Characterization of hepatitis C virus isolates

from chronically infected patients

Karakterisering van hepatitis C virus isolaten

bij chronisch geïnfecteerde patiënten

PROEFSCHRIFT

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Voor mijn ouders
Aan Inge en Miriam

Abbreviations

aa	amino acids
ALT	alanine aminotransferase
bp	basepairs
DNA	deoxyribonucleic acid
cDNA	complementary DNA
CAH	chronic active hepatitis
CPH	chronic persistent hepatitis
E	envelop
EIA	enzyme immunoassay
ELISA	enzyme linked immunosorbent assay
h	hour
HAV	hepatitis A virus
HBV	hepatitis B virus
HCV	hepatitis C virus
HDV	hepatitis D virus
HEV	hepatitis E virus
IFN	interferon
IRES	internal ribosomal entry site
IV	intravenous
LIA	line immuno assay
LiPA	line probe assay
LCR	ligase chain reaction
min	minute
M-MLV	Moloney murineleukemia virus
NANBH	non-A, non-B hepatitis
NASBA	nucleic acid system based amplification
NS	non-structural
nt	nucleotides
NTP	nucleotide triphosphate
ORF	open reading frame
PCR	polymerase chain reaction
PT	post transfusion
RIBA	recombinant immunoblot assay
RNA	ribonucleic acid
RT	reverse transcriptase
TMACL	Tetramethyl ammoniumchloride
UTR	untranslated region

Nucleotide codes (IUPAC-IUB)

A	adinine
C	cytosine
G	guanine
T	thymine
Y	C or T
R	A or G
M	A or C
K	G or T
S	G or C
W	A or T
H	A, C, or T
B	G, T, or C
V	G, C, or A
D	G, A, or T
N	G, A, T, or C

"Ik ben blij en ben er trotsch op te weten,
dat ik niets weet ... "

Nescio

CONTENTS

CHAPTER 1

General Introduction

INTRODUCTION

The function of the liver is to keep the human body in physiological equilibrium. This equilibrium is regulated by several metabolic pathways such as the production of plasma proteins and detoxification. Inflammation of the liver is known as hepatitis and as entity it has been recognized since the days of Hippocrates. The most important etiology of hepatitis is viral infection of the liver.

Five distinct hepatitis viruses are known. The hepatitis A virus (HAV) is orally transmitted and liver disease has a sudden onset after a short incubation period with an average of 4 weeks. HAV is a small cubic RNA virus of 27nm and belongs to the family of picorna viruses. Hepatitis B virus (HBV) is parenterally transmitted and onset of liver disease is usually slow after a long incubation period with an average of 12 weeks. The HBV virion is a spherical particle of 42nm, contains a partially double stranded DNA genome and is classified as a Hepadnavirus [Tiollais et al., 1985]. Hepatitis Delta virus (HDV) has been recognized as a defective RNA virus in the presence of HBV antigens [Rizetto et al., 1983].

Besides HAV, HBV and HDV another form of viral infections of the liver, with an intermediate incubation period between HAV and HBV, was postulated in the early 1970's [Prince et al., 1974; Feinstone et al., 1975]. These infections were mainly associated with blood transfusion and therefore, provisionally designated as post-transfusion non-A, non-B hepatitis (PT-NANBH). The name, PT-NANBH, reflects that the diagnosis was based on exclusion of HAV and HBV, as well as other hepatotropic agents such as HDV, Cytomegalovirus, Epstein-Barr virus, Varicella Zoster virus and Yellow Fever virus.

NANBH could be transmitted enterically as well as parenterally. Recently, hepatitis E virus (HEV), the enterically form of NANBH, was characterized. HEV is an RNA virus and belongs to the Caliciviridae [Reyes et al., 1990].

From non-A, non-B to hepatitis C

After the recognition of parenterally transmitted NANBH as a distinct entity [Feinstone et al., 1975], many attempts were made to characterize the viral agent. Over 50 serological or virological assays have been developed in the period from 1975 to the late eighties. None of them proved to be specific for NANBH in well characterized coded test panels [Purcell et al., 1994].

