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Distribution of Hepatitis C Virus Genotypes in Croatia – A 10 Year Retrospective Study of Four Geographic Regions

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ABSTRACT

The aim of this 10-year retrospective study was to investigate the distribution of HCV genotypes in patients with chronic hepatitis C monitored in the largest center for molecular diagnostics of HCV infection in Croatia. The study enrolled 1163 anti-HCV positive adults with detectable HCV RNA in the plasma. The patients were classified in four regions: Zagreb and surrounding continental area, Split, Slavonija and Rijeka. HCV genotyping was performed by using VERSANT HCV Genotyping Assay (LIPA) (Bayer Diagnostics, Puteaux Cedex, France). Statistical analysis was performed by using Statistica for Windows V. 5.1. The majority of HCV infections in the study population were caused by genotypes 1 (58.8% of infected patients) and 3 (35.6%). Percentages of patients infected with subtypes 1b and 1a were 37.4% and 13.1%, respectively. Genotypes 2 and 4 were present in a very low percentage of patients (2.2% and 3.4%, respectively) while genotypes 5 and 6 were not detected. Analysis of regional differences in the distribution of HCV genotypes revealed similar percentages of subtype 3a and 1b infections in the Split region while the majority of infections in other regions were caused by subtype 1b. Infections with genotypes 2 and 4 were present in less than 5% of patients in all geographic regions. Analysis of an association between risk factors for infection and distribution of genotypes and subtypes in a subset of patients from the Split region confirmed the association between IVDU and subtype 3a. We conclude that the prevalence of HCV genotypes and subtypes follows the pattern of other Southern and Eastern European Countries with the predominance of subtypes 1b, 3a and 1a.

Key words: hepatitis C virus, genotypes, subtypes, Croatia

Introduction

Infection with hepatitis C virus (HCV) is the major cause of chronic liver disease leading to liver failure and hepatocellular carcinoma¹. It is estimated that about 170 million persons worldwide are infected with HCV.

HCV is an enveloped Flavivirus with a single-stranded, positive-sense, non-segmented RNA genome of approximately 9,400 base pairs that codes for three structural (core, E1, E2) and seven non-structural (NS2-NS6B and p7) viral proteins². A very high degree of genetic heterogeneity in regions coding for both structural and non-structural proteins is one of the key features of this virus.

HCV variants are classified into 6 major genotypes that are associated with particular risk groups for infection and often exhibit specific distribution in different geographical regions. The majority of infections in Europe, USA and Japan are caused by genotypes 1, 2 and 3 but some genotypes show more restricted geographical distributions. For example, HCV genotype 2 predominates in western Africa while the majority of infections in central Africa are caused by genotypes 1 and 4^{3,4}. Genotypes differ from each other in nucleotide sequences by > 30% and can be further divided into more closely re-

lated subtypes that differ from each other by 20–25%⁵. Some HCV subtypes (1a, 1b and 3a) become widely distributed due to contaminated blood transfusion and needle-sharing in intravenous drug users (IDU) and are now responsible for the majority of infections in the developed world.

Determination of HCV genotypes is an important diagnostic tool for monitoring molecular epidemiology and determination of the source of HCV transmission, clinical management of chronic infection (response to antiviral therapy) and detection of multiple-genotype infection^{1,5,7}. Additionally, particular HCV genotypes are also associated with higher viral load, liver histology and severity of liver disease^{1,5,7}. HCV genotyping is also relevant for the ongoing efforts to develop an efficient HCV vaccine.

In our earlier study on the molecular epidemiology of HCV, we showed that the majority of infections in Croatia were caused by subtypes 1b, 3a and 1a⁶. We also reported a very low prevalence of subtype 2a and genotype 4 infections⁶. The aim of this 10-year retrospective study was to investigate the distribution of HCV genotypes and subtypes in patients with chronic hepatitis C monitored in the largest center for the molecular diagnostics of HCV infection in Croatia. We also compared HCV genotype distribution in four geographic regions in Croatia and analysed genotypes according to age, sex and risk factors for infection.

Materials and Methods

Study design and patients

This retrospective 10 year study was conducted at the Department for Viral Hepatitis and Outpatient Clinics for Viral Hepatitis at the University Hospital for Infectious Diseases „Dr. Fran Mihaljević“, Zagreb, Croatia between 1996 and 2005. We enrolled 1163 anti-HCV positive adults with detectable HCV RNA in the plasma (determined by the qualitative HCV RNA test COBAS AMPLICOR HCV version 2.0, Roche Diagnostics, Diagnostic System Pleasanton, CA). Median age of female patients (n=333) was 42 years (range 3–85 years). Median age of male patients (n=830) was 35 years (range 4–81 years).

