A comparative evaluation between real time Roche COBAS® TAQMAN 48 HCV and bDNA Bayer Versant® HCV 3.0

Cristina Giraldi[1], Alessandra Noto[1], Robert Tenuta[1], Francesca Greco[1], Daniela Perugini[1], Mario Spadafora[1], Anna Maria Lo Bianco[1], Olga Savino[1], Alfonso Natale[2]

[1]UOC Microbiologia e Virologia, PO "Annunziata" AO Cosenza;
[2]Bayer Diagnostics Italia

SUMMARY

The HCV virus is a common human pathogen made of a single stranded RNA genome with 9600nt. This work compared two different commercial methods used for HCV viral load, the bDNA Bayer Versant® HCV 3.0 and the RealTime Roche COBAS® TaqMan 48 HCV. We compared the reproducibility and linearity of the two methods. Seventy-five plasma samples with genotypes 1 to 4, which represent the population (45% genotype 1; 24% genotype 2; 13% genotype 3; 18% genotype 4) were directly processed with the Versant® method based upon signal amplification; the same samples were first extracted (COBAS Ampliprep - TNAI) and then amplified using RealTime PCR (COBAS® TaqMan 48).
The results obtained indicate the same performance for both methods if they have genotype 1, but in samples with genotypes 2, 3 and 4 the RealTime PCR Roche method gave an underestimation in respect to the Bayer bDNA assay.

KEY WORDS: Xxxxxxxxxxxxxxxxxx

Received February 8, 2006 *Accepted* August 4, 2006

INTRODUCTION

The Hepatitis C Virus (HCV) is a member of the flavivirus family and is a common human pathogen composed of a single-stranded RNA genome with approximately 9600 nucleotides. It is a blood-borne virus that enters the body through direct blood exposure. Via the bloodstream the HCV virus reaches the liver where the virus attacks cells in the liver, and replicates. HCV causes liver inflammation and kills liver cells. After a highly variable incubation period it causes an acute hepatitis which is usually asymptomatic. In the majority of cases (approximately 80%) the virus persists in the body because the infection does not clear up within six months, causing a progressive chronic infection of the liver Flamm, 2003. The disease progresses over a period of years, and may lead to serious liver damage, cirrhosis or liver cancer. Cirrhosis is a reason for liver transplants (Robertson *et al.,* 1998).
Phylogenetic analyses have divided all known HCV isolates into six groups, called clades, and into more than 70 subtypes (Pawlotsky, 2003, Simmonds, 1995, Simmonds, 1994, Simmonds *et al.,* 1993). Robertson *et al.* reorganized the different systems of nomenclature. They divided the genotypes as follows: genotypes 1, 2, 4 and 5 belong to clades 1, 2, 4 and 5 respectively, while genotypes 3 and 10 form clade 3 and genotype 6 together with genotypes 7, 8, 9 and 11 com-

Corresponding author
Cristina Giraldi
Laboratorio di Virologia e Microbiologia
P.O. Annunziata
Via F. Migliori, 1
87100 Cosenza, Italy
E-mail: gircri@virgilio.it

prise clade 6 Robertson *et al.,* 1998). The natural history of HCV infection can be followed by biomolecular tests which are necessary for the qualitative and quantitative evaluation of the viral genome and its genotype testing. Viral load tests measure the amount of HCV circulating in the blood. Therefore this assay is very important and must be performed on patients who will undergo medical treatment (Napoli *et al.*, 2003, Scotto *et al.,* 2005) at the baseline and during therapy to monitor its efficacy. The goal of this study was to compare two different methods used for the quantitative determination of HCV-RNA.

