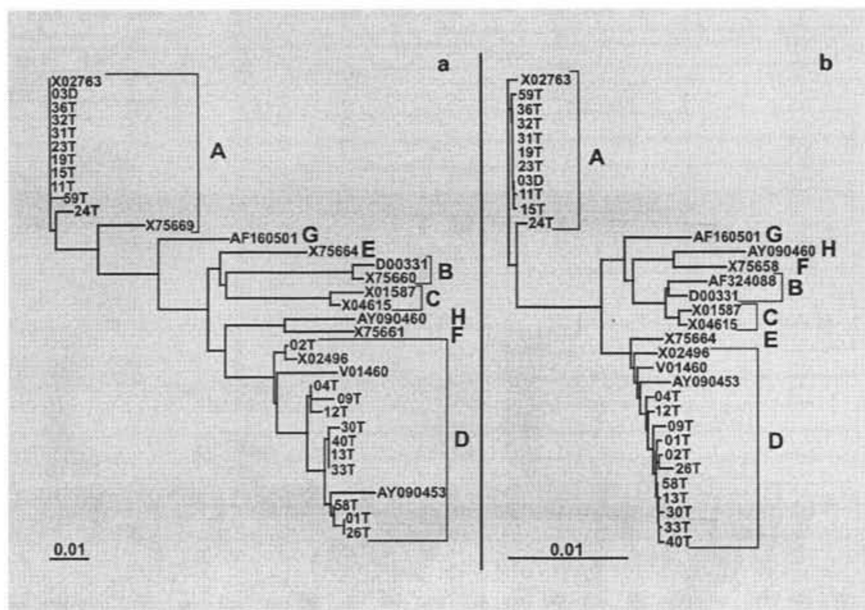

Fig. 1. Phylogenetic tree based on the 21 sequenced S (**a**) and C (**b**) genes. Database sequences (GenBank accession numbers are shown) representing genotypes A–H are included.

types [21]. It appeared that 8 HBV isolates were of HBsAg subtypes a*yw*3, one *ayw*2, and 3 *adw*3 (genotype D), whereas 9 HBV isolates belonged to the *adw*2 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 *ayw*2 (GenBank accession No. X02496) is most common in Latvia. However, the present study of renal transplant patients reveals a predominance of *ayw*3 (GenBank accession No. V01460) and *adw*3 (GenBank accession AY090453) subtypes of genotype D, whereas subtype *adw*2 (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-

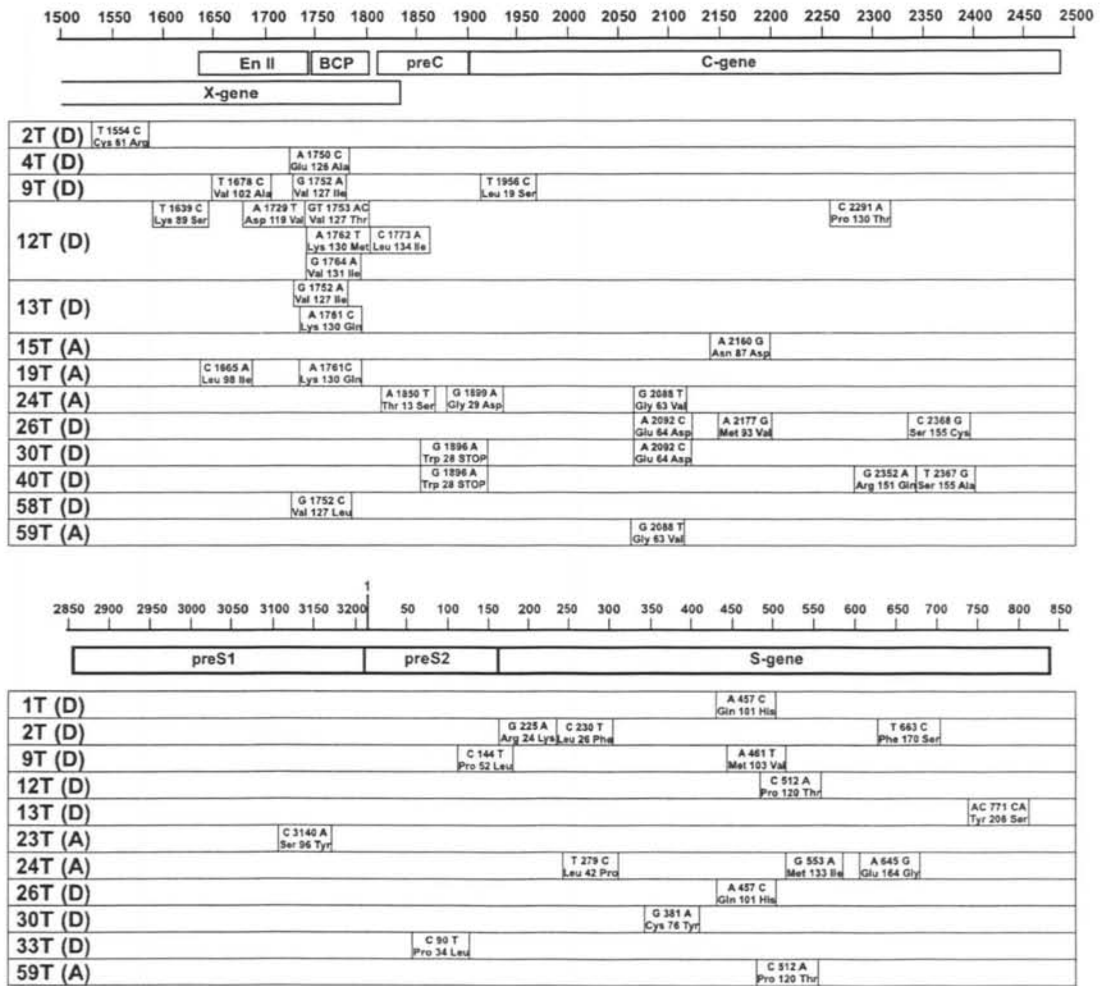

Fig. 2. Nucleotide sequences of DNAs isolated from patients' sera, numeration is given according to *adw*2 (X02763). Boxes show changes of nt (upper line) and aa (lower line) against the corresponding subtypes sequence.

tribute to the adaptive mechanisms decreasing the production and secretion of HBeAg and viral persistence.

The preC stop codon mutation G1896A was detected in 2 isolates: 30T and 40T. This mutation is common in HBeAg-negative HBV phenotype with aborted HBeAg expression and is known to cause chronic hepatitis [31], although G1896A mutations occurred in 15% [32] and 38% [33] of cases in HBeAg-positive patients. In our patient cohort, we also found HBeAg in both sera with the preC stop codon mutation. The presence of HBeAg in the sera could be explained by the appearance of wild-type HBV DNA along with the preC stop codon mutant, which is clearly evident from the sequencing data (fig. 3). Both HBV isolates belonged to genotype D and carried T at position 1858, which plays an important role in stabilizing the stem loop of the encapsulation signal ε which is critical for viral replication. At the same time this mutation rarely develops in genotype A because of the presence of C at position 1858 [34, 35]. A stem-loop stabilizing mutation G1899A was found in isolate 24T. In the absence of the G1896A mutation, but in the presence of the A1850T mutation, this led to the preC amino acid substitution, Thr13→Ser, with unknown consequences.

In general, transplanted patients show a relatively high level of the preC stop codon, as well as BCP mutations although such mutations are rare in Latvian HBV isolates from non-immunosuppressed patients, i.e. less than 1% [Sominskaya et al., unpubl. data]. High levels of such

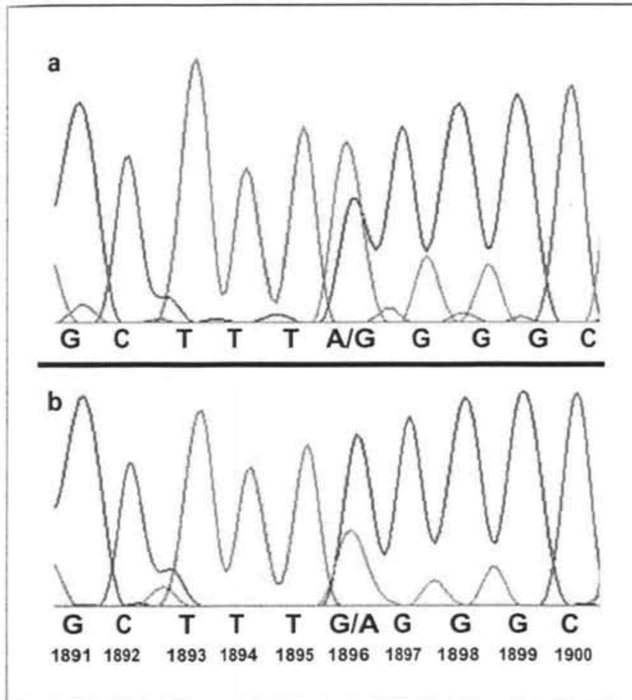

Fig. 3. Simultaneous presence of preC stop-codon mutant and wild-type DNA in the sera of patients 30T (**a**) and 40T (**b**). Results of automatic sequencing are shown.

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 Asn87Asp 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 Ser155Ala (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- to147-aa loop, and the Gly145Arg 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 Pro120Thr was described earlier [41]. Substitutions of Pro120 to Glu [42], or to Ser [43] have been previously found to be significant. The Met133Ile 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 (Arg24Lys, Leu26Phe, Leu42Pro, Gln101His (twice), Met103Val, Tyr206Ser. 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 Leu42Pro 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 (Ser96Tyr) and 2 in the preS2 (Pro34Leu and Pro52Leu).

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 sequences 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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An Outbreak of HBV and HCV Infection in a Paediatric Oncology Ward: Epidemiological Investigations and Prevention of Further Spread

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Hospital-acquired hepatitis B (HBV) and C virus (HCV) infections continue to occur despite increased awareness of this problem among the medical community. One hundred six patients were infected in a haematology oncology ward for children, over the time period 1996 to 2000. Serum samples from 45 such patients and 3 from infected medical personnel were used for nucleic acid amplification. HBV core, as well as HCV core and hypervariable region 1 (HVR1) nucleotide sequences, were analysed by phylogenetic tree analysis, in order to characterise the epidemiological pattern of viral transmission on the ward. Samples from 32 patients were positive for HBV-DNA or HCV-RNA by PCR. Ten patients were positive for both markers. Seventeen out of twenty-three HCV core gene sequences were found to be evolutionarily related and clustered separately from other local sequences in the phylogenetic tree, indicating nosocomial transmission. This was confirmed by analysis of HVR1 gene sequences. One nurse and one physician from the ward were HCV RNA positive, but their HCV sequences were not related evolutionarily to those of the patient cluster. Fifteen out of nineteen HBV core gene sequences were also clustered together and were positioned separately in the relevant tree. Epidemiological investigation excluded a common source infection and indicated that spread of infection was most likely due to inappropriate infection control measures on the ward. No obvious risk factors for transmission were identified during the retrospective survey in patients with related sequences, except use of multidose vials for saline and poor staff compliance with routine hand hygiene procedures. The preventive measures that were introduced reduced the incidence of infection significantly. No new cases of HBV infection and only three anti-HCV seroconversions occurred over a period of 19 months. The introduction and maintenance of strict prevention measures over a 2 year period, combined with HBV vaccination, reduced significantly the incidence of new HCV and HBV infections. ***J. Med. Virol. 69:331–338, 2003.*** © 2003 Wiley-Liss, Inc.