The patients were classified in four regions: Zagreb and surrounding continental area, Split, Slavonija and Rijeka. Percentages of patients from Zagreb and surrounding continental area, Split, Slavonija and Rijeka included in this study were 39.5%, 44.9%, 9.5% and 6.1%, respectively.

HCV genotyping

Plasma samples for the molecular diagnostics of HCV infection were collected as a part of the routine diagnostic follow-up of patients with chronic hepatitis C. The presence of HCV RNA in the samples was determined by the COBAS AMPLICOR HCV Test, version 2.0 (Roche Diagnostics, Diagnostic System Pleasanton, CA). HCV

genotyping was performed by using VERSANT HCV Genotyping Assay (LIPA) (Bayer Diagnostics, Puteaux Cedex, France) as recommended by the manufacturer. This line probe assay enables the identification of the following HCV genotypes and subtypes: 1, 1a, 1b, 1a/1b, 2, 2a/2c, 2b, 3, 4, 5a and 6a. HCV genotyping was done by using otherwise discarded HCV RNA 244-base pairs amplicons from COBAS AMPLICOR HCV 2.0 Test (Roche Diagnostics, Diagnostic System Pleasanton, CA). Molecular HCV assays were performed at the Laboratory for molecular diagnostics and cellular immunity, University Hospital for Infectious Diseases „Dr. Fran Mihaljević“, Zagreb, Croatia.

Statistical analysis

Statistical analysis was performed by using Statistica for Windows V. 5.1 (StatSoft, Inc., Tulsa, USA). Non-parametric Kruskal-Wallis and Mann-Whitney test were used.

Results

Distribution of HCV genotypes in Croatia

A ten year retrospective analysis on the distribution of HCV genotypes in 1163 Croatian patients with chronic hepatitis C showed that the majority of infections were caused by genotypes 1 (58.8% of infected patients) and 3 (35.6%) (Table 1). Percentages of patients infected with subtypes 1b and 1a were 37.4% and 13.1%, respectively. Genotypes 2 and 4 were present in a very low percentage of patients (2.2% and 3.4%, respectively) while genotypes 5 and 6 were not detected.

TABLE 1
DISTRIBUTION OF HEPATITIS C VIRUS (HCV) GENOTYPES AND SUBTYPES IN 1163 PATIENTS WITH CHRONIC HEPATITIS C IN CROATIA ANALYSED BETWEEN 1996 AND 2005

HCV genotype/subtype	Percentage of infected patients	Sex		Age in years median (range)
		Females (%)	Males (%)	
1	58.8	69.1 ^a	54.7 ^a	42 (3–84)
1a	13.1	13.2	13.0	32 (3–71)
1b	37.4	48.0	33.1	46 ^b (7–84)
2	2.2	3.9	1.6	33 (2–74)
3	35.6	24.0 ^a	40.2 ^a	32 (10–78)
4	3.4	3.0	3.5	31 (22–74)

^a difference between females and males is statistically significant (Mann-Whitney U test, $p < 0.05$ was considered significant)

^b significantly different compared with other groups (Kruskal-Wallis test and Mann-Whitney U test, $p < 0.005$ for all comparisons)

Regional distribution of HCV genotypes

Variability in the distribution of HCV genotypes in four geographic regions of Croatia is presented in Table 2.

The majority of HCV infections in three regions in Croatia (Zagreb and surrounding continental areas, Slavonija and Rijeka) were caused by genotype 1 (69.3%, 60.4% and 76.1%, respectively).

VERSANT HCV Genotyping Assay (LIPA) (Bayer Diagnostics, Puteaux Cedex, France) classifies patients infected with HCV genotype 1 as 1a, 1b, 1a/1b and 1. Subtype 1b was the predominant in Zagreb and surrounding continental areas, Slavonija and Rijeka (50.1%, 45.9% and 46.5% of infected patients, respectively) but not in the Split region (23.2%). Percentages of subtype 1a infections were lower compared with subtype 1b infections in all geographic regions of Croatia. Percentages of patients infected with HCV genotype 1 that our assay was unable to determine as 1a or 1b was less than 10% in regions. Only 3 patients were identified as infected with 1a/1b HCV subtypes.

The majority of infections in the Split region were caused by HCV genotype 3 (47.9% of infections) and genotype 1 (46.9%). Percentages of genotype 3 infections in regions Zagreb and surrounding continental areas, Slavonija and Rijeka region were 25.1%, 32.4% and 18.3%, respectively. More detailed analysis of genotype 3 infections (defined as 3a, 3b or 3) revealed that the vast majority of patients were infected with subtype 3a. Only 1 of 1163 analysed patients was infected with subtype 3b. Ad-

ditionally, 6 patients from the Split and Slavonia groups were defined as subtype 3 infections and could not be further classified with the employed assay.

Percentages of infections with genotypes 2 (mostly 2a/2c) and 4 were less than 5% for all regions.