MATERIALS AND METHODS

This research was performed in the Microbiology and Virology laboratory of the Annunziata Hospital of Cosenza. Seventy-five plasma samples from patients infected with HCV whose genotypes were 1, 2, 3 and 4 were collected and analysed. The genotype was determined using the Bayer Versant® HCV Genotype (LIPA) method following kit instructions.
The samples were simultaneously processed with the bDNA method (bDNA Bayer Versant® HCV 3.0) and with the Real Time method (RT Roche COBAS® TaqMan 48 HCV).
The extraction process was performed with COBAS® AmpliPrep Total Nucleic Acid Isolation kit. Both methods were used to evaluate reproducible results and to see if the two methods were linear and correlated. While the correlation between the two methods involved all the samples tested, only a few samples were used to examine reproducibility and linearity. The reproducibility was evaluated with tests performed in quadruplicate on four samples with different genotypes (1b, 2c, 3a 4). To obtain a comparable result for linearity, three dilutions were made (10^{-1}, 10^{-2} and 10^{-3}) on the same four samples which represented the four genotypes considered. For both methods the instructions of the kits were followed.

Versant® HCV RNA 3.0 Assay (bDNA)

The VERSANT HCV RNA 3.0 Assay (bDNA) is a sandwich nucleic acid hybridization procedure for the direct quantitation of hepatitis C viral (HCV) RNA in human serum and plasma. After HCV genomic RNA is released from the virions, the RNA is captured to a microwell by a set of specific, synthetic oligonucleotide capture probes. A set of target probes hybridizes to both the viral RNA and the pre-amplifier probes. The capture probes and the target probes bind to the 5' untranslated and core regions of the HCV genome. The amplifier probe subsequently hybridizes to the preamplifier forming a branched DNA (bDNA) complex.
Multiple copies of an alkaline phosphatase (AP) labeled probe are then hybridized to this immobilized complex. Detection is achieved by incubating the AP-bound complex with a chemiluminescent substrate. Light emission is directly related to the amount of HCV RNA present in each sample, and results are recorded as relative light units (RLUs) by the analyzer. A standard curve is defined by light emission from standards containing known concentrations of recombinant single-stranded phage DNA. Concentrations of HCV RNA in specimens are determined from this standard curve.

COBAS® TaqMan HCV Test

The COBAS TaqMan HCV Test is based on three major processes:
1) specimen preparation to obtain HCV RNA;
2) automated reverse transcription of the target RNA to generate complementary DNA (cDNA);
3) simultaneous PCR amplification of target cDNA using HCV specific complementary primers, and detection of cleaved dual fluorescent dye-labeled oligonucleotide detection probes that permit quantitation of HCV target amplified product (amplicon) and HCV