KEY WORDS: Hepatitis B virus; Hepatitis C virus; nosocomial transmission; paediatric oncology ward; phylogenetic tree analysis

INTRODUCTION

Patients with haematological malignancies are at a very high risk of HBV and/or HCV infection due to the large transfusional support they often need and the immunodeficiency status caused by the underlying disease and/or by cytostatic chemotherapy. In addition, immunosupression caused by chemotherapy increases the chronicity rate of viral hepatitis [Locasciulli et al., 1985] This problem has long been recognised [Locasciulli et al., 1983]. Screening of blood donors has led to a dramatic decrease in transfusion-associated infections. Moreover, vaccination against HBV is

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effective in preventing HBV infection in children with malignancies [Polychronopoulou-Androulakaki et al., 1996] and has been introduced successfully in many centres. Nevertheless, nosocomial outbreaks of HCV continue to occur even in highly developed clinical settings [Januszkiewicz et al., 1997; Widell et al., 1999; Knoll et al., 2001]. The high rate of chronic hepatitis in these patients is of major concern since the prognosis of childhood leukaemia has dramatically improved over the last three decades. Treatment outcomes have been controversial, and epidemiological preventive measures appear to be the most effective strategy.

Nucleotide sequences and phylogenetic tree analyses have been recently used extensively to characterise the transmission routes of HBV and HCV in different epidemiological settings. Phylogenetic tree analysis of HBV precore/core sequences was successfully applied to characterise the transmission of the virus within Gambian families [Dumpis et al., 2001]. HCV Core and HVR1 sequences have been used by different authors to confirm clusters of transmission during various outbreaks [Ross et al., 2000; Yerly et al., 2001].

Occasional testing for HBV and HCV markers in Latvian children on the haematology ward in 1996 led to the identification of a few cases, but the full extent of the problem was not recognised until 1998 when routine testing for hepatitis markers was introduced for all children admitted to the ward. A significant number of new cases of HBV and HCV infection were observed. Preliminary epidemiological investigations did not identify obvious risk factors. As a result, a retrospective epidemiological study was instigated based on phylogenetic tree analysis of viral sequences obtained from infected patients in an attempt to establish the relationship between circulating viral strains. In addition, an attempt was made to characterise the routes of transmission and risk factors involved. Concurrently, strict infection control measures were introduced to prevent new infections within the nosocomial setting.

MATERIALS AND METHODS

Patients

The study was undertaken in children on an oncology ward of the State University Hospital for Children. The ward had 30 beds with approximately 800 admissions per year. Most of the patients are admitted to the ward on multiple occasions. One hundred six cases of serologically confirmed hepatitis infection have been identified in patients with haematological malignancies since the beginning of 1996. Routine testing for hepatitis markers on admission was not started until 1998. Initial diagnostic samples were not stored and therefore repeated testing and confirmation of diagnosis by PCR was not possible. Forty-five children were available for interview and repeat testing, and therefore were included in the study. Simultaneously, all medical personnel on the ward were screened for anti-HCV and HBsAg. Two nurses and a doctor were found to be anti-HCV positive and were included in the study. This study was conducted with informed consent and approval by the Ethics Committee.

Serological Testing

All initial serological testing was performed in the certified clinical reference laboratory. Serological testing was repeated in all positive patients over the study period. Hepatitis B surface antigen (HBsAg) was detected with Enzygnost HBsAg 5.0 (Boehring, Marburg, Germany), whilst HBeAg and antibody to core antigen (anti-HBc) were detected with ELISA kits (Dia Sorin, Saluggia, Italy). Antibodies to HCV (anti-HCV) were detected with a second generation ELISA kit (Abbott Laboratories, Chicago, IL).

PCR Amplification for HCV Core and HVR1 Region

Amplification of the HCV core region was performed as described previously [Viazov et al., 1997]. cDNA synthesis for amplification of HVR 1 sequences was carried out using primer HVRO3 (5′-TCCGCA[C, T]GTCTT[A, G]GTGAACCC, nt 2007–2027, numbering according to Takamizawa et al. [1991], followed by nested PCR [Ross et al., 2002]. Primers HVRO5 (5′-TGGGATATGATGATGAACTGG, nt 1290–1310) and HVRO3 were used for the first round PCR, whilst primers HVRI5 (5′-CTAGTGGTGTCGCAG[C, T]T[A, G]CTC, nt 1326–1346) and HVRI3 (5′-CGCGTAATGC-CAGCAATA[A, T, G]GG, nt 1782–1802) for the second. Nineteen HCV isolates obtained from patients within a radius of approximately 10 kilometres from the hospital were used as controls. The HCV core and HVR 1 sequences obtained from the patient samples have been submitted to the GenBank nucleotide sequence database (accession numbers AY079244-AY079304).

PCR Amplification of the HBV Core Region

HBV-DNA was extracted from 50 μl of serum and resuspended in 20 μl of sterile distilled water as previously described [Karayiannis et al., 1995]. Five microlitres of this were used to amplify the core promoter/ pre-core/core region by PCR using primers M3 (5′-CTGGGAGGAGTTGGGGGA, nt 1732–1755) (12) and 5C (5′-CCCACCTTATGAGTCCAAGG, nt 2466–486) as described previously [Karayiannis et al., 1995]. If no product was obtained after the first round, second round amplification was performed under the same reaction and cycling conditions with primers M3 and 8C (5′-GTCCCTGGATGCTGGATCTTGCT, nt 21302154), and primers 6C (5′-GGCAAGCCATTCTTTGCTGGGG, nt 2070–2092) and 5C, generating two partially overlapping fragments and covering the entire region under study (nt 1732–2486). As area controls, 12 HBV isolates were obtained from patients within the same radius from the hospital as in the case of HCV cases. HBV core fragment sequences have also been submitted to GenBank (accession numbers AY079214–AY079243).

Sequencing and Phylogenetic Tree Analysis

Amplicons were excised from agarose gels following electrophoretic separation and purified with a DNA extraction kit (MBI Fermentas, Vilnius, Lithuania). Purified fragments were subjected to direct sequencing in both directions using an ABI Prism dye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA). Electrophoregrams were obtained using an ABI Prism 377 sequencer (Applied Biosystems). In both HBV and HCV cases, PCR primers were also used as sequencing primers. The sequences were edited manually by use of the Vector NTI Suite 7.0 software (InforMax, Inc., Bethesda, MD) and subsequently aligned in FASTA format. Phylogenetic relationships between HBV core, and HCV core and HVR1 sequences were reconstructed using the neighbour-joining (NJ) and maximum likelihood (ML) methods. All these analyses were conducted using the PAUP* package version 4.07b. Phylogenetic trees were drawn and rearranged using the Treeview 1.6 program.

Epidemiological Investigation

The medical histories of 17 infected children from the cluster with similar HCV core sequences were scrutinised by noting the time of admission, length of hospital stay and all parenteral exposures. Serum alanine transferase and bilirubin levels, as well as serological markers of hepatitis virus infection, were also examined.

Preventive Measures

Universal precautionary measures were introduced in the daily practice routines of all personnel on the ward. Adherence to guidelines for cleaning and disinfection procedures for instruments, equipment, and surfaces was monitored by a hospital epidemiologist and senior nurses. Particular attention was directed towards appropriate disposal of potentially infectious material in special containers. Special instructions for hand washing, and on the use and changing of gloves, were distributed to medical personnel. Gloves had to be changed after attending to each patient, and hand washing with alcohol-containing solution had to be applied before and after removal of gloves. The use of multiple dose vials of saline was abandoned. In most of the children who had not received HBV vaccination at birth, a full vaccination schedule was initiated on admission to the ward.

RESULTS

Serological Markers

Since 1996, when testing for hepatitis virus markers was introduced in the haematology oncology ward, 106 cases of HBV and HCV infection were recorded. Sixty-five children became HBsAg positive and seventy-one seroconverted to anti-HCV during or after hospitalisation. Thirty children were positive for both HBsAg and anti-HCV markers. Some patients died, many were lost to follow-up, and exact information on how many of them became chronic HBV or HCV carriers was not available.

HBsAg or anti-HCV positive serum samples from 45 children with various haematological malignancies were chosen for further testing. Their mean age at the presumed time of infection was 8.41 (range 2–16), and 36 (80%) of them were males. Their presumed time of infection was between 0.5 to 5 years before the start time of the survey. Varying serological profiles were found as shown in Table I. Thirty-four patients were chronic HBsAg carriers, forty-one were anti-HCV positive, whilst thirty of them were positive for both markers of infections. Nucleic acid amplification produced respective core amplicons from 19/34 (56%) HBsAg, and 23/41 (56%) anti-HCV, positive patients (Table I).

TABLE I. Serological and Routine PCR Profiles of the 45 Children Selected for the Study

Number of patients	Anti-HBc (total)	HBsAg	HBeAg	HBV-DNA	Anti-HCV	HCV-RNA
5	+	+	+	+	+	+
2	neg	+	+	+	+	+
3	+	+	neg	+	+	+
5	neg	+	neg	neg	+	+
2	neg	+	neg	neg	+	neg
7	+	+	+	+	+	neg
1	neg	+	+	+	+	neg
1	+	+	+	+	neg	neg
1	+	+	+	neg	+	neg
1	+	+	neg	neg	+	neg
2	+	+	neg	neg	neg	neg
1	neg	+	neg	neg	neg	neg
3	+	+	neg	neg	+	+
4	+	neg	neg	neg	+	+
3	+	neg	neg	neg	+	neg
3	neg	neg	neg	neg	+	+
1	neg	neg	neg	neg	+	neg
45	30	34	18	19	41	23

Phylogenetic Tree Analysis

Phylogenetic tree analysis of HBV core gene nucleotide sequences revealed two major clusters, representing genotypes A and D (Fig. 1). Sequences of the latter were predominant (16/19) and clustered separately from other local Latvian sequences, indicating a close evolutionary relationship and therefore likely transmission of the virus between patients of the cluster. Five isolates were completely identical. The three genotype A sequences were positioned in between the local sequences, with very small evolutionary distances between them. However, this was not sufficient to define a possible common source outbreak. A definitive statement on the transmission of HBV between these patients cannot be made based on the present phylogenetic tree analysis.

Phylogenetic tree analysis of HCV core gene nucleotide sequences (Fig. 2) showed that all isolates belonged to genotype 1b except one, which was 3a (12B). This individual had spent some time in a youth detention centre where tattooing was a common practice. Seventeen sequences formed a cluster, which indicated a very close evolutionary relationship, suggesting the possibility of a common source outbreak. Isolates from 12 patients were completely identical. The sequence from a child infected in 2001 (47B) was part of this big cluster indicating transmission. A second sequence (48B) from the same time of infection was positioned separately. Interestingly, some other sequences were positioned separately (14B, 4B, and 12B) and apparently could not be linked to the same transmission event, such as the big cluster. Sequences from a nurse (44B) and a physician (33B) were again positioned separately from the big cluster, although the possible relatedness of 33B to the other sequences could not be completely excluded.