Age, sex, risk factor for infection and HCV genotypes

Age and sex distribution in patients infected with different HCV genotypes is presented in Table 1.

Median age of patients infected with subtype 1b was significantly higher compared with patients infected with subtype 1a (median 32 years), genotype 3 (median 32 years) and genotype 4 (median 31 years) ($p < 0.05$ for all comparisons).

Male to female ratio in our patient group was 2.49. The most frequent HCV genotype in both female and male patients was genotype 1. Percentages of female (69.1%) and male (54.7%) patients with genotype 1 infection were significantly different ($p < 0.05$). Percentage of genotype 3 infections in male patients (40.2%) was significantly higher compared with female patients (24.0%).

In order to analyse the possible association between risk factors for HCV infection and distribution of genotypes, we analysed a subgroup of 125 patients from the Split region with complete anamnestic data (Table 3).

TABLE 2
HEPATITIS C VIRUS (HCV) GENOTYPES AND SUBTYPES IN FOUR GEOGRAPHIC REGIONS IN CROATIA

HCV genotypes/ subtypes	Number and percentages of patients infected with a particular genotype or subtype within regions			
	Zagreb and other continental regions	Split region	Slavonija	Rijeka region
1	41 (8.9%)	40 (7.7%)	7 (6.3%)	6 (8.5%)
1a	46 (10.0%)	82 (15.7%)	9 (8.1%)	15 (21.1%)
1b	230 (50.1%)	121 (23.2%)	51 (45.9%)	33 (46.5%)
1a/1b	1 (0.2%)	2 (0.4%)	0	0
total number of genotypes 1	318 (69.3%)	245 (46.9)	67 (60.4%)	54 (76.1%)
2	1 (0.2%)	1 (0.2%)	0	0
2a/2c	9 (2.0%)	5 (1.0%)	4 (3.6%)	3 (4.2%)
2b	2 (0.4%)	0	1 (0.9%)	0
total number of genotypes 2	12 (2.6%)	6 (1.2%)	5 (4.5%)	3 (4.2%)
3	0	3 (0.6%)	3 (2.7%)	0
3a	115 (25.1%)	246 (47.1%)	33 (29.7%)	13 (18.3%)
3b	0	1 (0.2%)	0	0
total number of genotypes 3	115 (25.1%)	250 (47.9%)	36 (32.4%)	13 (18.3%)
4	11 (2.4%)	14 (2.7%)	3 (2.7%)	0
4a	1 (0.2%)	0 (0)	0	0
4b	0	1 (0.2%)	0	0
4c/d	2 (0.4%)	5 (1.0%)	0	1 (1.4%)
4h	0	1 (0.2%)	0	0
total number of genotypes 4	14 (3.1%)	21 (4.0%)	3 (2.7%)	1 (1.4%)

TABLE 3
RISK FACTORS FOR INFECTION AND HEPATITIS C VIRUS (HCV) GENOTYPES AND SUBTYPES
IN 125 PATIENTS FROM THE SPLIT REGION

Risk factor for infection	Number of patients infected with a particular genotype or subtype within a risk group					Total number of patients
	1a	1b	2a/2c	3a	4	
IVDU	18	10	0	46	2	76
Transfusion	5	4	1	3	0	13
Sexual transmission	2	4	0	6	1	13
Medical procedure	1	5	0	2	1	9
Cosmetic procedure	3	0	0	5	0	8
Professional exposure	2	3	0	1	0	6

IVDU – intravenous drug user

The majority of HCV infections in IVDU group were caused by subtypes 3a (46 of 76 IVDU patients) and 1a (18 of 76 IVDU patients).

HCV infection in patients with a history of transfusion/blood product use (n=13 patients) were caused by subtypes 1a (n=5), 1b (n=4), 3a (n=3) and 2a/2c (n=1). HCV subtypes in patients with suspected sexual transmission of HCV (n=13 patients) were 3a (n=6), 1b (n=4), 1a (n=2) and 4 (n=1).

Discussion

This ten-year retrospective analysis on the distribution of HCV genotypes in 1163 patients with chronic hepatitis C in Croatia showed that the majority of infections were caused by genotypes 1 and 3. Genotypes 5 and 6 were not detected in Croatian HCV patients. Analysis of regional differences in the distribution of HCV genotypes revealed the predominance of genotype 3 in the Split region while the majority of infections in other regions were caused by subtype 1b. Infections with genotypes 2 and 4 were present in less than 5% of patients in all geographic regions. Analysis of an association between risk factors for infection and distribution of genotypes in a subset of patients from the Split region confirmed the association between IVDU and genotype 3.

