

Prevalence of Hepatitis C Virus (HCV) Genotypes and Increase of Type 4 in Central Italy: An Update and Report of a New Method of HCV Genotyping

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Abstract. Hepatitis C virus (HCV) genotyping is very useful for identifying the patients (type 1 and 4) that need more aggressive management. In recent years, genotype 4 has shown spread in different parts of Europe. The aim of this study was to update on the prevalence of HCV genotypes of 288 patients in Central Italy, to analyze the possible increase of genotype 4 and to evaluate a new simple genotyping method. A line-probe assay (LiPA, Bayer) was used based on the reverse hybridization of HCV genome fragments previously amplified and biotinylated by a polymerase chain reaction (PCR) assay, COBAS System Amplicor HCV monitor version 2.0 (Roche) or previously amplified by COBAS Ampliprep/TaqMan HCV test (Roche). This last method uses non-biotin-labeled primers, therefore we added for each sample 10 µl of amplified HCV products, 10 µl of denaturation solution and 10 µl of biotinylated-nested primers (Bayer) to utilize the genotyping procedure previously used. The results showed that the prevalence of type 1, 2 and 3 (48.2, 34.6 and 10.5%, respectively) as well as the prevalent subtypes, 1b and 2a/2c (30.7 and 27.2%, respectively) were similar to previous data. Type 1 and 2 were statistically associated with an older group of patients when compared with type 3 and 4 ($p<0.001$). Type 3 and 4 showed a significant prevalence of male patients compared to type 1 and in particular to type 2 ($p<0.014$). The prevalence of type 4 was 5.6% in 2004, 6.1% in 2005 and 9.9% from January to July 2006. Type 4 showed an increase of male prevalence over a 3-year period ($p<0.001$). In conclusion, subtype 1b and 2a/2c showed a very similar prevalence, age and gender distribution in Central Italy. The type 4 patient group was analyzed because an increase of this genotype (1.8 times) was detected.

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Hepatitis C virus (HCV) has been classified into six major genotypes (1-6) and about 80 subtypes (1b, 2a/2c, etc.) (1). Genotypes 1, 2, and 3 are found throughout the world whereas the other genotypes are relatively restricted to certain geographic regions of Africa, Asia and the Middle East (2). Genotype 4 is prevalent in North and Central Africa (Egypt shows the highest HCV genotype 4 prevalence of 91%) (2) and among immigrants, indigenous injection drug users or hemophiliacs of North America and Europe (2, 3). In recent years genotype 4 has spread to parts of world where it was previously rare, such as Spain (11.6%) (4) and Greece (13.2% or 15.2%) (5, 6). In Italy the prevalence of type 4 has reached 4.7% in Southern Italy (7), 4.5% in Central Italy (8) and 5% in children (9).

HCV genotyping is very useful for optimizing the antiviral therapy because it helps to identify the patients who need more aggressive management. In fact type 1 and type 4 are more frequently associated with a poorer prognosis than type 2, 3 or 5 leading much more quickly to chronic hepatitis, to cirrhosis and to hepatocellular carcinoma (10, 11).

We have previously shown the prevalence of HCV types in Central Italy over a four-year period (1999-2003) (8). Type 1 was detected in approximately half of the cases followed by type 2 (35% out of all cases). Type 3 and type 4 were reported much less frequently (12% and 4%, respectively). In particular type 4 was not common in our country but it was noted that its prevalence seemed to have increased (from 3.3% to 4.5%) (8).

Improvements in molecular biology procedures are continually being made to increase sensitivity and where possible automate the extraction, amplification and detection of HCV genotypes thus reducing the long, dangerous and time-consuming HCV RNA analysis.

At the beginning of 2006 a new and simple method for detecting HCV genotypes was introduced to our laboratory. This new method does not produce biotinylated products and precludes the direct use of amplicons in the HCV genotyping assay which were previously utilized in our laboratory.

Table I. HCV genotypes in Central Italy in 2005.

Genotypes	Prevalence		Gender		Mean age
	%	n.	Male	Female	
1	48.3	110	52%	48%	55.5 (19-85)
2	35.1	80	38%	62%	67.8 (25-87)
3	10.5	24	71%	29%	43.7 (33-56)
4	6.1	14	71%	29%	49.9 (38-71)
Total Cases		228			

p<0.001 (genotypes vs. mean age); p<0.014 (genotypes vs. gender).

The aim of this study was to update information on the prevalence of HCV types in Central Italy focusing on the possible increase of genotype 4. Additionally the results with the new method of detecting HCV genotypes are reported.

Patients and Methods

From January to December 2005, 370 HCV positive or borderline patients from Central Italy who attended the S.Giovanni-Addolorato-Calvary Hospital, Rome, as outpatients or inpatients were studied for HCV genotyping. Of these 228 patients (61.6%) showed a HCV positive viral load, an essential condition for identifying the HCV genotypes. The patients were 19 to 87 years old (mean age 58±15.45 years); 115 were men (50.4%) and 113 were women (49.6%). Moreover two other periods (from January to December 2004 and from January to July 2006) were considered in order to analyze the possible changes in the prevalence of genotype 4.