In order to characterise better the possible transmission of HCV between patients, the 15 viral isolates,

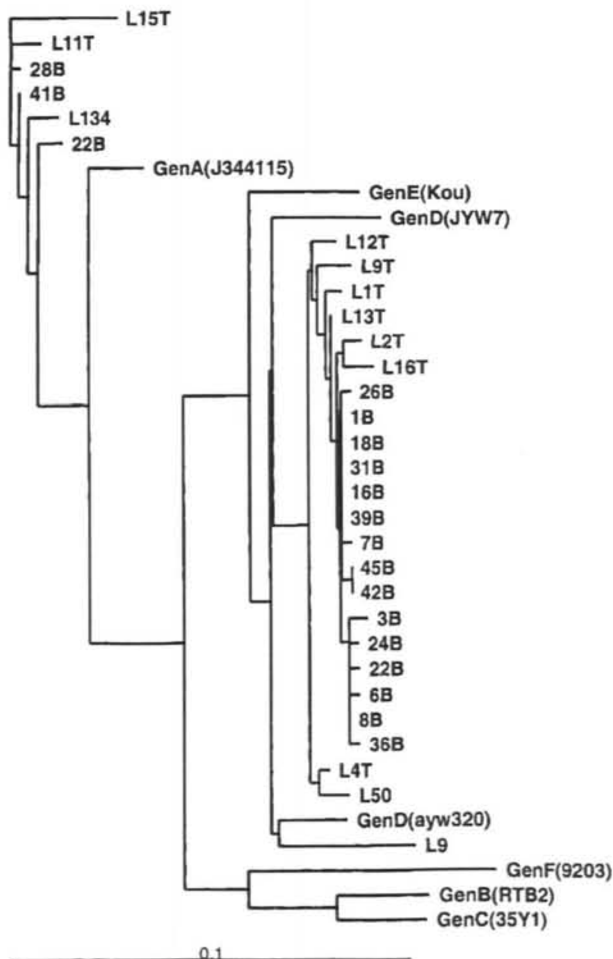

Fig. 1. Unrooted maximum likelihood (ML) phylogenetic tree of 37 HBV core (fragment length = 401 nt) sequences (Ts/Tv = 1,55; α = 0,32; ln likelihood = −1706.63). Sequences from children have extension B, whilst local sequences start with the prefix L. The ayw320 is the first HBV genome cloned in Latvia [Bichko et al., 1985].

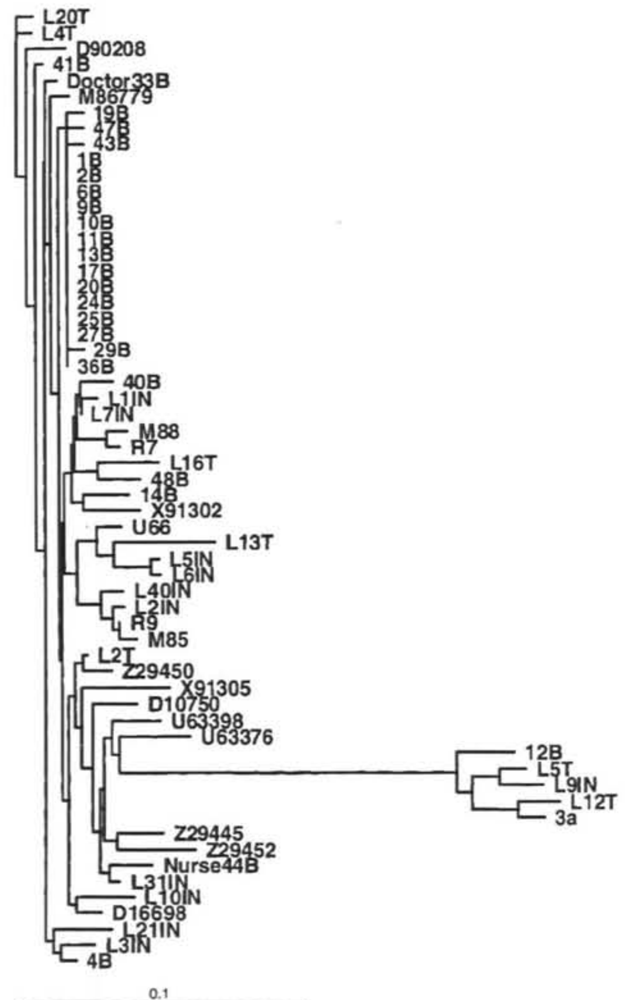

Fig. 2. Unrooted ML phylogenetic tree of 60 HCV core (fragment length = 216 nt) sequences (Ts/Tv = 3,4; α = 0,27; ln likelihood = −1008.95). Sequences from children have extension B, whilst local sequences start with the prefix L.

which clustered together in the phylogenetic tree based on core sequences, were re-analysed using sequences of amplicons from HVR1. Three more unrelated isolates from the ward were included for comparison. Phylogenetic tree analysis (Fig. 3) confirmed that the 15 isolates were closely related and in addition exhibited further clustering, which was used to obtain additional information in the epidemiological investigations.

Epidemiological Investigation

All medical personnel involved in the care of the patients were tested for HBsAg and anti-HCV antibodies. No HBsAg positives were identified. Two nurses and one physician were found to be anti-HCV positive. One of the nurses and the physician were HCV-RNA positive and their HCV C sequences were thus included in the phylogenetic analysis. None of them could be linked to any of the HCV infected patients as a source of infection. The nurse had been employed only recently and the doctor had no connection with most of the patients.

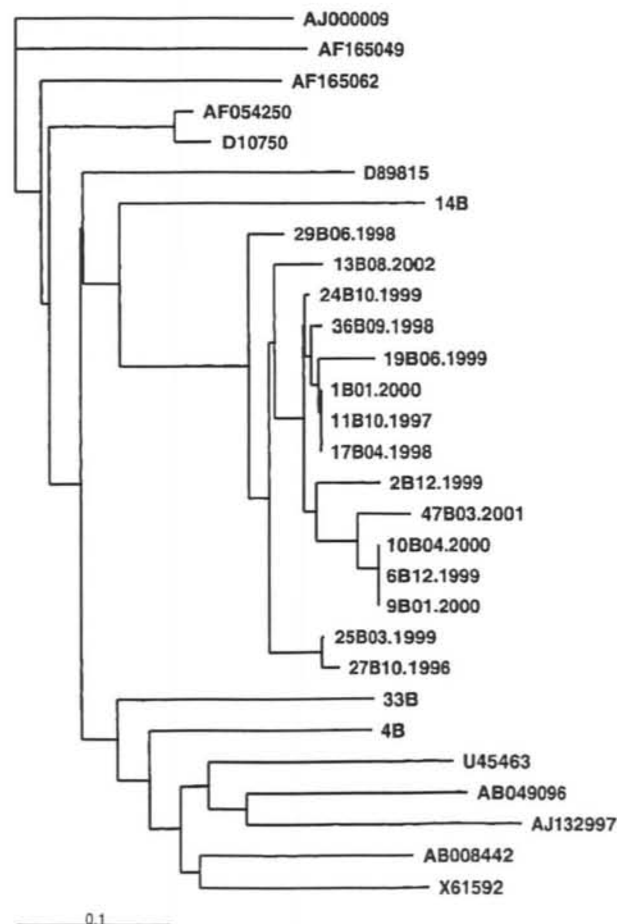

Fig. 3. Unrooted neighbour joining phylogenetic tree of 29 HCV HVR1 (fragment length = 81 nt) sequences. Sequences from children with extension B, followed by the time of diagnosis.

Blood products were supplied from the central blood bank. Serum samples from all blood donors in Latvia are tested for HBsAg and anti-HCV with conventional methods in a certified laboratory. Blood units supplied to the haematology ward were tested repeatedly and found to be negative. Unfortunately, any available serum samples could not be tested by PCR since these were not stored under the most appropriate conditions.

Seventeen HCV PCR positive patients with closely related HCV core sequences (Fig. 3) were selected for further epidemiological investigation (Fig. 4). Fifteen of them had haematological malignancies, one was a haemophiliac, and one patient had aplastic anaemia. Thirteen were males and four were females. The patient with haemophilia had not been admitted to the hospital previously but had received treatment on the ward. Times of hospital stay had frequently overlapped (Fig. 4), but a clear pattern of possible transmission did not emerge. In addition, the time between hospitalisation and diagnosis extended to over 2 years. All patients had received blood product transfusions. No correlation with the same batch of blood was found. Fourteen of them were undergoing chemotherapy at the time of infection. Twelve patients were boys and four were girls and therefore were hospitalised in different rooms. Particular attention was paid to patients from two clusters with identical HVR1 sequences. Patient 11B from the first cluster (Fig. 3) was a girl and patients 17B and 1B were boys and therefore could not have shared the same room. Patients 17B and 1B shared one room on 23-27.11.99 and on 06-07.12.00 (Fig. 4). Patients 9B, 10B, and 6B from the second cluster were boys (Fig. 3). Two of them had shared the same room.

Serum alanine transferase and bilirubin levels increased with the initiation of cytostatic therapy rather than during the appearance of hepatitis serological markers (data not shown) and therefore were not informative in establishing the exact time of the virus related hepatitis.

The use of multidose vials of isotonic solution for washing intravenous catheters and preparation of intravenous injections was common at the time of infection of all patients. Staff members however denied using the same syringe several times.

Impact of Preventive Measures

Infection prevention measures (see Methods) were introduced gradually over several months in the first half of 2000. Despite some irregularities due to poor staff compliance, eventually the routine work in the ward had changed significantly. A full vaccination schedule was initiated but was not completed in all admitted children over 3 years old, as some of them were discharged before the third dose was due. Routine serological testing that was introduced for all patients in 1998 revealed no new HBsAg cases since December 2000. However, three cases of anti-HCV positivity were registered in 2001, two of which were confirmed by PCR. No new cases of hepatitis infection have been recorded in 2002 (Fig. 5).

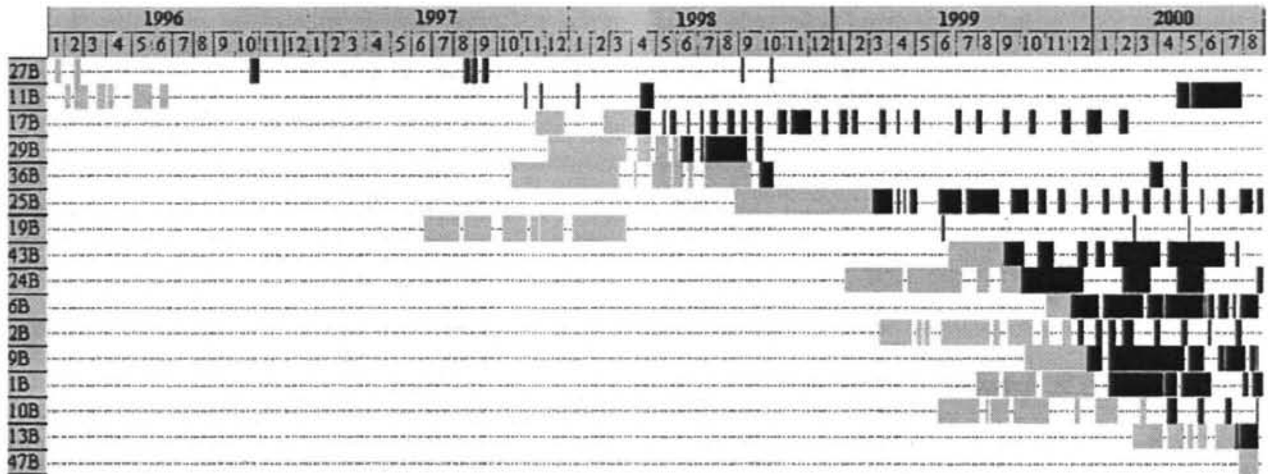

Fig. 4. Time and periods of hospitalisation of 16 patients with evolutionarily similar HCV core sequences. Grey colour, anti HCV negative; black colour, anti HCV or HCV PCR positive. Infection of 47B was detected in 2001.

