HCV non-1b genotypes in injecting drug users from Romania

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Abstract

Introduction: Chronic hepatitis C cases diagnosed in Romania were mostly related to unsafe parenteral treatments and blood transfusions; HCV genotype 1b was prevalent. During the last decade, an increasing number of HCV infections was reported among people who inject drugs (PWID). The aim of the current study was to test if this epidemiological shift triggered a diversification of the circulating viral strains.

Methodology: HCV genotypes were determined by reverse hybridization in 130 HCV-infected PWID (87.7% males; mean age 27.9 ± 6.7 years, injecting drugs for 8.1 ± 4.8 years).

Results: HIV-HCV co-infection was diagnosed in 80.8% of the subjects and 26.9% were HIV-HCV-HBV triple infected. Active HCV viral replication was present in 104 PWID (80%), more frequently in those HIV-co-infected (91.4% vs. 52% in HCV mono-infected, and 77.148.5% in HIV-HCV-HBV triple-infected, p = 0.0001). Non-1b genotypes were prevalent (54.8%), with subtype 1a the most commonly detected (24%), followed by genotypes 3a (14.4%) and 4 (7.7%). Mixed infections with genotypes 1a and 1b were found in nine subjects (8.7%). There was no difference in the genotypes frequencies based on HIV or HBV co-infection status, length of drug usage, or associated risk factors (tattoos, piercing, detention).

Conclusion: The continuous surveillance of HCV genotypes in PWID from Romania will add valuable information to the overall European epidemiological picture, with important therapeutic implications.

Key words: viral hepatitis C; injecting drugs; Romania.


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Introduction

In Europe, hepatitis C virus (HCV) transmission is mainly related to injecting drug use; nevertheless, in Romania, a country with a high HCV seroprevalence (3.3% in the general population), the majority of the diagnosed cases have been attributed to unsafe surgical procedures, parenteral treatments, and blood transfusions received before 1990 [1]. A change in this epidemiological picture occurred in the last decade, with an increasing trend of HCV infections reported among people who inject drugs (PWID): seroprevalence rates mounted from 47.6% in 2004 to 82.4% in 2012 [2]. This rise was driven both by the replacement of heroin with new psychoactive substances (NPS), traded as legal until 2011, and by the low coverage of harm-reduction measures. Human immunodeficiency virus (HIV) and hepatitis B virus (HBV) infections were scarcely detected in PWID in Romania until 2011, when an explosive increase in blood-borne viral infections (multiple hepatitis and HIV co-infection) was reported in drug users hospitalized as medical emergencies, mainly caused by acute intoxication or systemic infections secondary to drug-induced immunosuppression [3].

HCV genotype 1b is still considered to be prevalent in Romania [4], although a pilot study from 2011 [5] signaled the appearance of new genotypes in a small-size sample of injecting drug users treated in opioid substitution centers.

The aim of the current study was to identify if the continuous increase of PWID as a new risk-group for HCV infection in Romania has triggered a change in the circulating viral genotypes.

Methodology

Stored plasma samples from 130 PWID, newly diagnosed with HCV infection between 2011 and 2014 during hospitalization for systemic infections in a tertiary facility, were tested for HCV viral load, using real-time polymerase chain reaction (PCR) COBAS
Hepatitis C virus (HCV) genotypes in Romanian injecting drug users

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TaqMan HCV Test, version 2.0 (Roche Diagnostics, Mannheim, Germany), with a linear range between 25 and $3.91 \times 10^8$ IU/mL, and a lower detection limit of 25 IU/mL. HCV genotyping was performed in samples with detectable viral loads using a reverse hybridization line probe assay (VERSANT HCV Genotype 2.0 Assay [Siemens Healthcare, Erlangen, Germany]), according to the manufacturer’s instructions.

Serological markers of HBV and HIV infections were determined with third-generation commercial immunoenzymatic assay (DIA PRO Diagnostic, Milan, Italy). Chronic HBV infection was confirmed by the presence of HBsAg and IgG anti-HbcAb; HIV infection was confirmed by the presence of anti-HIV antibodies (enzyme-linked immunosorbent assay and Western blot); all positive samples were tested for HIV RNA, using real-time PCR (COBAS TaqMan HIV-1 Test, version 2.0 [Roche Diagnostics, Mannheim, Germany]), with a lower limit of detection of 20 copies/mL and a linear range of 20–10,000,000 copies/mL.

Statistical analysis was done using SPSS version 20.0. Baseline characteristics of the study participants were compared using the Chi-square test for categorical variables and the analysis of variance (ANOVA) test for continuous variables. A p value of < 0.05 was considered significant.