A line-probe assay (Versant HCV Genotype Assay LiPA HCV, Bayer, Tarrytown, NY, USA) was used based on the reverse hybridization of HCV genome fragments, previously amplified, to genotype-specific probes embedded on a nitrocellulose strip, followed by colorimetric revelation of the hybrids. The LiPA assay identifies the six HCV types and their most common subtypes (1a, 1b, 1a/1b, 1, 2a/2c, 2b, 2, 3a, 3b, 3c, 3, 4a, 4b, 4c/4d, 4e, 4f, 4h, 4, 5a, 6a, and 10a).

The HCV amplicons (244 bp), within the highly conserved 5'-untranslated region of the HCV genome, were obtained by a polymerase chain reaction (PCR) assay, COBAS System Amplicor HCV monitor version 2.0 (Roche Diagnostics Systems Inc., Branchburg, NJ, USA) or by a COBAS Ampliprep/COBAS TaqMan 48 HCV Analyzer (Roche Diagnostics Systems Inc.) (the linear range of quantification of these methods was: 600-700,000 IU/ml and 15-69,000,000 IU/ml, respectively). The COBAS Ampliprep/COBAS TaqMan HCV test was introduced in our laboratory in 2006.

RNA extraction and PRC amplification. The procedure was performed according to the manufacturer's instructions. Whole-blood specimens were collected from patients in sterile tubes using anticoagulant EDTA. After no more than four hours at room temperature, serum was separated by centrifugation (3,500 rpm for 10 min), placed in sterile tubes and frozen at -20°C. After having thawed and vortexed the serum, the HCV-RNA was manually

Table II. HCV subtypes in Central Italy in 2005.

Subtypes	Prevalence		Gender		Mean age
	%	n.	Male	Female	
1b	30.6	70	37%	63%	61.3 (24-85)
2a / 2c	27.1	62	37%	63%	67.2 (25-86)
1*	17.1	39	77%	23%	45.2 (19-72)
3a	10.5	24	71%	29%	43.7 (33-56)
2*	7.9	18	41%	59%	70.3 (45-87)
4c / 4d	3.0	7	57%	43%	52.7 (38-67)
4*	3.0	7	86%	14%	47.1 (42-65)
1a	0.4	1	/	/	/
Co-infection	0.4	1	/	/	/
Total Cases		229			

*Other cases not further subclassified.

extracted following a method previously reported for the COBAS System Amplicor HCV monitor 2.0 (8). The specimen preparation was semi-automated using the COBAS AmpliPrep/COBAS TaqMan 48 Analyzer and processed 850 µl of plasma utilizing a silica-based capture technique. The processed specimens were added automatically to the amplification mixture and transferred manually to the COBAS TaqMan 48 Analyzer which allowed both reverse transcription and PCR amplification to occur simultaneously with real-time detection of the amplicons. HCV positive and negative controls were included each time a test was performed.

Genotyping. The HCV genotypes were obtained as previously described with the Versant HCV Genotype Assay LiPA (Bayer) (8). In this study the various steps of HCV genotyping were performed by the Auto-LiPA (Bayer), a fully automated system for complete genotyping. In the case of the amplicons produced by the COBAS Ampliprep/COBAS TaqMan 48, which uses non-biotin-labeled primers, 10 µl of the amplified HCV products, 10 µl of denaturation solution and 10 µl of biotinylated-nested primers (Versant HCV Genotype Amplification Kit LiPA, Bayer) were added according to Beld *et al.* method (12). The specimens were mixed and immediately used for the detection of genotypes following the automated method with AutoLiPA, reported above.

Statistical analysis. Statistical analyses were performed using the SPSS and Epi Info software packages (SPSS for Windows, version 7.5, and Epi Info 6). Frequency tables were analysed using the Chi-square test, with the Pearson correlation being used to assess the significance of the correlation between the categorical variables. Differences in the means of age in the genotypes and subtypes were analysed using Student's *t* and ANOVA (analysis of variance) tests. In all tests, *p*-values <0.05 were regarded as statistically significant.

Results

The distributions of the genotypes among the patients in 2005 is reported in Table I, and Table II shows the results of

Table III. Genotype 4 over a 3-year period (2004-2006).

Years	Prevalence		Gender		Mean age
	%	n.	Male	Female	
2004	5.6	15 / 267	53%	47%	52.1 (33-78)
2005	6.1	14 / 228	71%	29%	49.9 (38-71)
2006*	9.9	15 / 151	73%	27%	45.3 (35-65)

*From January to July; $p < 0.001$ (3-year period vs. gender).

the HCV subtypes for the same time period. The type 3 cases are completely represented by subtype 3a.