DISCUSSION

An unusually high number of HBV and HCV infections was recorded in a Latvian paediatric oncology ward during the time period 1996–2000. Thus, an epidemiological investigation was initiated in order to identify risk factors for transmission and to institute effective preventive measures.

At the beginning of this investigation, medical personnel had to be excluded as a potential source of infection and were thus all screened for HBV and HCV markers. No HBsAg carriers were identified among the staff. A nurse and a physician, however, were found to be anti-HCV and HCV-RNA positive. Nevertheless, phylogenetic tree analysis failed to demonstrate any close genetic relatedness between their isolates and those seen in the patients. In any case, the nurse and the physician were not involved in the care of most of the patients surveyed. Therefore, it seemed very unlikely that they were the source of HCV infection on the ward.

Analysis of viral nucleotide sequences has been successfully used in the past for the characterisation of common source outbreaks of both HBV and HCV [Suzuki et al., 1994; Dumpis et al., 2001]. In this study, the phylogenetic tree analysis proved necessary in order to characterise the pattern of transmission of the hepatitis viruses on the ward. Phylogenetic tree analysis of 17 HCV core and 15 HCV HVR1 sequences confirmed that these patients were infected with the same viral isolate. The evolutionary distances between sequences were similar to those reported previously for epidemiologically linked HCV strains [Suzuki et al., 1994; Ross et al., 2000]. Thus, phylogeny clearly showed a high similarity between the viral sequences obtained from the patients on the ward, indicating nosocomial transmission.

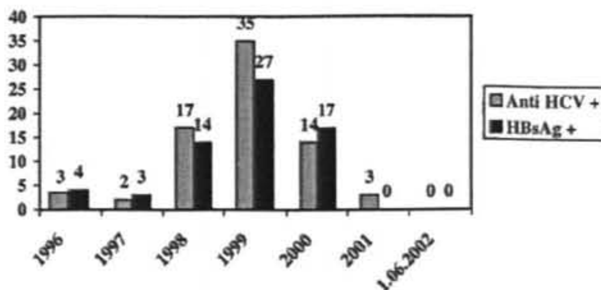

Fig. 5. Number of new cases of viral hepatitis (HBsAg positive and anti-HCV) in the childrens' haematology ward during the period 1996–2002.

Previous studies have shown that phylogenetic tree analysis of HCV HVR1 sequences clearly distinguishes between related and unrelated isolates of the same genotype. In our study, phylogenetic tree analysis of HVR1 sequences provided higher resolution than HCV core sequences.

Phylogenetic tree analysis indicated that some isolates were evolutionarily unrelated and came from different sources of infection such as blood transfusion. Screening by a second-generation anti-HCV assay may have possibly failed to identify some cases of infection, and thus contaminated blood could have been used for transfusion in the children. Nevertheless, this could not account for such a large number of infections. No more than one child received blood from the same donor. Transmission from the same batch was excluded.

Several reports have shown that even minor violations of safety procedures may result in the spread of hepatitis viruses [Widell et al., 1999; Ross et al., 2000]. Numerous breaches of standard infection control practices were detected during our epidemiological survey. Multidose vials of saline were used routinely for flushing of intravenous catheters and for preparation of intravenous infusions. Though staff denied using the same

syringe in multidose vials more than once, the heavy workload of these staff could have easily led them to contravene this rule. Changing of gloves after each patient was not strictly adhered to either.

The retrospective nature of the study did not provide us with opportunity to test multidose vials, needles, and medical devices for traces of hepatitis viruses. It is very difficult to assume that only inappropriate use of gloves could lead to an outbreak of such magnitude. Information on possible vial or equipment contamination would be essential.

Moreover, our epidemiological investigation had other shortcomings. A case control study was proposed at the initial stages, but this was abandoned due to difficulties in getting precise medical records and the very small probability that one risk factor would emerge as the cause. Instead, it was decided to focus only on the HCV cluster with evolutionarily related sequences by trying to find out what possible risk factors were common for these patients. The retrospective nature of the study did not provide an opportunity to test samples for HCV-RNA by PCR at the actual time of infection. In addition, at the beginning of the outbreak, serological testing was not used in the ward on a regular basis. This makes our estimated time of infection very inaccurate. It is also not clear whether the number of patients infected is an underestimate since some may have not been tested or became PCR negative by the time of our survey. Sequences from them could possibly provide the missing link between all patients from the cluster.

The epidemiological investigation was carried out only on the cluster of patients with similar HCV core sequences. We did not undertake a similar investigation for the HBV infected children since we assumed that the transmission route would be identical. In addition, many of the patients had cleared HBV infection (HBsAg negative and anti-HBc positive) and became HBV DNA negative. In the absence of sequence data, phylogenetic tree analysis was not possible.

The time of introduction of the preventive measures coincided with the time when most of the children admitted to the ward were already vaccinated against HBV. Vaccination of all newborns in Latvia was introduced in 1997 and most of the patients admitted to the ward were 3 years old or younger. This may have largely contributed to the total absence of new HBV infections after December 2000 and indicates that vaccine-induced protection remained high even in highly immunocompromised groups of patients. Nevertheless, susceptible children, who had received vaccine only on admission, did not become HBsAg carriers.

Retrospective examination of possible routes of HBV transmission was not feasible in a situation where nearly all patients showed serological signs of infection. Only 9% (4/45) of surveyed patients were not positive for markers of HBV infection (HBsAg, anti-HBc). Since the prevalence of infection in the general population is assumed to be less than 5%, it is reasonable to assume that the higher rates of infection in hospitalised patients are due to nosocomial transmission. Usually the risk of HCV infection is lower than that for HBV for the same route of transmission. Some of the children from the big cluster shared the same rooms at different times of hospitalisation. Boys and girls are usually hospitalised in different rooms. This observation has lead to the suggestion that infection could occur outside the patients' rooms and may relate to some malpractice by medical staff at the ward level.

It is difficult to explain the large variety of serological findings in different patients with HBV infection (Table I). Commercial high quality antigen and antibody detection tests (see Methods) were employed in a certified clinical reference laboratory where cross-contamination or specimen carry-over was very unlikely. In addition, samples from most of the patients were tested repeatedly. We can only speculate that in patients that received immunosuppressive therapy (steroids and cytostatics), production of anti-HBc antibodies is somehow suppressed. A commercial assay was not employed for HBV PCR testing and our assay was not designed for high-sensitivity testing. This could explain why only 56% of patients were found to be viraemic. Though anti-HCV testing was done by second generation ELISA and positive results were not confirmed by RIBA, we do not think that the low sensitivity of the PCR in anti-HCV positive patients was due to low specificity of the ELISA. This could be also explained by shortcomings of our PCR method because it was designed to obtain sequences with sufficient length for phylogenetic analysis. It is very unlikely that so many patients could clear the infection, though this cannot be excluded. Another explanation for this observation could be that in the case of HBV and HCV co-infection, the lower sensitivity of the HCV PCR observed in HBsAg positive patients may have been due to suppression of replication of one virus by the other.

We also acknowledge that some cases of HCV infection may be anti-HCV negative as a result of immunosupression and therefore could not be detected by ELISA. Possible use of a commercial PCR diagnostic assay might possibly help to identify new cases. Nevertheless, we assume that the number of such patients would be small and would not influence the outcome of our investigation and intervention.

The intervention measures designed to prevent all possible transmission routes proved to be successful. Since the exact mode of transmission of HBV and HCV in children with haematological malignancies were not documented, preventive measures had to be targeted at the three speculative routes: hand-borne transmission through health-care workers, sharing of equipment by patients, and use of multidose vials for saline, and other solutions. Adherence to the new guidelines was carefully monitored and as a result, the newly implemented hygienic standards reduced the incidence of HBV and HCV infection quite significantly. Three new cases of HCV (two confirmed by PCR) and none of HBV infection in a 2-year period can be considered as a success in implementing infection control measures.

In conclusion, a major outbreak of HBV and HCV infection in a paediatric oncology ward has been characterised. Our findings strongly implicate malpractice by medical staff as having facilitated viral transmission within the ward. This study emphasises the importance of adequate infection control practices and implementation of universal precautionary measures in controlling the outbreak of hepatitis virus infection. It indirectly confirms the high efficacy of HBV vaccine in children with haematological malignancies. Increased awareness of medical staff about nosocomial transmission of HCV and HBV can lead to significant reduction of new cases in endemic countries.

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Hepatitis C virus molecular epidemiology in Latvia

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Abstract

The aim of this study was to identify the hepatitis C virus (HCV) genotypes distributed in Latvia and to estimate their prevalence in various risk groups. HCV genotypes of 65 isolates were estimated using amplification and direct sequencing of PCR fragments from the core region of HCV genome. Phylogenetic analysis of the genotypes was conducted. Genotype 1b was identified in about 85 % of cases, genotype 3a in about 10 %, and HCV genotypes 1a and 2c were present only in some isolates. A similar epidemiological distribution is typical for the former Soviet Union Republics. Our study suggests that contaminated blood products may be main route of HCV infection in Latvia.

Key words: genotypes, HCV, prevalence.

Introduction

Hepatitis C virus (HCV) is the major agent of parenterally transmitted non-A, non-B hepatitis worldwide. Although representative prevalence data are not available from many countries, it is known that about 3 % of the world's population are infected with HCV. It is estimated that 170 million people worldwide are at risk of liver cirrhosis and hepatocellular carcinoma due to chronic infection with HCV (Cohen 1999). HCV causes 20 % of acute hepatitis cases, 70 % of all chronic hepatitis cases, 40 % of all cases of cirrhosis of the liver, 60 % of hepatocellular carcinomas, and 30 % of liver transplants in Europe (European Association for the Study of the Liver 1999).

Molecular characterization of HCV revealed the existence of a positive sense RNA genome of approximately 9400 bases in length. The complete genome sequence has been determined in different HCV isolates worldwide, which indicated substantial nucleotide sequence variability throughout the viral genome (Choo et al. 1991). HCV exhibits enormous genetic diversity: the comparison of published sequences of HCV has led to the identification of distinct HCV genotypes that may differ from each other by as much as 33% over the entire viral genome (Okamoto et al. 1992). The variability within the HCV genome has formed the basis for several genotyping systems. The current, most commonly used classification system has been proposed by Simmonds et al. (1994). HCV is classified into six major types (genotypes 1 to 6) and numerous subtypes (e.g., genotype 1a, 1b), which differ in diversity, geographical distribution, and transmission routes. Genotypes 1 to 3 are distributed widely around the world, while others have a more restricted distribution. For example, types 5 and 6 are only found in specific geographical (Simmonds 1999).

The genotypes of HCV appear to differ in serological reactivity and in treatment, although their role in variation of disease progression remains unclear (Poynard et al. 2003). Any successful HCV vaccination or control strategy, therefore, requires an understanding of the nature and variability of the epidemic behavior among subtypes.

The aim of this study was to identify hepatitis C virus genotypes distributed in Latvia and to estimate their prevalence in various risk groups.

Materials and methods

Patients

In total, 65 anti-HCV positive sera were included in this work: 23 sera from patients with chronic HCV infection, 18 sera from patients after kidney transplantation, 5 sera from patients undergoing dialysis and 19 sera from patients from the pediatric oncology ward. All sera were tested for the presence of anti-HCV antibodies using a second generation ELISA kit (Abbott Laboratories, Chicago, IL).