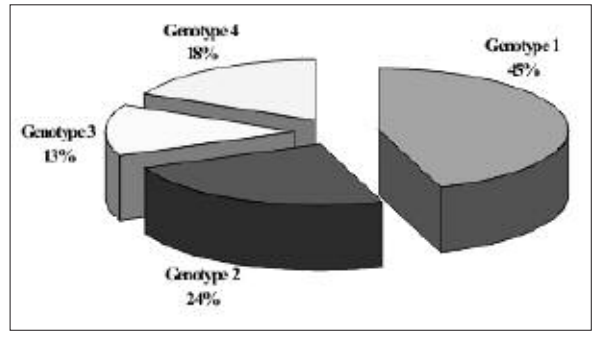

FIGURE 1 - *Genotype, local distribution.*

TABLE 1 - *Sample, results and relative genotypes*

#	*Genotype*	*Taqman (log IU/ml)*	*bDNA (log IU/ml)*	#	*Genotype*	*Taqman (log IU/ml)*	*bDNA (log IU/ml)*
54	4c/4d	4,35	5,11	1715	3a	4,54	5,14
79	4c/4d	3,77	4,46	1753	3a	4,29	4,88
113	2	5,31	5,21	1816	4	4,51	5,86
114	1b	5,61	6,47	1983	2	4,45	5,56
115	1b	5,66	5,11	2189	1b	3,73	4,91
125	2a/2c	4,73	4,95	2335	4	5,47	6,42
130	1b	6,59	6,11	2336	2	5,67	6,39
225	2c	5,45	5,78	2459	3a	5,85	5,85
279	2	6,34	5,69	2472	4	6,37	6,06
281	2a/2c	5,29	5,36	2531	3a	5,10	6,38
282	3a	4,97	6,19	2564	1b	5,24	6,42
300	4c/4d	4,96	5,36	2735	1b	5,43	6,47
301	2a/2c	6,30	6,01	2791	3a	5,43	6,39
313	2a/2c	6,23	6,11	2936	1b	4,69	5,53
374	2c	4,63	5,40	2985	1a	3,53	4,53
382	3a	5,80	5,58	2986	2	2,89	3,68
437	2c	6,07	5,67	2987	4	4,92	5,98
563	4	7,25	6,72	3018	4	5,36	5,97
648	1b	6,13	6,43	3097	4	5,16	5,92
730	4	5,29	6,57	3121	2c	5,37	5,98
900	1b	3,22	2,87	3418	1b	4,27	5,06
918	1b	3,60	3,92	3562	3	3,58	4,03
918	1b	3,60	3,92	3647	1b	2,98	3,24
932	1b	4,08	4,25	3654	3a	4,45	6,31
964	1b	4,06	4,25	3661	2a/2c	6,47	6,29
1081	1b	5,08	4,72	3683	1	6,43	6,32
1121	1b	4,91	5,44	3697	2a/2c	6,40	6,29
1170	1b	5,11	5,51	3708	1a	5,35	5,34
1183	4c/4d	5,91	5,56	3712	1b	4,35	4,43
1235	2c	6,59	5,78	3715	1b	3,50	3,45
1339	4	5,99	5,81	3723	1b	4,45	6,04
1348	2c	5,97	5,85	3729	2a/2c	4,89	6,09
1360	1b	5,35	5,91	3742	1b	4,69	6,09
1369	4	5,68	6,36	3758	1b	4,81	5,97
1632	4	6,19	6,75	3761	3a	3,99	5,13
1648	3a	4,95	5,04	3762	1b	3,08	4,22
1713	2c	5,87	5,43	3772	1b	2,52	3,22

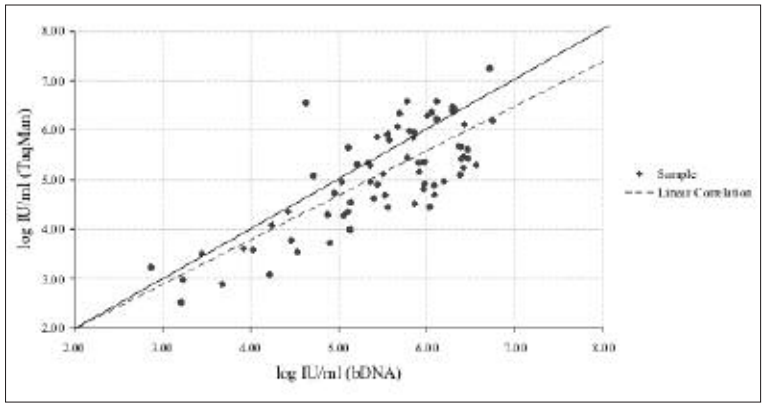

FIGURE 2 - *The graph shows the linear regression obtained from the correlation of the viral load results performed with bDNA Bayer and RT Roche. The equation of the straight line obtained is y= 0.9032x + 0.1668, and its correlation coefficient R2 is 0.611.*

Quantitation Standard RNA, which is processed, amplified, and detected simultaneously with the specimen.

Commercially available standards containing human plasma spiked with recombinant HCV phage DNA at different IU/ml were used (HCV RNA Test Panel; Bayer Corporation, 511 Benedict Avenue Tarrytown, NY 10591-5097 USA, 914 631-8000). We used four standards which were diluted by a factor of six on account of the need to have a sufficient amount of the sample to perform both methods. After dilution, we obtained the following standards: the first had 125.693 IU/ml, the second 8.126 IU/ml, the third 845 IU/ml and the last 211 IU/ml. The first and the second standards were tested in duplicate, the last two in single.