Results

Sociodemographic characteristics of the patients and the prevalence of multiple blood-borne co-infections

The cross-sectional study of the 130 PWID showed that a vast part of the study subjects were young males (87.7%, mean age 27.9 ± 6.7 years), from urban areas (89.2%) who were presently unemployed (88.5%). The mean estimated duration of drug use was 8.1 ± 4.8 years (Table 1); 87.7% were currently injecting both heroin and NPS, and 86.9% were sharing injecting equipment frequently. Of the 130 HCV-infected patients, 80.8% were co-infected with HIV, and 26.9% were also chronic HBV carriers (Table 1).

The age of the hospitalized injecting drug users increased from 24.8 ± 5.2 years in 2011 to 29.7 ± 6.6 years in 2014 (p = 0.003), with a consistent longer period of drug abuse (7.1 ± 4.8 years in 2011 vs. 10.9 ± 4.5 years in 2014; p = 0.007). The prevalence of the HIV co-infection increased from 76% in 2011 to 100% in 2013 (p < 0.0001). HIV co-infection was newly diagnosed in almost all patients (only two had a positive HIV test in the previous two years) and seemed to be recently acquired, since 53.3% of HIV infected PWID had CD4 cell count higher than 500 cells/mL. There were no significant differences between associated risk factors detected in HCV mono-infected and HCV-HIV or HCV-HBV-HIV co-infected patients (Table 1). Most PWID were unaware of their infection status, and all were treatment naive.

HCV genotypes in PWID

Active HCV viral replication was detected in 104 patients (80%). HIV-HCV co-infected patients tended

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Total N = 130</th>
<th>HCV mono-infected N = 25</th>
<th>HCV-HIV coinfected N = 70</th>
<th>HCV-HBV-HIV coinfected N = 35</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age at drug initiation (years ± SD)</td>
<td>19.1 ± 6.4</td>
<td>18.2 ± 5</td>
<td>19.2 ± 6.7</td>
<td>19.1 ± 4.1</td>
<td>0.6a</td>
</tr>
<tr>
<td>Mean length of drug use (years ± SD)</td>
<td>8.1 ± 4.8</td>
<td>7.1 ± 4.6</td>
<td>8.3 ± 4.7</td>
<td>8.5 ± 4.1</td>
<td>0.4a</td>
</tr>
<tr>
<td>Invasive dental treatments</td>
<td>62 (47.7)</td>
<td>12 (48)</td>
<td>34 (48.6)</td>
<td>16 (45.7)</td>
<td>0.9b</td>
</tr>
<tr>
<td>Surgical interventions/ transfusions</td>
<td>48 (36.9)</td>
<td>9 (36)</td>
<td>24 (34.3)</td>
<td>15 (42.9)</td>
<td>0.7b</td>
</tr>
<tr>
<td>Tattoos</td>
<td>100 (76.9)</td>
<td>19 (76)</td>
<td>52 (74.3)</td>
<td>29 (82.9)</td>
<td>0.6b</td>
</tr>
<tr>
<td>Piercing</td>
<td>71 (54.6)</td>
<td>14 (56)</td>
<td>38 (54.3)</td>
<td>19 (54.3)</td>
<td>0.9b</td>
</tr>
<tr>
<td>Detention</td>
<td>88 (67.7)</td>
<td>18 (72)</td>
<td>50 (71.4)</td>
<td>20 (57.1)</td>
<td>0.3b</td>
</tr>
<tr>
<td>Unprotected sexual contacts</td>
<td>103 (79.2)</td>
<td>20 (80)</td>
<td>57 (81.4)</td>
<td>26 (74.3)</td>
<td>0.7b</td>
</tr>
<tr>
<td>Mean HCV-RNA (IU/mL)</td>
<td>2.2±10^6 ± 4±10^4</td>
<td>4.8±10^6 ± 1.1±10^4</td>
<td>3.7±10^5 ± 5.8±10^5</td>
<td>7.6±10^5 ± 8.6±10^5</td>
<td>0.03a</td>
</tr>
<tr>
<td>Undetectable HCV-RNA</td>
<td>26 (20)</td>
<td>12 (48)</td>
<td>6 (8.6)</td>
<td>8 (22.9)</td>
<td>0.0001b</td>
</tr>
<tr>
<td>High HCV-RNA &gt; 600,000 (IU/mL)</td>
<td>67 (51.5)</td>
<td>3 (12)</td>
<td>47 (67.1)</td>
<td>17 (48.6)</td>
<td>0.0001b</td>
</tr>
<tr>
<td>Non-1b HCV genotypes</td>
<td>57 (43.8)</td>
<td>10 (40)</td>
<td>29 (41.4)</td>
<td>18 (51.4)</td>
<td>0.6a</td>
</tr>
</tbody>
</table>