Experimental infections of chimpanzees with contaminated sera [Alter et al., 1978] or clotting factor VIII preparations [Bradley, 1979 and 1981] were successful. After approximately a decade of studies in chimpanzees, obtained physico-chemical data resulted in the hypothesis that the causative agent might belong to the Togaviridae [Bradley et al., 1985]. Togaviruses are small enveloped viruses and contain a single stranded, positive sense RNA genome.

Plasma samples from a persistently infected chimpanzee [Bradley et al., 1985] were pooled and subsequently used for construction of a CDNA expression library. Expressed viral antigens were detected by serum from chronically infected PT-NANBH patients. The first clone, designated as clone 5-1-1, was found in 1987 [Houghton et al., 1990; Choo et al., 1989] and used as a probe for detection of overlapping clones covering the entire viral genome [Choo et al., 1991].

The hepatitis C virus genome

Sequence analysis of the HCV genome revealed that the genomic organization and hydrophobicity profile were similar to viruses belonging to the family of Flaviviridae. Therefore, HCV was classified as a separate genus within this family [Miller et al., 1990].

The HCV genome is a single-stranded, positive-sense RNA molecule of approximately 9,400 nucleotides (nt). It contains one large open reading frame (ORF) which encodes for a polyprotein of 3,010-3,033 amino acids [Kato et al., 1990; Takamizawa et al., 1991]. This polyprotein is cleaved into functional proteins by host and viral proteases. The N-terminal region encodes for the structural core and envelope proteins (E1 and E2/NS1). The C-terminal region encodes for the non-structural (NS) proteins which are involved in the replication