RESULTS

The data obtained for the genotypes show a characteristic local distribution with a prevalence for genotype 1. Of the patients tested we obtained genotype 1 in 45% of the samples, genotype 2 in 24%, genotype 3 in 13% and genotype 4 was

TABLE 2 - *Reproducible analysis*

#	*bDNA (log IU/ml)*	*Average and ΔS*	*Taqman (log IU/ml)*	*Average and ΔS*
1	6.31 6.29 6.32 6.29	6.30 ± 0.01	6.45 6.47 6.43 6.40	6.44 ± 0.03
2	6.38 6.42 6.47 6.39	6.42 ± 0.03	5.10 5.24 5.43 5.43	5.30 ± 0.14
3	5.98 5.97 5.92 5.98	5.96 ± 0.03	4.92 5.36 5.16 5.37	5.20 ± 0.18
4	6.04 6.09 6.09 5.97	6.04 ± 0.05	4.45 4.89 4.69 4.81	4.71 ± 0.16

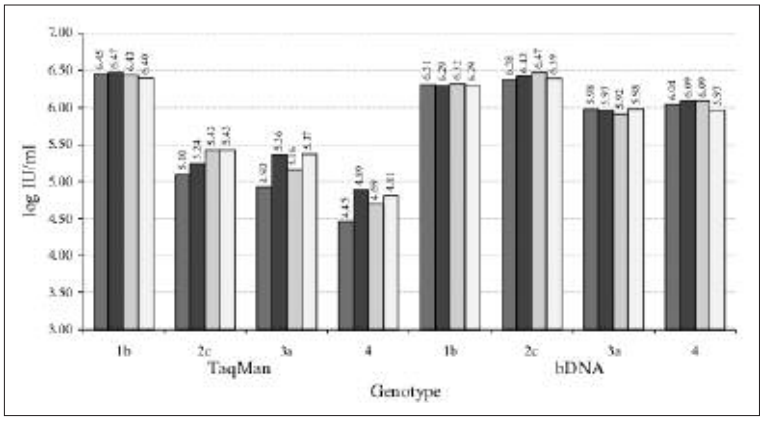

FIGURE 3 - *This graph shows the results of the intra-assay variability. The histograms indicate the value expressed in log IU/ml obtained in the tests performed in quadruplicate (represented by the four different colors) for the four samples chosen. On top of each histogram is the result obtained. On the left side of the graph the results obtained with TaqMan are shown while on the right side the results with bDNA are reported.*

present in 18% of patients (Fig.1). Table 1 reports the results of the seventy-five patients tested for genotype and viral load determination with both methods: bDNA Bayer Versant® HCV 3.0 and Real Time Roche COBAS® TaqMan 48 HCV. The two tests differ from each other for the type of amplification used. Branched DNA utilizes signal amplification, while the second method is based upon target amplification. The results of viral load are expressed in International Units/ml (log IU/ml) and were analysed and correlated by linear regression (Fig. 2). The correlation coefficient R^2 calculated upon seventy-five samples of plasma processed simultaneously with bDNA and Real Time was 0.611 with a slope of 0.903. We also used linear regression on four subgroups, obtained by dividing the samples by their genotype so that a genotype-dependent result could be obtained. For genotype 1, the correlation coefficient R^2 was 0.749, with a slope of 0.935; genotype 2 had an R^2 of 0.723 and a slope of 0.904; genotype 3 had an R^2 of 0.946 and a slope of 0.785 and last but not least genotype 4 had an R^2 of 0.645 with a slope of 0.829. The graphs depicting this analysis for each genotype have not been reported. After having chosen the

TABLE 3 - *Dilution factor*

Genotype	*Dilution*	*bDNA log (IU/ml)*	*Taqman (log IU/ml)*
1	-	6.30	4.44
	1:10	5.34	5.35
	1:100	4.43	4.35
	1:1000	3.45	3.50
2	-	6.42	5.30
	1:10	5.53	4.69
	1:100	4.53	3.53
	1:1000	3.68	2.89
3	-	5.96	5.20
	1:10	5.06	4.27
	1:100	4.03	3.58
	1:1000	3.24	2.98
4	-	6.04	4.71
	1:10	5.13	3.99
	1:100	4.22	3.08
	1:1000	3.22	2.52