Amplification of fragments of HCV and HBV genomes

HCV-RNA was extracted from 100 μl of serum with a commercially available DNA/RNA isolation kit based on phenol/chloroform extraction ("Litech", Moscow, Russia). Amplification of HCV core region 476-725 nt fragment (numbering according to Takamizawa et al. 1991) was performed by "in house" nested RT-PCR. cDNA synthesis for amplification of core sequences was carried out using primer AS1 (5'ATGTACCCC ATGAGGTCGGC3'). Primers 2S (5'TAGATTGGGTGTGCGCGCGA3',) and 1AS were used for the first round PCR, whilst primers 3S (5'CGCGCGACTAGGAAGACTTC3'), 4S (5' TGTGTGCGCGACGCGTAAA3') and 5AS (5'GCAYGTRAGGGTATCGATGA CYT3') for the second.

Sequencing of the PCR fragments

Products of the PCR were excised from the agarose gel and purified using the DNA Extraction Kit (MBI Fermentas, Vilnius, Lithuania). Purified fragments were subjected to direct sequencing in both directions using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems, Foster City, USA), and electrophoregrams were obtained using an ABI Prism 377 sequencer (Applied Biosystems). PCR primers 3S and 5AS served as sequencing primers.

The sequences were edited manually by BioEdit Sequence Alignment (Hall, 1999) and subsequently aligned in the FASTA format (http://ngfnblast.gbf.de/docs/fasta.html). The phylogenetic tree was constructed using the DNA-distance algorithm and the neighbor-joining method in the PHYLIP package (Felsenstein 1989).

Results

Phylogenetic analysis of HCV core region nucleotide sequences showed that 57 isolates belonged to genotype 1b, six isolates to genotype 3a, one isolate to genotype 1a and one isolate to genotype 2c (Fig. 1). Distribution of HCV genotypes in isolates from different patient groups are shown in Table 1.

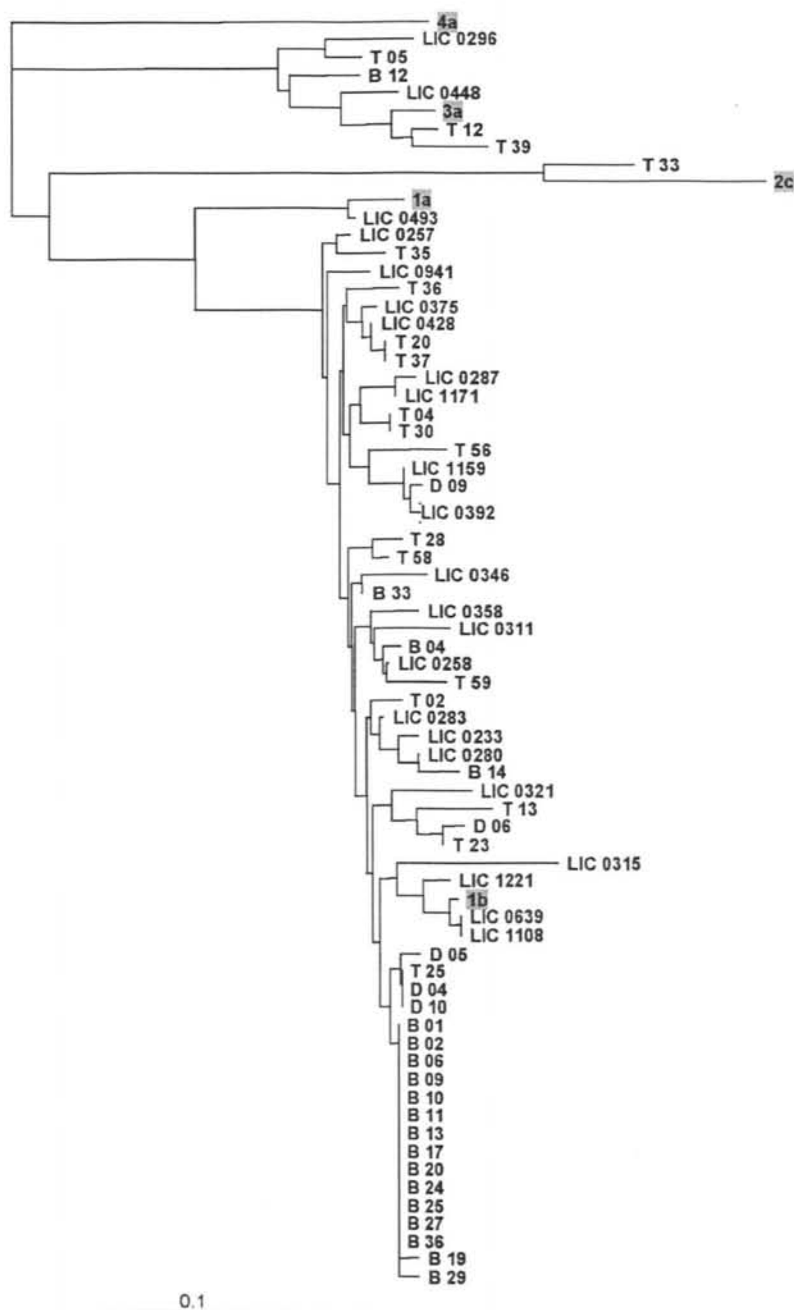

Fig. 1. Phylogenetic tree of 70 HCV core region sequenced fragments. Sequences from patients with chronic HCV infection have the prefix LIC, sequences from patients after kidney transplantation have the prefix T, sequences from patients ongoing dialysis have the prefix D and sequences from patients from the pediatric oncology ward have the prefix B. Reference sequences representing published genotypes 1a, 1b, 2c, 3a and 4a are included.

Table 1. Distribution of HCV genotypes in different groups of patients

Group of patients	1b	1a	2c	3a
Patients with chronic HCV infection	20	1	–	2
Patients after kidney transplantation	14	–	1	3
Patients undergoing dialysis	5	–	–	–
Patients from the pediatric oncology ward	18	–	–	1
Total	57	1	1	6

Discussion

The obtained results showed a strong predomination of genotype 1b in all groups of patients: 87 % in cases of chronic HCV infection, 78 % in posttransplantation, 100 % during dialysis treatment and in 95 % of cases in patients from the pediatric oncology ward. Not all of the analyzed groups are equally representative. As it was shown earlier (Dumpis et al. 2003), most of the examined patients from the pediatric oncology ward were infected in an outbreak. According to phylogenetic analysis, 13 of the 19 patients were infected with the same HCV genotype 1b isolate. On the other hand, the rate of evolution to chronicity after acute exposure to HCV was 92 % in patients exposed to HCV genotype 1b infection, compared with 33 % to 50 % in patients exposed to other genotypes (Zein 2000). The distribution of the HCV genotypes in patients undergoing dialysis and after kidney transplantation is similar to the distribution among non-renal patients in the same country (Fabrizi et al. 2001). Taking all of these obstacles into consideration, about 85 % of HCV isolates present in Latvia may belong to genotype 1b. Genotype 1b is seen more often in patients who acquired HCV through blood transfusion of unscreened blood products and medical procedures (Zein 2000), and unfortunately it shows relatively poor response to treatment by traditional anti-viral drugs (Poynard et al. 2003).

The genotype 3a may be present in about 10% of the Latvian patients infected by HCV. According to published data, this genotype is most common among intravenous drug users and shows a good response to interferon therapy (Zein 2000).

In a contrast with West-Europe countries (Zein 2000), HCV genotypes 1a and 2 are not common in Latvia. A similar epidemiological distribution was also described in other former Soviet Union Republics and some of Asian countries (Lvov et al. 1996; Viazov et al. 1997; Kurbanov et al. 2003). We believe that, at present in eastern neighbour countries, the main transmission pattern of HCV epidemic in Latvia is 1b genotype infection that spreads through blood transfusion and medical procedures.

Thereby, complete and proper screening of blood products, improving conditions needed for sterilisation of medical instruments, and using disposable syringes on the one hand, and management of measures targeted to users on the other, are two main directions that are important for limiting HCV transmission and the prevention of new outbreaks in Latvia.

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Hepatīta C vīrusa molekulārā epidemioloģija Latvijā

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Kopsavilkums

Darba mērķis bija identificēt Latvijā izplatītus hepatīta C vīrusa genotipus, kā arī novērtēt to izplatību dažādās riska grupās. Pielietojot HCV genoma *core* reģiona fragmenta PCR amplifikāciju, sekvinēšanu un iegūto sekvenču filoģenētisko analīzi, HCV genotipu noteica 65 paraugiem. 1b genotipu atrada apmēram 85 % gadījumu, 3a genotipu – apmēram 10 % gadījumu, HCV genotipus 1a un 2c atrada tikai dažos paraugos. Līdzīga epidemioloģiskā situācija ir raksturīga bijušajām PSRS valstīm. Mūsu pētījumi ļauj secināt, ka Latvijā HCV izplatās ar inficētiem asins materiāliem.

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STRUCTURAL FEATURES OF HEPATITIS B VIRUS FROM LONG-TERM IMMUNOSUPPRESSED PATIENTS IN LATVIA

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The aim of our work was to identify and characterise hepatitis B virus (HBV) genomes from kidney transplantation patients admitted to the P. Stradiņš Clinical University Hospital Transplantation Centre. The HBV serotypes and genotypes and localisation of mutations in structural HBV genes (S, C genes) and non-structural gene (X gene) were determined.

After analysis of the obtained sequences we found various mutations in both S and C genes and in the X gene. 62.5% of HBV DNA-positive samples from patients after kidney transplantation belonged to the D genotype ayw3 *serotype and 37.5% belonged to the A genotype* adw2 *serotype. These data show HBV DNA of the genotype D to be more common for Latvia. Very important substitutions were detected in the preC region position 1896 G/A (correspond to 28aa), causing a tryptophan change to a stop codon. Such a mutation is common for the HBeAg negative HBV phenotype with aborted HBeAg expression and is known to cause chronic hepatitis. In serum from one patient, we found a double mutation in the X gene core promoter region: position 1762 A/T and 1764 G/A. These point mutations caused substitution of amino acids at codon 130 and 131: 130aa lysine for methionine and 131aa valine for isoleucine. Patient 43T had a mutation in position 82aa of the C gene: arginine substitution to serine (mutation affects the B cell epitope—major immunodominant region of the core protein). Another core variant was detected in patients 12T and 22T: 130aa proline substitution to threonine. This mutation lies within an important immunological region (another B cell epitope), 129–132aa. It has been reported that this mutation is associated with exacerbation of chronic hepatitis and hepatocellular carcinoma. Deletions or insertions were not found. The greatest part of HBV DNA-positive samples had substitutions within the X gene basic* core *promoter or/and enhancer II region.*

The impact of the found mutations on the clinical outcome needs further study.

Key words: *hepatitis B virus, genotype, subtype, mutations.*

INTRODUCTION

One third of the global population has been acutely infected with HBV; less than 1% of patients with acute infection will develop fulminant hepatitis (Lee, 1997). Approximately 350 million people are chronic carriers of HBV (Maynard, 1990; Zuckerman, 1996). Of these, only 1–2% will annually spontaneously clear hepatitis B surface antigen (HBsAg) (Sampliner *et al.*, 1979). Ultimately, more than half of the HBsAg-positive patients will die of hepatocellular carcinoma or liver failure (Beasley *et al.*, 1981). Viral and host factors, such as age, immunosuppression, and sex contributes to the patient outcome (Lee, 1997; Lok *et al.*, 1990; Fattovich *et al.*, 1991).

HBV is a virus with partially double stranded circular 3.2 kb long DNA genome. It belongs to *Hepadnaviridae* family. HBV has four genes (*S*, *C*, *X*, and *P*), encoding at least eight proteins; among these proteins are three surface proteins (LHBs, MHBs, and SHBs) encoded by gene *S*, two core-derived proteins (HBc and HBe) encoded by gene *C*, and Pol and HBx proteins encoded by the non-structural genes *P* and *X*, respectively (Kann and Gerlich, 1998; Nassal, 1999; Seeger and Mason, 2000).

