

Molecular Epidemiology of Hepatitis C Virus Strains from Romania

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Abstract

Background & Aims: A high seroprevalence of Hepatitis C Virus (HCV) infection has been reported in Romania, with limited data on the viral subtypes' distribution. In order to detect any changes in the genetic composition of the epidemic, a survey on the recent profile of circulating HCV genotypes was conducted. **Methods:** 241 hepatitis C infected patients with active viral replication diagnosed between September 2004 - October 2008 were included in a retrospective study. Genotyping using commercial Line Probe Assay (Innogenetics) was confirmed by sequencing of Core PCR products followed by phylogenetic analysis. **Results:** HCV subtype 1b was found in 92.6% of the samples, subtype 1a in 5.4 % of the samples, subtype 4a in 1.2%, and subtype 3a in 0.8% of the samples. Chronic hepatitis C infections with subtype 1b were found in women aged 40-60 years old with a history of blood transfusions received during surgical/obstetrical interventions. No geographical clustering was evident for HCV 1b sequences. The new emerging non-1b genotypes were detected mainly in younger patients with a history of intravenous drug use. The genetic distances among the HCV 1a strains are very homogeneous and small, with a high sequence identity with other European strains, suggesting the recent entrance of this subtype in Romania from singular or limited sources of infection. **Conclusion:** The introduction of new HCV genotypes in Romania stimulates a continuous epidemiological surveillance, suggesting shifts in the transmission pathways and risk factors, with the possible emergence of recombinant strains in patients with multiple infections.

Key words

Hepatitis C virus – genotyping methods – molecular epidemiology – risk factors.

Introduction

Hepatitis C virus (HCV) is the subject of intense research and clinical investigations due to its worldwide prevalence and major role in chronic liver disease. Phylogenetic analysis of HCV genomes has led to a classification of HCV into six different genotypes that differ from each other by 31%-33% on the nucleotide level, and have a regional specific distribution, genotypes 1a and 1b being the most frequently encountered in Europe, United States, and Japan. HCV subtypes 2a and 2b are commonly found in North America, subtype 2c in Northern Italy, while genotype 4 is predominant in North Africa (especially in Egypt) and the Middle East; genotypes 5 and 6 are commonly reported in South Africa and Hong Kong, respectively. Genotype 3a is endemic in South East Asia, and seems to be dominant in intravenous drug users (IDUs) in Europe and the United States [1].

Viral genotype is the most important factor in assessing the optimal treatment duration for HCV infection, with additional guidance provided by the on-treatment response, rapid virological response being an earlier predictor of treatment success, and early virological response - an accurate marker of treatment failure. HCV genotypes can be ranked, in a decreasing order of susceptibility to interferon-based treatment, as follows: genotypes 2, 3, 4 and 1 [2, 3].

In the latter years it has been reported that, especially in genotypes 1 and 4, single nucleotide polymorphisms (SNPs) located near the gene for interleukin-28B (IL28B) are strongly associated with the likelihood of achieving a sustained virological response [4, 5], as well as with the viral kinetics during treatment [6], and the natural clearance of HCV infection [7]. Infection with HCV genotype 1b has been strongly associated with more severe liver disease and higher risk for the development of hepatocellular carcinoma than infection with other HCV genotypes [8]. HCV genotype 3 was recently associated with faster fibrosis progression compared to other genotypes [9, 10].

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Romania remains a high prevalence region for HCV infection, with a general prevalence rate of 3.23% in adult population, and significant differences between rural and urban areas (3.8% versus 2.68%) [11]. However, limited data on the distribution of the viral genotypes and subtypes in our country are available. In a recent Romanian report gathering data from two studies, genotype 1 or 1b were almost exclusively found in Romanian patients with chronic hepatitis C (99.13% of 461 patients from ENMS study had genotype 1 without subtype identification, and 93.46% of 153 patients in ACHIEVE study had subtype 1b) [12].

Nevertheless, the HCV epidemic in Europe is changing, most of the countries from this area experiencing drug-use related outbreaks [13]. As a consequence, the aim of our study was to evaluate the current profile of circulating HCV genotypes and subtypes in Romania and their association with transmission risk factors, in order to promptly detect any changes in the genetic composition of the Romanian epidemic.