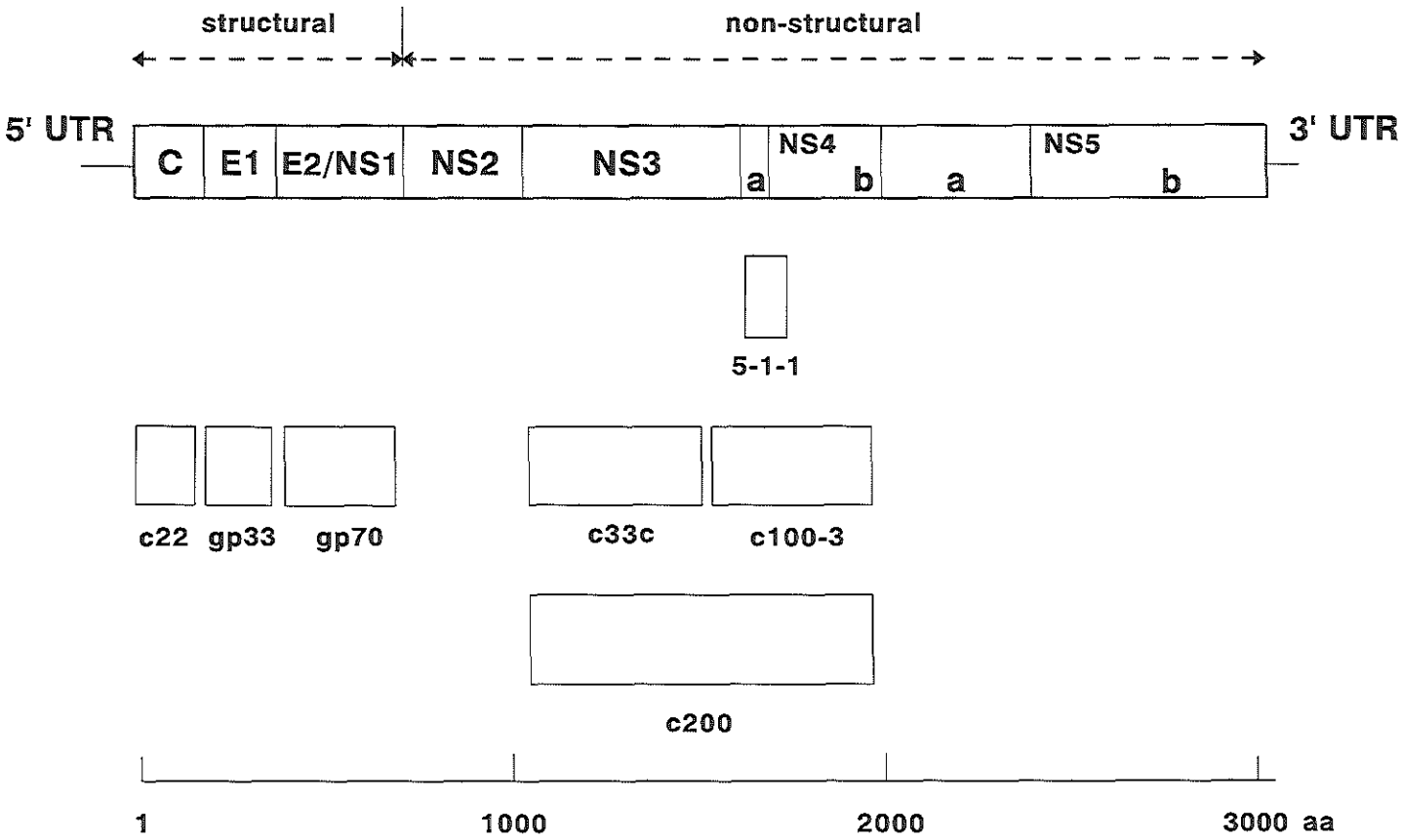


Figure 1. Outline of the HCV genome

cycle (Figure 1).

The RNA genome contains an untranslated region (UTR) at both ends. The 5'UTR is involved in initiation of translation [Han et al., 1991]. The putative secondary structure of this region has been determined and one of the stem-loop structures was characterized as an internal ribosomal entry site (IRES) [Brown et al., 1992; Tsukiyama-Kohara et al., 1992]. The 3'UTR appeared to be variable in length as well as sequence and this region is probably involved in termination of translation and initiation of replication [Kato et al., 1990; Choo et al., 1991, Han et al., 1991; Takamizawa et al., 1991; Okamoto et al., 1991 and 1992a].

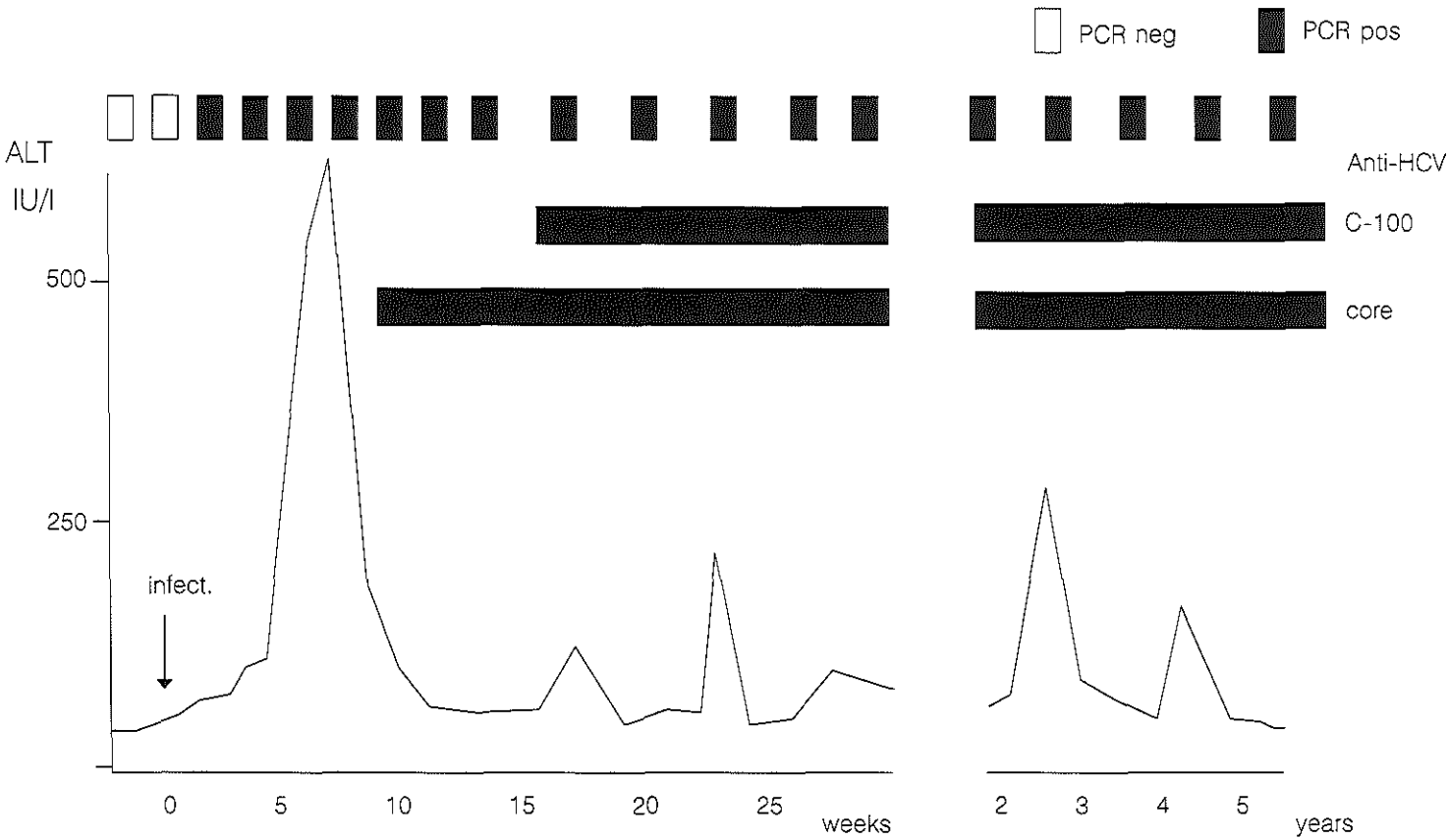
Clinical characteristics of HCV infection