Initially, serological grouping revealed a basic set of HBV subtypes: *adw*, *ayw*, *adr*, and *ayr*, which represented the immunological properties of surface protein as a combination of a common immunodominant determinant "a" with at least two mutually exclusive subdeterminants *d* or *y*, and *w* or *r*. In total, nine serotypes: *ayw*1, *ayw*2, *ayw*3, *ayw*4, *ayr*, *adw*2, *adw*4, *adrq-*, *adrq+* have been described (Blitz *et al.*, 1998; Courouce-Pauty *et al.*, 1983). Genetic classification of HBV based on the sequence data has defined seven geno-

types (A–G) (Okamoto *et al.*, 1988; Norder *et al.*, 1990). The above studies allow a more accurate follow-up of the geographical distribution of HBV genotypes (Lindh *et al.*, 1997; Norder *et al.*, 1993; Telenta *et al.*, 1997). HBV genotypes do not correspond equivocally with the serotypes mentioned above. The correlation of the four serotypes with the seven genotypes has been studied previously (Norder *et al.*, 1992; Stuyver *et al.*, 2000). Genomes encoding *adw* are found in genotypes A–C, F and G, while the genomes encoding both *adr* and *ayr* occur in genotype C, along with *adw*. Genomes encoding *ayw* are found only in genotype D.

Over the past years, increasing attention has been focused on the contribution of variant HBV strains to the clinical course of acute or chronic infection. Mutant HBV can display enhanced virulence with increased levels of HBV replication, resistance to antiviral therapies (e.g., interferon-α or nucleoside analogues), facilitated cell attachment/penetration, or alteration of epitopes important in the host immune response. Several viral mutations appear to be associated with increased virulence including those in the precore, core, and pre*S2* regions. Also, viral mutations can influence response to treatment: pre*core* or *core* mutations may modulate response to interferon-α, and treatment-emergent polymerase mutations can result in reduced sensitivity of viral replication to antiviral nucleoside analogues and diminished therapeutic response. Mutations in the immunodominant region of HBsAg S domain make vaccines against HBV non-effective because of immune escape (Hunt *et al.*, 2000).

It has been found that immunosuppressed patients have a high variability of HBV. Some authors report that up to 100% of immunosuppressed patients had deletions and/or insertions in the *C* gene and pre*S* region (Gunter *et al.*, 1995; Prekschat *et al.*, 1999).

The aim of our work was to identify and characterise HBV genomes from kidney transplantation patients admitted to the P. Stradiņš Clinical University Hospital Transplantation Centre: determination of HBV serotypes and genotypes and localisation of mutations in structural HBV genes (*S*, *C* genes) and non-structural gene (*X* gene).

MATERIALS AND METHODS

Patients. Serum samples from 41 patients (after kidney transplantation) were used for virological assays. Sixteen of them were anti-HCV (anti hepatitis C virus antibodies) positive, meaning that these patients had coinfection of HCV.

Serological tests. Patient sera were tested for HBsAg and for total anti-HBc. HBsAg positive samples were tested for HBeAg and anti-HBe. HBsAg was detected with an Enzygnost HBsAg 5.0 ELISA Kit (Behring, Marburg, Germany), HBeAg, anti-HBe and anti-HBc were detected with ELISA kits (DiaSorin, Saluggia, Italy).

Amplification of fragments of HBV genomes. HBV DNA's were extracted from 50ģl of serum with a commercially available DNA-RNA isolation kit based on phenol/chloroform extraction (Lyteh, Moscow, Russia). Amplification of HBV genome fragments was performed by PCR methods. We amplified whole pre*S*/*S*, pre*C*/*C* regions and a fragment of the *X* gene that included the basic *core* promoter and enhancer II. When it was not possible to amplify the whole fragments, we performed nested PCR with inner primers. For amplification of the whole pre*S*/*S* region, 17p 5' TTATTTACATACTCTTTGGAAGGC 3' (Preikschat *et al.*, 1999) and 8.15p 5' AATGTATACCCAAAGACAG 3' primers were used. For nested PCR on *S* domain S1p 5' TTGTTGACAAGAATCCTCACAATACC 3' (Preikschat *et al.*, 1999) and S2p 5' GCCCTACGAACCACTGAACAAATGG 3' (Preikschat *et al.*, 1999) primers, and for amplification of whole pre*C*/*C* region—M3 5' CTGGGAGGAGTTGGGGGA 3' (Karayiannis *et al.*, 1995) and 19p 5' AGGTACTGTAGAGGAATAAAGCCC 3' (Preikschat et al., 1999) primers. For nested PCR on the preC/C region we used C1" 5' GTTCACCTCACCATACTGCACTCAGGC 3' (Preikschat *et al.*, 1999) and C2' 5' GAGTTCTTCTTCTAGGGGACCTGCCTCG 3' (Preikschat *et al.*, 1999) primers and for amplification of *X* gene fragment- 21p 5' CGTTCCAGCCGACCACGGGGCGC 3' (Preikschat *et al.*, 1999) and 2-Sp 5'AAAAAGTTGCATGGTGCTGG 3' primers (Gunter *et al.*, 1995).

Sequencing of the PCR fragments. Products of the PCR were excised from the agarose gel and purified using a DNA Extraction Kit (MBI Fermentas, Vilnius, Lithuania). Purified fragments were sequenced in both directions using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems, Foster City, USA), and electrophoregrams were obtained using an ABI Prism 377 sequencer (Applied Biosystems, USA).

In all cases PCR primers were used as sequencing primers. An additional primer, 14p 5' CTGTAACACGAGCAGGGGTCCTAG 3' (Preikschat *et al.*, 1999), was used for sequencing of pre*S* region complementary strand. The sequences were edited manually by Vector NTI Suite 7.0 software (InfoMax Inc, Bethesda, USA) and subsequently aligned in FASTA format. The genotypes and serotypes of HBV were determined by comparison of the obtained sequences with standard sequences from GeneBank.

RESULTS

Sera from 31 patients after kidney transplantation were HBsAg positive; 20 of them were HBeAg positive in ELISA tests (see Table 1). Twenty-four sera were HBV DNA positive in PCR, all of them were HBsAg positive and only two were HBeAg negative in the ELISA tests (Table 1).

HBV DNA from 15 patients belonged to the HBV genotype D, serotype *ayw*3 (Galibert *et al.*, 1979) (accession number V01460). HBV DNA from nine patients belonged to the

Table 1

RESULTS OF ELISA TESTS, PCR, AND SEQUENCING ANALYSIS OF PATIENTS SERA USED IN THE STUDY

Code	a-HCV	HbsAg	HBeAg	anti-HBe	anti-HBc	S	C	X	Genotype/serotype
1T	–	+	+	–	+	+	+	+	D/*ayw*3
2T	+	+	+	–	+	+	+	+	D/*ayw*3
3T	–	+	–	+	+	–	–	–	–
4T	+	+	+	–	+	+	+	+	D/*ayw*3
5T	+	+	–	+	+	–	–	–	–
6T	–	+	–	+	+	–	–	–	–
9T	–	+	+	–	+	+	+	+	D/*ayw*3
10T	–	+	–	+	+	–	–	–	–
11T	–	+	+	–	+	+	+	+	A/*adw*2
12T	+	+	+	–	+	+	+	+	D/*ayw*3
13T	+	+	+	–	+	+	+	+	D/*ayw*3
14T	–	+	+	–	+	+	+	+	A/*adw*2
15T	+	+	+	–	+	+	+	+	A/*adw*2
16T	–	+	+	–	+	+	+	+	D/*ayw*3
17T	–	–	–	–	–	–	–	–	–
18T	–	–	–	–	–	–	–	–	–
19T	–	+	+	–	+	+	+	+	A/*adw*2
20T	+	–	–	–	–	–	–	–	–
21T	+	–	–	–	–	–	–	–	–
22T	+	+	+	–	+	+	+	+	D/*ayw*3
23T	–	+	+	–	+	+	+	+	A/*adw*2
24T	–	+	–	+	+	+	+	–	A/*adw*2
25T	+	–	–	+	–	–	–	–	–
26T	–	+	–	+	+	+	+	–	D/*ayw*3
27T	–	+	+	–	+	+	+	+	D/*ayw*3
28T	–	+	–	+	+	–	–	–	–
29T	–	+	–	+	+	–	–	–	–
30T	–	+	+	–	+	+	+	+	D/*ayw*3
31T	+	+	+	–	+	+	+	+	A/*adw*2
32T	–	+	+	–	+	+	+	+	A/*adw*2
33T	–	+	+	–	+	+	+	+	D/*ayw*3
34T	+	–	–	–	–	–	–	–	–
35T	+	–	–	–	–	–	–	–	–
36T	+	+	+	–	+	+	+	+	A/*adw*2
37T	+	–	–	–	–	–	–	–	–
38T	+	+	–	+	+	–	–	–	–
39T	+	–	–	–	–	–	–	–	–
40T	–	+	+	–	+	+	+	+	D/*ayw*3
41T	+	–	–	–	–	–	–	–	–
42T	–	+	+	–	+	+	+	+	D/*ayw*3
43T	–	+	+	–	+	+	+	+	D/*ayw*3

HBV genotype A, serotype *adw*2 (Valenzuela *et al*., 1980) (accession number X02763) (see Table 1).

After sequencing and comparison of the obtained sequences with Gene Bank data, we found various mutations in both *S* and *C* genes and the *X* gene (Figure.1).

HBV DNA from sera of 20 patients contained different mutations.

DISCUSSION

62.5% of HBV DNA positive samples from patients after kidney transplantation belonged to the D genotype *ayw*3 serotype and 37.5% belonged to the A genotype *adw*2 serotype. These data show that the HBV DNA of the genotype D is more common for Latvia, although the genotype A is also widespread. In some cases the found mutations were common only for the A or D genotypes. Two muta-