Methods

Patients

We conducted a retrospective cohort study, including HCV infected patients from Bucharest, Timisoara and surrounding areas diagnosed between September 2004 and October 2008. Two hundred and forty-one patients with active viral replication (positive HCV viral load) were included in the genotyping analysis. Viral load was tested by RT-PCR (Cobas Amplicor HCV Monitor, vers 2.0, Roche, Germany, with a linear range between 600 - 700 000 IU/mL, and a lower detection limit of 600 IU/mL).

All patients answered a standardized questionnaire at enrollment, on potential risk factors for HCV infection, including receipt of blood products, minor and major surgical interventions, multiple parenteral treatments, intravenous drug use (IDU), body piercing, occupational exposure, and data about sexual behavior. Written informed consent was obtained from the patients, and the study was approved by the Bioethic Committee of the Stefan S. Nicolau Institute of Virology.

HCV genotyping

Genotyping was initially performed using commercial Line Probe Assay (VERSANT™ HCV Genotype 2.0 Assay, Siemens, Germany), a reverse hybridization line probe assay in which two distinct biotinylated DNA fragments of 240 and 270 base pairs, representing 5'UTR and core regions are hybridized to immobilized oligonucleotide probes that are specific for the same regions of different HCV genotypes.

Genotype confirmation was carried out by sequencing in the core genomic region. The protocol of viral RNA isolation and reverse transcription was done as previously described [14]. A heminested PCR system was designed to amplify a 422 bp fragment from HCV core gene. First round of PCR was carried out in a final volume of 50 microliters consisting of: 1.5 U GoTaq Flexi (Promega), 400 nM CO-1S primer, 400 nM CO-2AS, 0.2 mM of each dNTP, 1.5 mM MgCl₂, 5 µL of 10X reaction buffer, 5µL of cDNA and water for the final volume. For the second PCR, 2 microliters of Mix 1 were added in PCR Mix 2 containing CO-1S and CO-3AS primers (400 nM each). Oligonucleotides used for amplification are listed in Table I. The same thermic profile was used for both rounds of the PCR: initial denaturation (94°C, 3 min); 20 cycles consisting of: denaturation (95°C, 30 sec), annealing (60°C to 51°C, decreasing 1°C for every 2 cycles), extension (72°C, 30 sec); final extension (72°C, 5 min).

The resulted amplicons were gel purified with a commercial kit (Wizard SV Gel and PCR Clean-up System, Promega, Germany), then sequenced on a 3100-Avant Genetic Analyzer (Applied Biosystems). The HCV genotype was identified using the Basic BLAST software from the National Center for Biotechnology Information site.

Phylogenetic analyses of the core sequences were conducted with Mega 4 software - Molecular Evolutionary Genetics Analysis software [15]. UPGMA (Unweighted Pair Group Method with Arithmetic Mean) phylogenetic tree was obtained, and core region sequences from the phylogenetic analysis were deposited in EMBL/GenBank (access numbers: AM114084 – AM114101, AM422808 – AM422831, FR850661-FR850667 and FR854392).

Statistical analysis was performed by univariate analyses, using Fisher's exact test and Pearson's chi-square test for proportion. All p values were two-tailed. Data were analyzed with SPSS, version 11.

Results

Demographic and virological characteristics of the study subjects

The female/male ratio was 1.62, female gender representing the majority – 61.9%; 83.8% of the cases came from an urban area. Mean age was 42.6 ± 14.9 years, most of the cases clustered in the age groups 20 to 59 years (82.6%) (Fig. 1). Distribution by gender and age group shows that female HCV infected patients were significantly older than males (72.5% of the females vs. 39.1% of the men were older than 40 years, $p < 0.001$, OR = 4.9% CI = 2.05-7.63).

Overall, the percentage of patients with low and high viremia (using a cut-off of 600.000 IU/mL) was quite similar

Table I. Primers used for core fragment amplification (primer positions refer to H77 strain, accession number AF011752).

Primer	Sequence 5'→3'	Polarity	Position
CO-1S	TAG GGT GCT TGC GAG TGC CCC	External sense	297-317
CO-2AS	ATG TAC CCCATG AGG TCG CC	Internal reverse	732-751
CO-3AS	AGG GTA TCG ATG ACC TTA C	Internal reverse	700-718

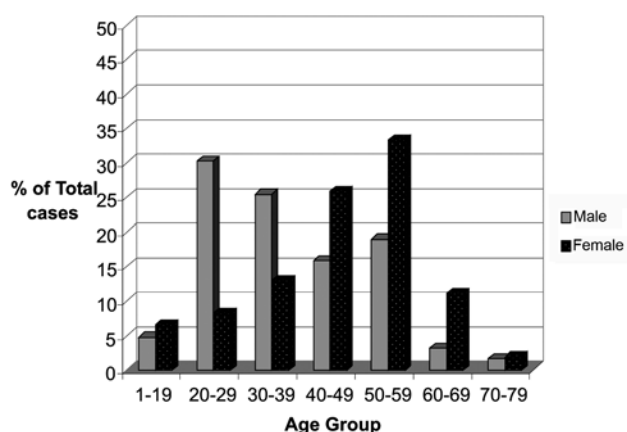


Fig 1. HCV distribution by gender and age group.