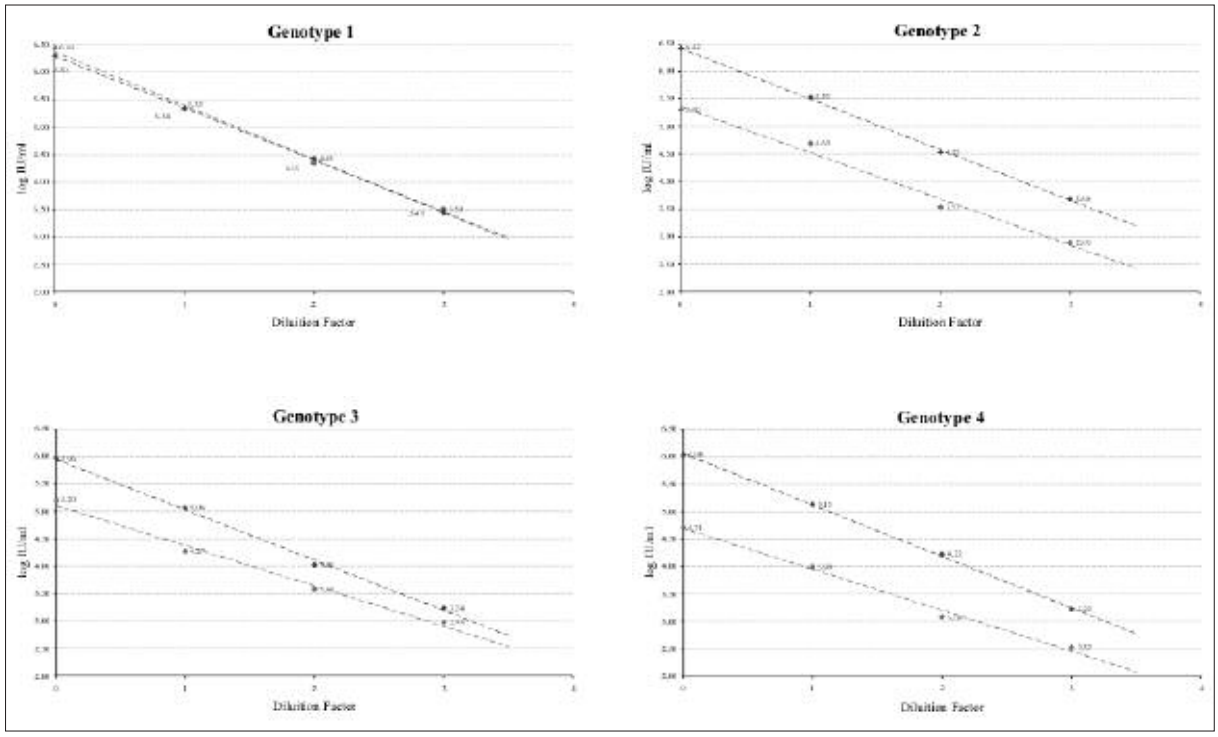

FIGURE 4 - *Dilution factor. Linearity assays performed on 4 samples. The 2 colors show the different methods used. Blue corresponds to bDNA (log IU/mL), while red shows the TaqMan assay.*

four samples representing the four genotypes found in our study (genotype 1, 2, 3 and 4), we performed a reproducible analysis of the methods. The same sample was processed independently four times to evaluate intra-assay variability with both methods. The results, obtained for the genotype 1 sample using bDNA, expressed in log IU/ml were 6.31-6.29-6.32-6.29 with an average standard deviation of the population (DS) equal to 6.30±0.01. With the TaqMan method the results were 6.45-6.47-6.43-6.40 with an average of 6.44±0.03. The genotype 2 sample gave the following results: 6.38-6.42-6.47-6.39 with an average of 6.42±0.03 and 5.10-5.24-5.43-5.43 with an average of 5.30±0.14 for bDNA and TaqMan. The results obtained with bDNA for the genotype 3 sample were 5.98-5.97-5.92-5.98 with an average of 5.96±0.03 while with