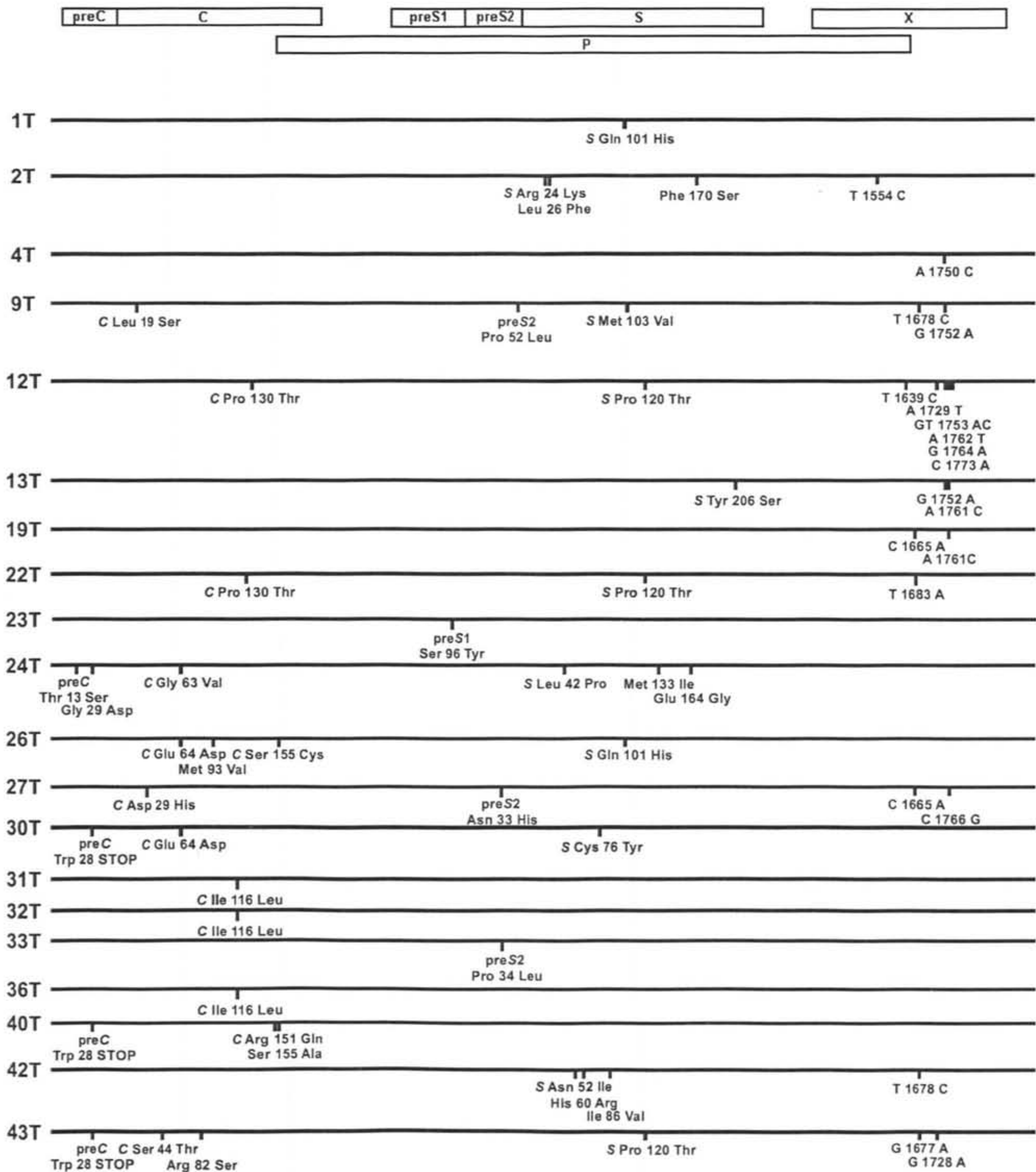

Fig. 1. Mutations found in immunosuppressed patients from Latvia: codes of the patients (numbers and letters on the left side of the figure) and substitutions of amino acid codons (on the left side on the scale; *C* and *S* genes) and positions (on the right side on the scale; *X* gene).

tions were found in the *X* region positions (1678 and 1752): position 1678 T to C and position 1752 G to A in DNA from patients 9T and 42T, and 9T and 13T, respectively (all D genotype) (Figure 1).

One more mutation that was found only in the D genotype was located in the S domain: A to C replacement caused a glutamine switch for histidine at codon 101 (1T and 26T).

We found several mutations only in the D genotype. HBV DNA from patients had mutations in the *X* region position 1728 G/A (43T) and 1766C/G (27T). It has been reported that a mutation at position 1766 (C to T) can be found simultaneously with a mutation at position 1768 (C/G), which affects the basic *core* promoter (BCP) (Gerner *et al.*, 1999). However, in our case we found a mutation at position 1766 without a change at position 1768. Literature data show that

a double mutation (1766 and 1768 position) results in enhanced promoter activity and rarely is present in chronic carriers; possibly the *core* promoter activity is increased by altering transcription factor binding, suggesting that this variant may be involved in the pathogenesis of frequent HBV reactivations (Gerner *et al.*, 1999). HBV DNA samples 13T and 40T had additional mutations in the X gene: 13T had a mutation at position 1761 (A to C) (in BCP) and 43T, at position 1677 (G to A).

HBV DNA from three sera samples (D genotype) had a substitution of codon 120 in the S domain (samples 12T, 22T and 43T; see Figure 1). In this case, proline was changed to threonine. A proline change to glutamine in the same position has been previously reported (Kfoury *et al.*, 2001). We found also substitutions of some of codons in the S domain in other patients, but a mutation was not found in the group-specific "a" determinant 124–147 region. Only in one case in HBV DNA from patient 24T (A genotype) we detected a substitution of codon 133, which caused a metionine change to isoleucine. Codon 103 in S domain had a mutation that led to a methionine substitution to valine in patient 9T. It has been demonstrated that substitution of amino acids adjacent to the 124–147 codon region can influence group-specific "a" determinant structural configuration, resulting in severe antigenic changes of the immunodominant region as well as in the subtype serology (Kfoury *et al.*, 2001).

Patient 9T had a substitution in the preS2 domain 52aa: proline to leucine, 27T– 33aa: asparagine to histidine and 33T–34aa: proline to leucine.

A very important substitution was detected in the pre*C* region position 1896 G/A (corresponds to 28aa), causing a tryptophan change for a stop codon. This mutation was detected in DNA of three patients: 30T, 40T, and 43T (genotype D). Such a mutation is common for the HBeAg negative HBV phenotype with aborted HBeAg expression and is known to cause chronic hepatitis (Parvez *et al.*, 2001). The destabilised C:G base pairing in the lower stem of the epsilon-hairpin due to a G1896A substitution is reportedly compensated by a second C1858T mutation and suggested to play an important role in enhanced selection of the HBe negative variant (Parvez *et al.*, 2001). However, in our case we did not find a compensatory mutation in position 1858. Moreover, all patients had HBeAg. The pre*C* G1896A mutation has been reported to occur in 38% of cases in HBeAg-positive patients (Luo, 1999), which can be explained by the existence of wild type of HBV in the sera of these patients. Analysis of the *C* gene showed several mutations, some of these affecting immunologically important regions of HBcAg. Patient 9T had a substitution of codon 19: leucine for serine, within the core CTL epitope 18–27 codons. Patient 43T had a mutation in position of 82aa: arginine substitution to serine (mutation affects the B cell epitope of core protein). Another variant was detected in patients 12T and 22T: 130aa proline substitution to threonine. This mutation lies within the important immunodominant region (B cell epitope) 129–132aa, and it has been observed to be associated with exacerbation of chronic hepatitis (Okumura *et al.*, 1996; 2001) and hepatocellular carcinoma (Takahashi *et al.*, 1998). In two cases we found substitutions in codon 155 of the core protein. Patient 26T had a 155 serine codon substituted to cysteine codon, and patient 40T had a serine substituted for alanine.

In serum from 12T patient we found a double mutation in the *X* gene *core* promoter region: position 1762 A/T and 1764 G/A. These point mutations caused substitutions of amino acids at codons 130 and 131: 130aa lysine for methionine and 131aa valine for isoleucine. These changes, considered as "hot spot mutations," were found in strains of HCC patients carrying an *ayw* serotype (D genotype), but not in patients with chronic hepatitis B (Venard *et al.*, 2000).

In conclusion, of 31 HBsAg positive samples, 24 samples were HBV DNA positive. HBV DNA from sera of 20 patients contained different point mutations. Deletions or insertions were not found. In three cases, stop codons were found within preC domain, all in samples with the D genotype ayw3 serotype.

The greatest part of HBV DNA positive samples had substitutions within the *X* gene basic *core* promoter and/or enhancer II region.

Further study is needed to determine clinical outcome of the found mutations.

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HEPATĪTA B VĪRUSA STRUKTURĀLĀS ĪPATNĪBAS IMUNOSUPRESĒTAJOS PACIENTOS NO LATVIJAS

Mūsu darba mērķis bija identificēt un raksturot hepatīta B vīrusa (HBV) genomus no pacientiem pēc nieru transplantācijas P. Stradiņa klīniskās universitātes slimnīcas Transplantācijas centrā: Tika noteikti HBV serotipi un genotipi, kā arī lokalizētas strukturālo (*S*, *C*) gēnu un nestrukturālā gēna (*X* gēna) mutācijas.

Pēc iegūto nukleotīdu secību analīzes tika atrastas vairākas mutācijas abos *S* un *C* gēnos un arī *X* gēnā. 62,5% no HBV DNS pozitīviem paraugiem no pacientiem pēc nieru transplantācijas piederēja D genotipam *ayw*3 serotipam un 37,5% piederēja A genotipam *adw*2 serotipam. Šie dati apstiprina to, ka Latvijai ir raksturīgs D genotipa HBV. Ļoti nozīmīga aizvietošana tika konstatēta pre*C* rajonā 1896. pozīcijā G/A (atbilst 28. as). Tā izraisīja triptofāna kodona maiņu uz stop kodonu. Šī mutācija ir raksturīga HBeAg negatīvam HBV fenotipam ar defektīvu HBeAg ekspresiju un sekmē hroniska hepatīta attīstību. Viena pacienta seruma HBV DNS X gēna kora promotera rajonā tika atrasta dubultmutācija: 1762. pozīcijā A/T un 1764. pozīcijā G/A. Šādas punktveida mutācijas radīja aminoskābju maiņu 130. un 131. kodonā: 130. as lizīns uz metionīnu un 131. as valīns uz izoleicīnu. 43T pacientam noteicām mutāciju kora gēna 82. kodonā: arginīna aizvietošana uz serīnu (mutācija ietekmē B šūnu epitopu–kora proteīna galveno imunodominanto rajonu). Cits kora variants tika identificēts 12T un 22T paraugos: izraisīja 130.as prolīna maiņu uz treonīnu. Šī mutācija skar imunoloģiski svarīgu rajonu (citu B šūnu epitopu) no 129. līdz 132. as. Šo mutāciju saista ar hroniskā hepatīta saasināšanos un hepatocelulāro karcinomu. Delēcijas vai insercijas netika atrastas. Lielākā daļa HBV DNS pozitīvu paraugu saturēja variācijas X gēna kora promotera un/vai enhansera II rajonā.

Lai spriestu par mutāciju ietekmi uz infekcijas klīnisko ainu, ir nepieciešama tālāka šīs problēmas izpēte.

Vīrushepatīta B specifiskā diagnostika: dažas problēmas šodienas skatījumā

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Patoloģijas, ko izraisa hepatīta vīrusi, pirmkārt, hepatīta B (HBV) un hepatīta C (HCV) vīrusi, pieder p izplatītākajām un bīstamākajām visā pasaulē. Piemēram, tikai HBV nēsātāju skaits vien pasaulē pārsniedz 250 n jonus, un apmēram 1/3 no tiem, attīstoties patoloģiskajam procesam, mirst aknu cirozes vai primārās hepatokarc nomas rezultātā. Latvijā HBV nēsātāju skaits 1997.gadā sasniedza 517 (jaunatklāto nēsātāju skaits pieaudzis par 33,6%, salīdzinot ar iepriekšējo gadu). 1997.gadā Latvijā reģistrēti 375 vīrushepatīta B (VHB) slimnieki, pie tar jāatzīmē, ka akūta VHB gadījumā letalitāte kā aknu komas rezultāts ir 1%. Šinī pašā gadā reģistrēti arī 45 nedifc rencēti vīrushepatīti. Jāpiezīmē, ka ne visi vīrushepatīti, tai skaitā arī VHB, Latvijā tiek reģistrēti, jo daļa gadījun norit subklīniski. Vēl vairāk, situāciju sarežģī tas, ka tieši pēdējos gados pasaulē, arī Latvijā, strauji pieaug netra cionālo un nediagnosticējamo vīrushepatītu īpatsvars, ko izraisa jaunradušās mutantās hepatīta vīrusu formas, tai skaitā HBV formas, un savvaļas un vairāk pētītie un zināmie vīrusi tiek pakāpeniski izspiesti no populācijas.