(51.5% vs. 48.5%). No significant differences in viral load values were recorded with respect to the patients' age: high viral loads were present in 51.1% of the young patients (≤ 40 years), and in 44.9% of older patients ($p=0.63$, $OR=1.34$, $95\%CI=0.75 - 2.41$) (Table II).

HCV genotypes

HCV subtype 1b was the most commonly encountered, accounting for 92.6% of the cases, followed by subtype 1a in 5.4%, subtype 4a in 1.2%, subtype 3a in 0.8% of the cases, respectively. Concordant results were obtained by the commercial Line Probe Assay and by a sequencing method in the core region for all tested samples.

Molecular characterization was carried out by phylogenetic reconstruction and genetic distance calculation in the core region. The phylogenetic analysis shows that subtype 1b HCV, the most frequent in our population, presented similarity with strains found worldwide, with no specific geographic clustering (data not shown). The UPGMA phylogenetic tree presented in Fig. 2 included HCV strains from Romanian patients compared with genotyped HCV strains from GenBank.

The genetic distances among the HCV 1a sequences are very homogeneous and small, ranging from 0.0000 to 0.0318. High sequence identity was found in some Romanian 1a isolates with strains from Russia (X71407), Switzerland (EU155347) and UK (FN666308), suggesting an epidemiological link.

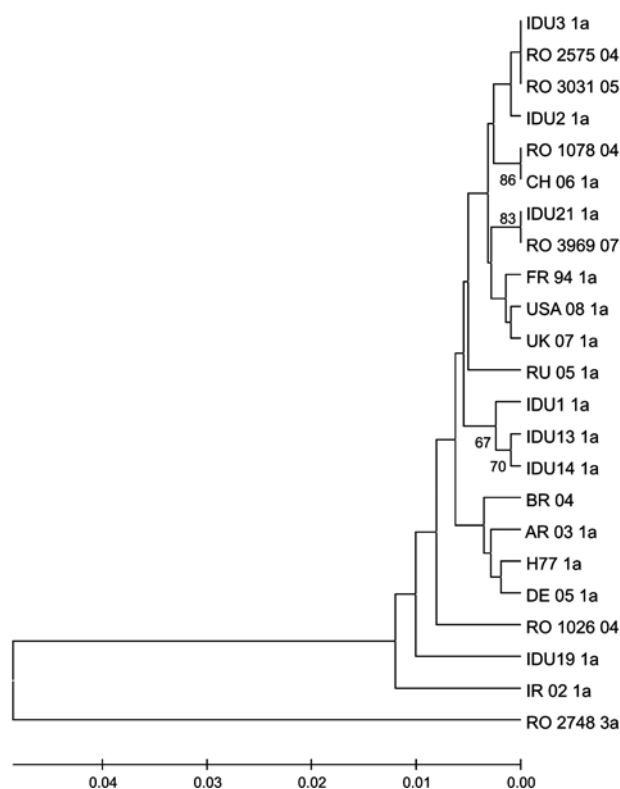


Fig 2. Phylogenetic analysis in the core region of Romanian HCV 1a strains compared with other 1a strains from GenBank using the following accession numbers: AF359345 (AR_03); EF032889 (BR_04); AF512996 (IR_02); EU155355 (CH_06); L12353 (FR_94); EU781767 (USA_08); EU687193 (DE_05); FN666297 (UK_07); X71407 (RU_05). The phylogenetic tree was created with the UPGMA method using Maximum Likelihood model in the Mega 4.0 software.

Patients infected with HCV genotype 1a were significantly younger than those infected with other genotypes (Table II): 84.6% of cases with genotype 1a were < 40 years old, as compared with 36.8% of other genotypes ($p=0.007$, $OR=7.4$). Also, mean ages for patients infected with HCV genotypes 1a and 3a were lower when compared with those infected with subtype 1b and 4a (mean age 28.4 ys. \pm 11.5 and 24.5 ys. \pm 0.5, versus 43.5 ys. \pm 14.6 and 46.5 ys. \pm 30.4, respectively; $p < 0.001$).