HCV: hepatitis C virus; HBV: hepatitis B virus; HIV: human immunodeficiency virus; ^aValues are numbers (%) unless otherwise mentioned; ^bANOVA test; ^cChi-square test for independence, p < 0.05 was considered significant.
to present more frequently with active HCV replication (91.4% vs. 52% in HCV mono-infected, and 77.1% in HIV-HCV-HBV triple-infected; \( p = 0.0001 \)) and with high levels of HCV viral load (> 600,000 IU/mL) compared to those HCV mono-infected or triple infected with HBV (73.4% vs 23.1% and 62.9%, respectively; \( p = 0.002 \)) (Table 1). The level of HCV viral replication was not influenced by the degree of immunosuppression and was not correlated with the degree of HIV replication (data not shown). Non-1b genotypes were present in 54.8% of the cases (57/104 patients with active viral replication), with subtype 1a being the most commonly found (24%), followed by genotypes 3a (14.4%) and 4a (7.7%). Genotype 1b infection was found in 36.5% of the cases, and mixed infections with genotype 1a and 1b were found in nine subjects (8.6%). Patients infected with HCV subtype 1b had higher levels of HCV RNA than those infected with non-1b genotypes (mean viral load \( 3.5 \times 10^6 \pm 6.04 \times 10^5 \) vs. \( 1.7 \times 10^5 \pm 2.2 \times 10^5 \); \( p = 0.005 \)).

There was no difference in the genotypes’ frequencies based on the HIV/HBV co-infection status or the length of drug usage and presence of associated risk factors (tattoos, piercing, detention) (Table 2).

**Discussion**

This study shows the emergence of non-1b HCV genotypes in PWID. Most of these infections were fortuitously diagnosed during hospitalization for other life-threatening conditions associated with drug use. This is a consequence of the limited accessibility to healthcare of this vulnerable population who often lack identity papers and do not benefit from social insurance. Improving access to testing, especially in high-risk populations, is compulsory, as it allows the timely detection of asymptomatic infections, potentially preventing further transmission both inside vulnerable communities and in the general population. According to some estimates, only a quarter of the chronic hepatitis C cases in Romania are diagnosed; this confirms the delayed detection of infection, with associated hindered access to care. A national program for screening and treatment of HCV infection must be based on an accurate assessment of the real burden of the HCV infection. The high rate of HIV/HBV co-infections found in this study, which are associated with a higher level of HCV viral replication, are further complicating factors, inducing an accelerated progression of liver fibrosis and higher mortality rates. HIV co-infection is also associated with a diminished treatment response rate [6] that worsen the prognosis in these patients, who have reduced compliance to therapy compared to mono-infected patients. In our study, the HCV mono-infected PWID had lower percentages of active viral replication, a fact that might indicate either a recent seroconversion associated with an early decline of the viral load, as has been previously suggested [7], or a spontaneous clearance of the virus. A longitudinal follow-up of these patients will help clarify the infection status; nevertheless, in practice, this can be a challenging task due to their low access to medical services.

Surveillance of circulating HCV genotypes in high-risk groups versus the general population reflects the dynamic evolution of HCV infection in a geographical area and the potential for cross-border viral transmission, associated with migration and increased travel. Non-1b genotypes were detected in more than half of the cases included in this study, the most frequent being 1a and 3a, the subtypes that prevail in

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Table 2. HCV genotype distribution in 104 study patients with detectable HCV RNA