Pirmais netradicionālo hepatītu vīrusu patoloģiju izplatīšanās iemesls ir meklējams straujā vīrusu mainības paātrinājumā. Nesenie pētījumi pierādīja, ka HBV genoms ģenētiski nav nemaz tik stabils, kā domāja agrāk, bet ir iespējamas mutācijas, un tās notiek visos HBV gēnos un regulējošos elementos (1,2). Šodien jau skaidrs, k dažādi faktori (bioloģiski, ķīmiski, fizikāli) var radīt šīs HBV mutācijas. Kā svarīgākos der atzīmēt: imunizāciju (vakcināciju) pret VHB, ilgstošu imūnsupresiju, ilgstošu HBV un saimnieka organisma mijiedarbību, citu vīrusu vai citu mikroorganismu klātbūtni, dažādus pretvīrusu līdzekļus, citostatikus, interferonus, citas ķīmiskas un toksiskas vielas, kā arī radiāciju - ultravioleto starojumu un jonizējošo starojumu (3,4,5,6,7,8,9).

Tikai pašā pēdējā laikā, sākot ar 1995.-1996.gadu, notiek priekšstatu veidošanās par HBV struktūru, HB genoma lokalizāciju un ilgstošu (gadu desmitiem ilgu) izdzīvošanu un funkcionēšanu vīrusa nēsātāju organismā. Plaša strukturālo metožu ieviešana Rietumu medicīnas praksē pēdējos gados ļāva identificēt HBV izmainītās formas un pierādīt, ka tās ir atbildīgas par virkni netradicionālo un nediagnosticējamo aknu patoloģiju, kā akūto, tā hronisko. Abas patoloģiju formas, īpaši hroniskās, noved pie tālākas mutanto HBV formu izplatīšanās ar neprognozējamām epidemioloģiskām sekām, jo pierastās vīrusu formas tiek strauji nomainītas ar mutantām infekciozo aģentu dabīgajā cirkulācijā. Mutācijas skar, pirmkārt, B šūnu imūndominantos rajonus strukturālajos proteīnos, a tradicionālajiem imunoloģiskajiem testiem padarot tos nediagnosticējamus. Tās skar arī citus funkcionāli svarīgu struktūrproteīnu rajonus (T helperšūnu un citotoksisko limfocītu epitopus, šūnu pazīšanas signālus), kā arī attiecī gus rajonus nestrukturālos vīrusu proteīnos. Tas piedod vīrusam jaunas, neprognozējamas īpašības, bieži virulen un patoloģijas bīstamāko seku (letalitātes) pieauguma virzienā (10). Otrkārt, vispārējā cilvēku imunoloģiskā stā sa pasliktināšanās stimulē HBV, un vēl jo vairāk, to mutantu izraisīto infekciju netipisko gaitu un hronizāciju (11,12,13). Tāda hronizācija savukārt veido auglīgu vidi mutanto HBV genomu selekcijai un replikācijai. Līdz to HBV nēsātāju imunoloģiskie defekti provocē vēl lielāku netradicionālo un grūti diagnosticējamo VHB spek

HBV specifisko diagnostiku veic, pacientu asins serumā nosakot virusa antigēnus vai antivielas, kā arī vīrusa DNS. Virusa proteinu diagnostika parasti balstās uz monoklonālām anti-HBV antivielām. Ja HBV genomā notiek mutācijas, kuras izmaina virusa proteinu epitopu struktūru (14), monoklonālās antivielas nespēj atpazīt izmainītos virusa proteinus. Īpaši nopietni tas ir gadījumos, kad preparāts satur tikai vienu monoklonālo antivielu veidu. No teiktā izriet galvenā problēma, ar ko šodien nākas sastapties VHB specifiskās diagnostikas jomā. Šajos gadījumos jālieto polimerāzes ķēdes reakcija (PCR), kas spēj noteikt virusa DNS. Atsevišķas mutācijas HBV genomā nespēj ietekmēt to jutīgumu, tāpēc ar PCR palīdzību izdodas noteikt diagnozi gadījumos, kad ar tradicionālām imūnķimiskajām metodēm tas nav iespējams. Dažreiz hronisko slimnieku HBV genomā notiek plašas delēcijas, kuras skar strukturālos proteinus. Tādos gadījumos slimība notiek īpaši smagi. Pirmkārt, izmainītais viruss spēj veiksmīgi izvairīties no slimnieka imūnsistēmas. Otrkārt, var gadīties, ka defektīvie virusa proteīni nespēj veidot pilnvērtīgas virusa daļiņas. Rezultātā tās nesekretējas, bet paliek šūnās un negatīvi ietekmē šūnas metabolismu. Ar PCR palīdzību ir iespējams savlaicīgi noteikt delēcijas mutantu rašanos un paredzēt slimības attīstības gaitu. Mutācijas lokalizētas, pirmām kārtām, HBV strukturālajos gēnos (virsmas antigēnos preS1, preS2 un S, iekšējā antigēnā C), kā arī nestrukturālajos gēnos (polimerāzē P, transkativatorā X), bet galvenā vērība tika pievērsta HBV strukturālo gēnu imūndominantajiem rajoniem: preS1 rajonam 20-50, preS2 - 1-20 un 40-55, HBsAg - 110-150, preC - 1-29, HBcAg - 70-90 (galvenais imūndominantais rajons c1/e1) un 126-140 (e2).

~~VHB~~ specifiskās diagnostikas grūtības rada ne tikai HBV genomā notiekošās mutācijas. Šeit svarīgi atzīmēt arī citus apstākļus un faktorus, kas nereti traucē virushepatītu, VHB tai skaitā, precīzu un savlaicīgu specifisko laboratorisko diagnostiku: 1) HBV replikācijas persistenci perifēro asiņu šūnās; 2) HBV marķeru sorbciju perifēro asiņu šūnās; 3) superinfekciju ar citiem hepatīta virusiem, citiem vīrusiem vai citiem mikroorganismiem; 4) ksenobiotiķu ierosināto citohroma P450 sistēmas indukciju un sekojošu imūnsistēmas disfunkciju; 5) no augšminētajos punktos atzīmētajiem faktoriem gala rezultātā atkarīgo HBV replikācijas un HBV marķeru kinētikas īpatnības slimnieka organismā.

Metodes

No esošām datu bāzēm un žurnālu publikācijām izanalizēti apmēram 200 zināmo hepatīta B virusa un to mutantu genomu. Balstoties uz iegūtiem datiem, PCR-diagnostikai izvēlēti sekojoši virusa genoma reģioni: centrālais hidrofīlais reģions S-antigēna gēnā (190 - 748 nt, numerācija dota saskaņā ar (15)), centrālais imūndominantais reģions korantigēna gēnā (2043 - 2431 nt) un kora promotoru un X-gēna fragmentu saturošais reģions (1505 - 1825 nt).

Praimeri tika sintezēti ķīmiski LU BMC oligonukleotīdu sintēzes grupā prof. E.Stankēvičas vadībā un pārbauditi uz PHB320 plazmīdas, kas satur klonēto HBV *ayw* genomu (16).

Labākās praimeru kombinācijas tika izmantotas rutīnās diagnostikas mērķiem ar dažāda kontingenta slimnieku un asins bankas asinīm.

Izmantoto praimeru sekvences:

S-gēns:
S1': 5'- CTCGTGTTACAGGCGGGGTTTTTC - 3'
S2': 5'- AAACCGAAAGTCAATATACCTACTAC - 3'
C-gēns:
C1'': 5'- TTGTTGACAAGAATCCTCACAATACC - 3'
C2'': 5'- GCCCTACGAACCACTGAACAAATGG - 3'
C-gēna promotors:
p21 5'-CGTTCCAGCCGACCACGGGGCGC-3'
p2-S 5' - AAAAAGTTGCATGGTGCTGG - 3'

Rezultāti un diskusija

Kopā tika izpētīti 55 slimnieku asins seruma paraugi ar neidentificētu hepatītu vai ar neparastu seroloģismarķeru kompleksu. DNS tika izdalīta, izmantojot standarta metodes. Izdalītā DNS tika pārbaudīta ar PCR palīdzību, izmantojot iepriekšminētos praimerus. Iegūto PCR produktu garums tika salīdzināts ar pozitīvās kontroles šūnu agarozes gēlā. Paraugu pētīšanas rezultāti apkopoti 1.tabulā. Tajā parādītas arī slimnieku diagnozes, kā arī slimnieku asins serumā atrastie seroloģiskie marķeri.

1.tabula. *Asins seruma testēšanas rezultāti slimniekiem ar HBV infekciju.*

Diagnoze (sākotnējā)	kopā	HBsAg	a-HBc	a-HBC IgM	HBeAg	a-HBe	a-HCV	HBV DNS
VH neidentificēti	11	1	4	0	0	3	0	2
Akūts VHB+VHC	26	24	24	25	2	4	24	24
Hronisks VHB	8	8	6	1	2	2	2	8
Aknu ciroze	5	5	2	1	0	4	2	4
Nieru transplantācija	5	3	1	0	3	0	1	5

1.tabulā atspoguļoto datu analīze liecina, ka HBV seroloģisko marķeru dizains ne visos gadījumos atbilst tradicionālajam HBV spektram, kas raksturīgs attiecīgajai kliniskajai diagnozei. Tai pašā laikā, izmantojot PCR tehniku, tika pārliecinoši pierādīta HBV DNS (svarīga HBV replikācijas marķera) klātbūtne, pie tam daudz vairākos gadījumos nekā cits tradicionāli laboratoriski nosakāmais HBV replikācijas procesa markeris - HBeAg.

Divos gadījumos tika konstatētas PCR produktu garuma novirzes no normas. Iespējams, tas ir saistīts ar HBV C gēnā notikušām delēcijām. Viens no paraugiem pieder slimniekam ar hronisko hepatītu B, otrais – slimniekam ar neidentificētu hepatītu. Turpmākajā darbā paredzēta abu neparasto PCR produktu nukleotīdu secības noteikšana (sekvenēšana).

Provizoriskie dati liecina, ka ar šodien tradicionālām metodēm izvērtējot katra VHB slimnieka asinis nosakāmo HBV marķeru spektru, būtu obligāti jāņem vērā HBV mutantu klātbūtnes iespējamība, kā arī citi šī raksta ievaddaļā minētie faktori. To ignorēšana var radīt (un arī rada) nopietnas nepilnības HBV infekcijas specifiskajā diagnostikā.

Abstract

Specific diagnostics of viral hepatitis B - some problems in today's view.

The constant influence of hazardous environmental and genetic factors as well as the wide usage of modern medical methods (immune modulators, antivirals, organ transplantation, widely distributed vaccination against hepatitis B etc.) created the substantial background for the replacement of traditional HBV genomes by the mutant ones in the natural circulation of infectious agents. Mutations affecting as usual immunodominant structural HBV proteins (a) convert them for undiscoverable diagnostically by traditional approaches, (b) add them new unexpected properties which lead always to enhancement of virulence and, thus, negative sequels of the disease. The present project in generally proposes 1) elucidation of mutant HBV forms occuring in Latvia, their identification and structural characterization, 2) explanation of some other HBV specific laboratorial diagnostics difficulties associated with a) HBV replication persistence in peripheral blood cells, b) sorbtion of HBV and its markers by blood cells, c) cytochrome P450 system induction and, thus, the immune system disfunction, d) other hepatitis viruses, other viruses and microorganisms superinfection, e) virologic (HBV) and HBV markers kinetics in patients with HBV infection.

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