Table II. Characteristics of study subjects by age (expressed as percentage, %)

Characteristics	Age (years)		OR	95%CI	P value	
	≤ 40 ys.	> 40 ys.				
Gender	Male	60.9	39.1	4.9	2.05 - 7.63	< 0.001
	Female	27.5	72.5			
Viral load	< 600.000	48.9	55.1	1.34	0.75 - 2.41	0.63
	≥ 600.000	51.1	44.9			
Genotypes	1a	84.6	15.4	7.4	1.56 - 35.5	0.007
	Other genotypes	36.8	63.2			
Risk factors	Parenteral risk	39.3	60.7	0.39	0.31 - 0.49	< 0.001
	Drug use	100	0			

Risk factors associated with HCV infection

Only 21.2% of the patients reported a single risk factor for acquisition of HCV infection, while the majority had multiple associated risk factors (in the decreasing order of prevalence: multiple invasive dental procedures – in 62.2% of the cases, multiple parenteral treatments – in 37.8%, previous major surgery – in 21.6%, transfusion – 13.7%, body piercing and cosmetic interventions – in 9.1%, multiple sexual partners and sexual transmitted diseases in the past – in 1.2% of the cases).

Women reported more frequently than men exposure to parenteral risk factors - transfusions, major surgery, multiple parenteral treatments (68.5% vs. 43.5%; $p < 0.001$, RR = 1.513, 95%CI = 1.2–1.905). Surgical interventions are more important for HCV genotype 1b acquisition ($p < 0.001$, RR = 1.546, 95%CI = 1.256–1.09).

Intravenous drug use was reported by 17 patients (7.1% of the total cohort). All these subjects were significantly younger than those exposed to parenteral interventions (mean age 28.4 ys. versus 43.5 ys., 95%CI = [-15.77–14.91], $p < 0.001$). Men were more prone to report drug use: 13% from the total number of the male patients, compared to only 3.4% of the total number of female subjects reported drug use ($p = 0.007$, OR = 0.23, 95%CI = 0.08–0.68). Intravenous drug use was more frequently associated with genotype 1a than genotype 1b infections (84.6% vs. 19.7%; $p = 0.003$, OR = 20.4, 95%CI = 6.70–575).

Discussion

Both the genotyping and phylogenetic analysis' results from our study revealed that while subtype 1b is still dominant in the Romanian hepatitis C epidemic, infections with newly introduced genotypes (1a, 3 and 4) are emerging. Patients infected with the non-1b genotypes are younger than the rest and come from urban areas, where IDU is rapidly spreading. In 2009, a routine monitoring for the prevalence of drug related infectious diseases, showed HCV infection values among drug users from Romania over the European average [16].

Due to the permanent people migration both between rural and urban areas inside Romania and abroad, this may have important consequences for the overall epidemiological trend of the HCV epidemic in Romania, where a higher HCV prevalence in rural areas compared to urban ones was reported [11]. Although in our study the number of subjects from rural area was small (39 patients - 16.2% of the total cohort), all were infected with genotype 1b previously described as prevalent in Romania, most probably reflecting the past iatrogenic transmission of HCV. Correlation of the infecting genotypes with the potential risk factors for the HCV acquisition can promptly detect changes in the epidemic evolution. As long as many patients from our study present multiple associated risk factors, underreporting of illicit drug use cannot be excluded. According to the Romanian National Anti-Drug Agency, in Romania, heroin is used especially by young people (almost 70% of the

total demands for substitution treatment in heroin addicts are registered among people younger than 29 years) and the onset of heroin use appears at a young age: 42% of the subjects are in the age group 15 -19 years [16]. Exposure to intravenous drugs, tattoos and piercing can play an important role in the acquisition of new HCV genotypes, previously absent from this region.

The phylogenetic analysis shows that the prevalent HCV 1b strains isolated from our patients are genetically related to a variety of HCV sequences from different geographic regions, with no specific clustering. This observation is in accordance with reports from other European countries [13], suggesting a large international transmission network, involving inadequately screened blood products and unsterilized needles and syringes. The older age of the patients from our study infected with this genotype also supports the idea of an early introduction of subtype 1b in our country.