TABLE 4 - *Evaluation of commercial standards*

		bDNA		*Taqman*	
Standard	*log Std*	*log IU/ml*	*CV (%)*	*log IU/ml*	*CV (%)*
Std 1	5.10 5.10	5.08 5.09	0.22	4.56 4.76	3.03
Std 2	3.91 3.91	3.44 3.45	0.29	4.43 4.12	5.13
Std 3	2.93	2.81	-	3.17	-
Std4	2.32	<2.79	-	2.34	-

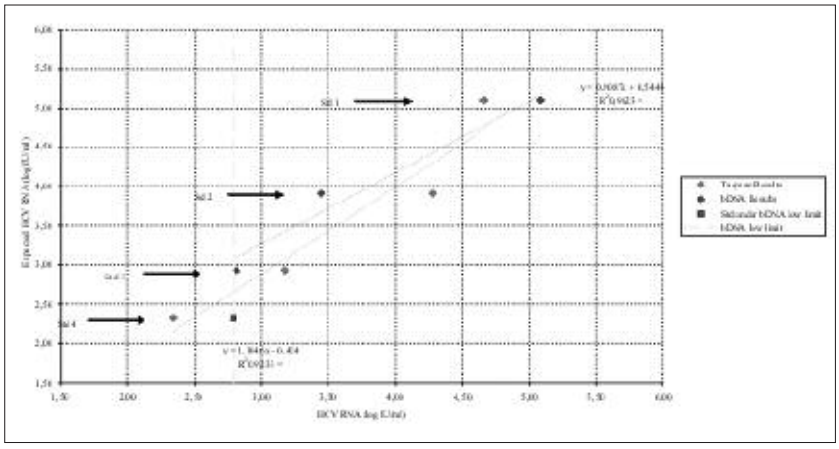

FIGURE 5 - *Plot of measured against expected log (IU/ml) of HCV RNA for the HCV standards for both methods. For the first two standards (1 and 2) the average obtained for the two values is reported. Linear regression for the values obtained with bDNA was calculated omitting the fourth standard because the expected value is less than the low limit of linearity of the method.*

TaqMan the results were 4.92-5.36-5.16-5.37 with an average of 5.20±0.18. The genotype 4 with bDNA gave the following results: 6.04-6.09-6.09-5.97 with an average of 6.04±0.05 and with TaqMan the results were 4.45-4.89-4.69-4.81 with an average of 4.71±0.16. These results are seen in table 2 and reported in figure 3.

Another parameter useful in evaluating the performance of a quantitative examination is linearity. To obtain this result, we used 4 samples representing the 4 genotypes analysed. The four samples were diluted 10^{-1}, 10^{-2} and 10^{-3}. The results have been inserted in table 3 and are reported in the following graphs (Fig. 4).

Moreover we tested the six standards to evaluate the performance of the two methods with a commercial panel of HCV. The results reported in table 4 indicate the concentration values expressed in log IU/ml. We obtained percentage values of CV for the first two standards tested in duplicate. bDNA gave a range of 0.22-0.29 while Taqman had a range between 3.03 and 5.13. The measured versus expected values are plotted in figure 5.