Distribution of HCV genotypes worldwide is variable and often associated with specific risk factors for infection (for review see Simmonds et al, 2004)⁷. The commonest HCV subtype worldwide is subtype 1b whereas subtype 1a is also widely distributed in northern Europe and USA and is often associated with IVDU^{8,9–11}. Genotype 2 is predominantly found in older patients from Mediterranean countries and Far East^{12,13}. IVDU is an important risk factor for infection with genotypes 3 (in Europe) and 6 (in Asia)^{8,13–15}. Genotype 5 is widely distributed in Africa. Genotype 4 is often found in Middle East but can be found in other regions such as Belgium, Greece and France^{9,11,16,17}.

Countries of Central and Southern/South-Eastern Europe are characterised by the high prevalence of subtype 1b, particularly in the hyperendemic areas (for example

Central and Southern Italy)^{13,17–20}. Our results showed that, similarly to neighbouring countries, subtype 1b predominates in Croatian patients with chronic hepatitis C.

The second most frequent HCV genotype in our study population was genotype 3 that is often associated with IVDU. In Western Europe, HCV subtypes 1a and 3a predominate within IVDU groups but subtypes 4d and 2b have also entered this population as well^{8,21}. Recent reports described the spread of HCV genotype 4 in some areas of Europe, particularly south of Belgium¹⁶. Unlike in Western Europe, infections with genotypes 2 and 4 in Croatia remain uncommon.

Our analysis of HCV genotype regional distribution showed that the predominant subtype in three out of four Croatian regions is 1b. However, subtype 3a predominates in the Split region near the Adriatic coast. Analysis of genotype/subtype association with risk factors for infection in the subgroup of patients from the Split region confirmed the strong association between subtype 3a infection and IVDU.

The results of our current study are in concordance with the results of our previous report on the molecular epidemiology of HCV infection in Croatia that was based on the analysis of 203 anti-HCV positive patients⁶. Subtype 1b remained the predominant HCV subtype in Croatia, followed by subtypes 3a and 1a. Genotypes 2 and 4 remained a rare finding in Croatian HCV-infected patients. However, we observed an increase in the percentage of patients infected with HCV subtype 3a compared with the earlier study. Increasing percentages of subtype 3a infections will not only change the molecular epidemiology of HCV infection in Croatia but will have an important impact on the overall efficacy of antiviral therapy in our patients.

In conclusion, the prevalence of HCV genotypes/subtypes follows the pattern of other Southern and Eastern European Countries with the predominance of subtypes 1b, 3a and 1a. A retrospective 10 year analysis revealed increasing percentages of 3a infections and very low prevalence of genotypes 2 and 4 in Croatian HCV patients.

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DISTRIBUCIJA GENOTIPOVA HEPATITIS C VIRUSA U HRVATSKOJ: DESETOGODIŠNJE RETROSPEKTIVNO ISTRAŽIVANJE U ČETIRI REGIJE

SAŽETAK

Cilj ovog desetogodišnjeg retrospektivnog istraživanja bio je analizirati distribuciju genotipova HCV-a u bolesnika s kroničnim hepatitisom C koji su praćeni u najvećem centru za molekularnu dijagnostiku HCV infekcije u R. Hrvatskoj. U istraživanje smo uključili 1163 anti-HCV pozitivne odrasle osobe s mjerljivom HCV RNA u plazmi. Pacijenti su klasificirani u četiri regije: Zagreb i okolna kontinentalna područja, Split, Slavonija i Rijeka. Genotipovi HCV-a određivani su primjenom VERSANT HCV Genotyping Assay (LIPA) (Bayer Diagnostics, Puteaux Cedex, France). Rezultati su statistički obrađeni primjenom programa Statistica for Windows V. 5.1. Većina pacijenata uključenih u istraživanje bili su zaraženi genotipovima 1 (58,8% pacijenata) i 3 (35,6%). 37,4% pacijenata bilo je zaraženo genotipom 1b dok je infekcija genotipom 1a dokazana u 13,1% bolesnika. Broj bolesnika s genotipovima 2 i 4 (2,2% i 3,4%) bili su vrlo mali. U našoj skupini bolesnika nismo utvrdili genotipove 5 i 6. Analiza regionalne distribucije HCV genotipova pokazala je sličnu zastupljenost subtipova 3a i 1b u Splitu dok je u većine bolesnika iz drugih regija dominirala infekcija subtipom 1b. U svim analiziranim regijama genotipovi 2 i 4 bili su otkriveni u manje od 5% bolesnika. Analiza povezanosti rizičnih čimbenika za infekciju HCV-om i distribucije genotipova i subtipova u grupi bolesnika iz Splita potvrdila je povezanost između IVDU i subtipa 3a. Zaključujemo da je prevalencija genotipova i subtipova HCV-a u R. Hrvatskoj slična onoj u drugim zemljama južne i istočne Europe te da dominiraju subtipovi 1b, 3a i 1a.