By contrast, infections with HCV subtype 1a seem to have been recently introduced in Romania (over the past 10 years). In our study, subtypes 1a and 3a were detected in younger patients, mainly associated with a history of IDU during the preceding years. The very low genetic distance between the Romanian 1a sequences from our patients (median value for isolates under 0.033) point to a single or limited source of infection [17]. Moreover, some Romanian HCV 1a isolates revealed a high sequence identity with strains from other European countries (Russia, UK, Switzerland), suggesting an epidemiological link.

The co-circulation of multiple HCV subtypes raise the possibility of recombinants emergence, as has been already described for HCV subtypes 1b/1a and 2k/1b [18, 19]. Detection of non-1b HCV genotypes in Romania may also lead to major shifts in the dominant subtype distribution and a possible expansion of previously minor subtypes. In Western Europe infection with HCV subtype 1a has already overtaken those with genotype 1b. In addition, a slight increase in the proportion of genotype 3 infections (ranging from 12 to 35%) was seen especially in IDUs, as shown by several reports from United Kingdom [20], Austria [21], Germany [22, 23] and Italy [24].

Similar information has been also gathered from Eastern European countries. In the Czech Republic, an increase in the prevalence of genotypes 1a (13.3%) and 3a (19.7%) was associated with the considerably increased incidence of HCV in IDUs over the last 15 years [25]. In Poland the relative proportion of genotype 1b has decreased (57.5%) in favor of genotype 3a (31.3%), mostly, but not exclusively among IDUs [26]. Recent reports from Slovakia detected a high HCV genotype 3 prevalence in IDUs (60.9%) [27], and in Russia genotype 3a appears to have outstripped 1a in younger people and IDUs (56.9% and 11.9%, respectively) [28].

The Romanian HCV genotype 4 strains seem to be imported from Egypt, as shown by the close genetic homology with the reference strain ED43 from an Egyptian politransfused patient in Cairo in the '80s. Generally, HCV type 4 circulates mostly in the Middle-East and

Africa, but recent studies indicate spread of this genotype to several European countries, particularly among IDUs and in immigrants from Italy, France, Greece and Spain, with a prevalence of 10 - 24% of the HCV infections [2]. Circulation of genotype 4a in Romania was previously reported using a genotyping assay by restriction fragment length polymorphism in the HCV 5' untranslated region and sequencing in the NS5B region [14].

The recent introduction of new HCV genotypes in Romania stimulates a continuous alert, suggesting shifts in the transmission pathways, with the possible expansion of a previously minor subtype or with the emergence of recombinants in people with multiple infections.

Identification of HCV genotypes is also of utmost importance for the therapeutical management of patients. While patients with HCV genotypes 2 and 3 can be cured with standard therapy in about 80% of cases, the rate of sustained virologic response for patients with HCV genotype 1b remains approximately 40%. Furthermore, subtype 1a rather than 1b and subtype 2a rather than 2b are likely to respond better to IFN-based therapy [3, 9, 10].

An important achievement in the treatment of chronic hepatitis C infection is now available: two HCV-specific protease inhibitors, boceprevir (VICTRELIS™) and telaprevir (INCIVEK™) were approved by the Food and Drug Administration (FDA) in May 2011 for the treatment of HCV genotype 1 infection. The triple therapy - combination of pegylated interferon and ribavirin (PegIFN/RBV) with a protease inhibitor - was shown to significantly increase the success rate in treatment-naïve HCV infected patients (from 38-44% obtained with PegIFN/RBV alone, to 63-75%) [29, 30], as well as in nonresponders or relapsers to previous PegIFN/RBV therapy (from 17-21% to 59-66%) [31]. This will be an important asset for Romanian patients, as the number of annually treated patients remained relatively stable between 2002 and 2007 (1,813 patients treated with PegIFN and RBV in 2002 and 2,446 in 2007) [32]. Moreover, a recent study has signaled the relative young age of Romanian patients infected with genotype 1b with severe chronic HCV hepatitis and possible cirrhosis' occurrence that are still on the waiting lists for antiviral treatment [33]. It would be interesting to evaluate comparatively the long term evolution of young patients infected with non-1b HCV genotypes, who are candidates for shorter-time therapy with important implications for both the quality of life and treatment costs. The continuous surveillance of HCV genotypes and the early recognition of multiple or/and new risk factors will add valuable information to the overall European epidemiological picture, with important implications for the improved design of treatment regimens and prevention campaigns.

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Conflicts of interest

Nothing to declare.

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