DISCUSSION

By analysing the general correlation obtained comparing both methods (Fig. 2) and looking at the pattern of linear regression we can deduce that when viral load increases the TaqMan method underestimates the result with respect to the result obtained by bDNA. We noted that the underestimation is concentrated on the samples with genotypes 2, 3 and 4 (Fig. 6). We previously saw that this data was confirmed by the reproducibility and linearity testing (Figs. 3 and 4). Moreover the analyses regarding the reproducibility and linearity of the tests demonstrated that while for genotype 1 we obtained an excellent performance with both methods, for geno-

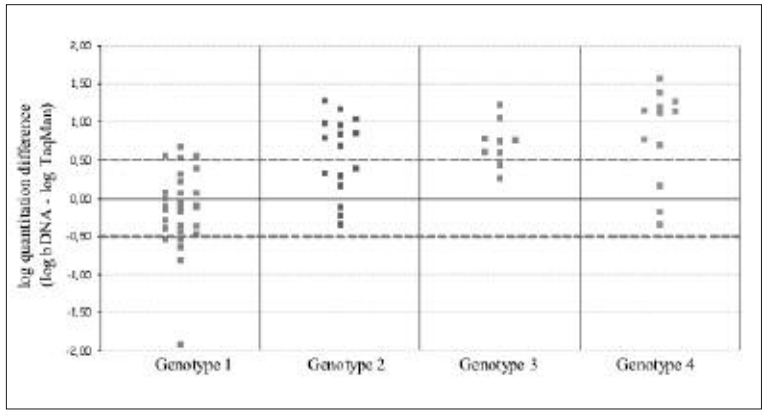

FIGURE 6 - *Difference of quantitative analysis. The graph was obtained analysing for each genotype the difference between bDNA and TaqMan results for viral load determinations.*

types 2, 3 and 4 the TaqMan method gives a greater variability in results. Instead, bDNA produces constant results. The results obtained in our laboratory confirm the results presented by Marcel Beld in November 2004 during the AASLD in Boston.

REFERENCES

Flamm SL. (2003). Chronic hepatitis C virus infection. *Journal of the American Medical Association,* **289**: 2413-2417.

Napoli, N., Giannelli, G., Parisi, C.V., Antonaci, A., Maddalena, G. and Antonaci, S. (2005). Predictive value of early virological response to treatment with different interferon-based regimens plus ribavirin in patients with chronic hepatitis C. *New Microbiologica,* **28** (1): 13-21.

Pawlotsky, J.M. (2003). Hepatitis C virus genetic variability: pathogenic and clinical implications. *Clinical Liver Disease,* **7**: 45-66.

Robertson, B., Myers, G., Howard, C., Brettin, T., Bukh, J., Gaschen, B., Gojobori, T., Maertens, G., Mizokami, M., Nainan, O., Netesov, S., Nishioka, K., Shin, T., Simmonds, P., Smith, D., Stuyver, L. and Weiner, A. (1998). Classification, nomenclature, and database development for hepatitis C virus (HCV) and related viruses: proposals for standardization. International Committee on Virus Taxonomy. *Archives in Virology,* **143:** 2493-2503.

Scotto, G., Fazio, V., Palumbo, E., Cibelli, D.C., Saracino, A. and Angarano, G. (2005). Treatment of genotype 1b HCV-related chronic hepatitis: efficacy and toxicity of three different interferon alfa-2b/ribavirin combined regimens in naive patients**.** *New Microbiologica*, **28** (1): 23-9.

Seeff, L.B. and Hoofnagle, J.H. (2003). Appendix: The National Institutes of Health Consensus Development Conference Management of Hepatitis C 2002. *Clinical Liver Disease,* **7**: 261-287.

Simmonds, P. (1995). Variability of hepatitis C virus. *Hepatology,* **21**: 570-583.

Simmonds, P., Alberti, A., Alter, H.J., Bonino, F., Bradley, D.W., Brechot, C., Brouwer, J.T., Chan, S.W., Chayama, K., Chen, D.S., Choo, Q.L., Colombo, M., Cuypers, H.T.M., Date, T., Dusheiko, G.M. and Esteban, J.I. (1994). A proposed system for the nomenclature of hepatitis C viral genotypes. *Hepatology,* **19**: 1321-1324.

Simmonds, P., Holmes, E.C., Cha, T.A., Chan, S.W., McOmish, F., Irvine, B., Beall, E., Yap, P.L., Kolberg, J. and Urdea, M.S. (1993). Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. *Journal of General Virology,* **74** (Pt 11): 2391-2399.