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HCV-1b infected patients</th>
<th>HCV non-1b infected patients</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age at drug initiation (years ± SD)</td>
<td>19.1 ± 6.18</td>
<td>19.5 ± 6.70</td>
<td>0.8( ^a )</td>
</tr>
<tr>
<td>Mean length of drug use (years ± SD)</td>
<td>9.2 ± 5.7</td>
<td>10.1 ± 4.5</td>
<td>0.5( ^a )</td>
</tr>
<tr>
<td>Invasive dental treatments</td>
<td>15 (31.9)</td>
<td>22 (38.5)</td>
<td>0.6( ^b )</td>
</tr>
<tr>
<td>Surgical interventions/ transfusions</td>
<td>14 (29.8)</td>
<td>25 (43.9)</td>
<td>0.2( ^b )</td>
</tr>
<tr>
<td>Tattoos</td>
<td>36 (76.6)</td>
<td>43 (75.4)</td>
<td>0.9( ^b )</td>
</tr>
<tr>
<td>Piercing</td>
<td>25 (53.2)</td>
<td>35 (61.4)</td>
<td>0.5( ^b )</td>
</tr>
<tr>
<td>Detention</td>
<td>27 (57.4)</td>
<td>37 (64.9)</td>
<td>0.6( ^b )</td>
</tr>
<tr>
<td>Unprotected sexual contacts</td>
<td>37 (78.7)</td>
<td>41 (71.9)</td>
<td>0.5( ^b )</td>
</tr>
<tr>
<td>Mean HCV-RNA (IU/mL)</td>
<td>( 3.5 \times 10^6 \pm 6 \times 10^5 )</td>
<td>( 1.7 \times 10^6 \pm 2.2 \times 10^5 )</td>
<td>0.005( ^a )</td>
</tr>
<tr>
<td>HCV-HIV coinfected (64 HCV-RNA detectable)</td>
<td>27 (57.4)</td>
<td>37 (64.9)</td>
<td>0.6( ^b )</td>
</tr>
<tr>
<td>HCV-HBV-HIV coinfected (27 HCV-RNA detectable)</td>
<td>12 (25.5)</td>
<td>15 (26.3)</td>
<td>0.9( ^b )</td>
</tr>
</tbody>
</table>

HCV: hepatitis C virus; HBV: hepatitis B virus; HIV: human immunodeficiency virus; \(^1\)Values are numbers (%) unless otherwise mentioned; \(^a\)unpaired t test; \(^b\)Chi-square test, \( p < 0.05 \) was considered significant.
PWID in Europe. Subtype 1a is predominant in Spain [8] and Portugal [9], countries with an important number of Romanian migrants, as well as in the Netherlands [10], while subtype 3a dominates in France [11], Belgium [12], and Cyprus [13], the latter a country that employs a high number of Romanian workers. Genotype 4 is continuously increasing in prevalence in southern Europe, especially in Greece [14], as well as in Italy [15], Portugal [9], and Spain [8].

The emergence of new genotypes has important therapeutic implications. From the clinician’s point of view, the introduction of the highly efficient direct-acting antivirals (DAAs) with pan-genotype activity might render the genotype determination futile. Nevertheless, developing economies such as Romania can hardly sustain the cost of DAAs; therefore, the standard of care still relies on the association of pegylated interferon and ribavirin, which has a genotype-dependent response rate. As a consequence, the correct identification of circulating genotypes can greatly influence the rational design of national treatment strategies. Even if the new DAAs, which can be associated with sustained virological response in 90% of the cases, will be introduced and made available for hard-to-reach populations such as PWID, the selection of resistance mutations that pre-exists at different rates for different genotypes [16] cannot be overlooked. Accordingly, the infecting genotypes will remain an important factor in treatment decisions.

This study has several limitations, especially related to the relatively low number of patients, the cross-sectional nature of the study, and the lack of national coverage. The studied cases came from a single tertiary facility; nevertheless, the geographical coverage is fair, as this is one of the largest infectious diseases care settings in Romania, receiving patients from half of the capital city, Bucharest, and from six southeastern counties in Romania, a total of more than 2.9 million inhabitants (15% of the Romanian population). In Romania, samples from ambulatory care are usually transferred to tertiary facilities, which account for more than a third of the current healthcare expenditure. Moreover, harm reduction services and blood-borne pathogens testing for PWID are concentrated in the capital city, and the reported seroprevalence rates for drug-related infectious diseases are derived from limited samples (300–400 PWID) collected in these areas. Data on the number of PWID and on the seroprevalence of infectious diseases at the national level are scarce, mostly because of the limited availability of both suitable data sources, and of treatment outside the capital city. The latest reported data are from 2013, with 6,288 injecting drug users (5.3 per 1,000 inhabitants 18–49 years of age) reported in Bucharest (based on the treatment multiplier method), with an increasing trend in HCV prevalence, but without data on the circulating HCV genotypes [17]. The findings of the current study cannot be generalized at the national level, nevertheless they signal a subtle switch from the long-term evolving infections caused by HCV genotype 1b nosocomially transmitted in older patients, to those recently acquired, in much younger subjects, associated with drug abuse. Long-term, country-wide longitudinal studies will confirm if the increase in injecting drug use triggers a constant change in the profile of the HCV circulating strains in Romania.

Conclusions
The continuous surveillance of HCV genotypes and the early recognition of multiple or and new risk factors, such as high-risk drug use, 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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**Conflict of interests:** No conflict of interests is declared.