From 2004 to 2006, 23 of the type 4 cases (52.3%) showed the subtype 4c/4d, 20 cases (45.4%) type 4* not further classified and one case (2.3%) subtype 4e (a young woman injection drug user). The subtype 4c/4d (mean viral load: 1,742,449 IU/ml) included 14 men (60.9%) and nine women (39.1%), three human immunodeficiency virus (HIV) infected patients (13.0%) and one African patient (4.3%). The unclassified type 4* cases (mean viral load: 334, 380 IU/ml) included 15 men (75.0%) and five women (25.0%), nine HIV patients (45.0%) and six African patients (30.0%).

The prevalence of genotype 4 over a three-year period (2004-2006) is reported in Table III.

No differences were detected between the non-biotinylated amplicons at room temperature (immediately or after one night) and those frozen at -20°C for some days. However, amplicons, denaturation solution and nested primers were always mixed immediately before the beginning of the different steps of HCV genotyping.

Discussion

In recent years the type 1 HCV genotype (48.2% of cases) was prevalent in Central Italy, in particular subtype 1b (30.7% of cases) and type 2 (34.6% of cases), almost completely represented of the subtype 2a/2c (27.2% of cases), were the next most common. The subtypes 1b and 2a/2c had similar distributions of prevalence, sex and mean age (see Table II) nevertheless they demonstrated different responses to antiviral therapy. Women were prevalent (67%) in these two subtypes and they were of an older age than patients in the other subtypes. Other studies have also reported that subtypes 1b and 2a/2c were associated with older age (6, 7, 13). Moreover, a statistically significant correlation between type and sex ($p < 0.014$) was demonstrated.

Our results confirm the data of Ansaldi *et al.* that suggest two transmission patterns co-existent in Italy (13). "The first pattern is characterized by infections with subtypes 1b and 2c, mainly in adults older than 60 years consistent with

health care-related practices in the past. The second is characterized by subtype 3 and 4 infections, mainly found in the 31-60 years age group, consistent with intravenous drug use and immigration" (13). The epidemiological changes due to decreased iatrogenic transmission and blood products transfusion can be observed in children and young adults (9, 14). Types 1 and 2 are still prevalent in adolescents and young adults in Italy, but types 3 and 4 are rapidly increasing among children (9). Moreover in Europe two epidemics of HCV subtypes linked to intravenous drug use have been observed, the first encompassed subtypes 1a and 3a (4, 14, 15) and the second was more recent and involves the spread of type 4 as detailed below. In our experience subtype 1a was rare but subtype 1* (not further classified) represented the third largest group in our study (17.1%) and seemed to be very similar to subtype 3a regarding distribution of sex and age (Table II). In our opinion subtype 1* corresponds to 1a of the literature and the difference of subclassification depends on the interpretation of positive lines on the strips of the HCV genotyping assay.

In Central Italy genotype 4 has increased three fold from 1999 to 2006 (3.3% from 1999 to 2002 and 9.9% in 2006) (8) and 1.8 fold from 2004 to 2006 (Table III). Hayashi *et al.* (3) have reported that genotype 4 (in particular subtype 4a) may be present in Japanese and Italian hemophiliacs who received clotting factor from foreign countries. The type 4 genotype was represented mainly by two subtypes, 4c/4d and 4*. These two subtypes seem to be different but further studies with a large number of patients are required to verify these categories. In fact subtype 4* encompassed HIV positive patients (mainly injection drug users or homosexuals) in almost half of cases, African patients in 30% of cases and it demonstrated a younger mean age than subtype 4c/4d (47.1 vs. 52.7 years). Moreover, the mean viral load was very different between the two subtypes of type 4. These subtypes may have different epidemiological sources as well as intravenous drug use, sexual transmission and immigration for subtype 4* or endemic small areas for subtype 4c/4d (7, 13). In fact subtype 4* showed the major increase in recent years in Central Italy (in our previous study, 88% of type 4 cases were subtype 4c/4d, in the present study they accounted for 52% of cases) (8).

Co-infection with more than one HCV genotype was confirmed to be a rare event in Italy (0.4% of cases, Table II). Moreover genotype 5 and 6 were not detected.

To our knowledge, the present study was the first to detect the HCV genotype both by COBAS Ampliprep/TaqMan 48 HCV amplicons and with the HCV genotyping assay Lipa without an additional amplification step, according to Beld *et al.* method (12). In our hospital laboratory practice real-time PCR was introduced for its accuracy, rapidity and the advantage of semi-automation in

analyzing the viral target sequences (16) and proved to be highly compatible with HCV genotyping.

In conclusion, further studies with large numbers of patients are required to evaluate the recent epidemic of HCV including the spread of genotype 4 and to examine the epidemiological characteristics of its subtypes. Moreover new efforts should be made to enhance real-time PCR and to determine a more accurate test for the identification of rare HCV genotypes and subtypes.

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