In the acute phase of HCV infection the clinical symptoms are indistinguishable from HAV and HBV. Jaundice has been observed in approximately 10% of the infections [Miyamura et al., 1990; van der Poel et al., 1991a]. HCV RNA is detectable within the first week of infection and elevation of liver-specific enzymes such as alanine aminotransferase (ALT) is observed with an average 8 weeks peak after infection. The first HCV antibodies are directed to the core antigen and become detectable at the onset of ALT elevation. HCV C100-3 antibodies are usually found after the ALT peak (Figure 2). Approximately 80% of the HCV infected patients develop a chronic infection [Alter et al., 1989; van der Poel et al., Lancet 1991b; Gerber et al., 1993] with characteristic fluctuations of the ALT level. HCV has been recognized as a significant risk factor in the development of cirrhosis and liver cancer [Bruix et al., 1989; Colombo et al., 1989; Kew et al., 1990; Saito et al., 1990], although there is no evidence for direct oncogenic activity of HCV.

Diagnosis of HCV infection

After discovery of HCV, immunoassays became available for specific screening of HCV instead of diagnosis by exclusion. In the first generation anti-HCV assays the recombinant proteins 5-1-1 and C100-3, both derived from the NS4 region, were used in radio immunoassays (RIA) for the detection of HCV anti-

Figure 2. Clinical pattern chronic HCV infection



bodies. By this assay, HCV antibodies were detected in approximately 80% of chronically infected PT-NANBH patients and in 58% of those with no identifiable exposure to the virus [Kuo et al., 1989]. Additionally, in prospective PT-NANBH studies, 60-80% of the implicated blood donors were anti-HCV positive [van der Poel et al., 1990; Esteban et al., 1990; Alter et al., 1989]. These initial results suggested that the first generation assay could be improved.

The second and third generation screening assays were extended with recombinant antigens core (C22) and NS3 (C33c) and synthetic peptides derived from the NS5 region. These antigens were also used in a HCV specific recombinant immunoblot assay (RIBA; Ortho Diagnostics, Raritan, N.J., USA) for confirmation of anti-HCV screening results [Follett et al., 1991; van der Poel et al., 1991b; Nakatsuji et al., 1992; García-Samaniego et al., 1993]. Similarly, screening results can be confirmed by a Line Immuno Assay (LIA; Innogenetics NV, Ghent, Belgium) which contains several peptides derived from the structural regions core and E2 as well as the nonstructural regions NS3, NS4 and NS5.

In HCV diagnosis antibody assays can only provide evidence of previous exposure to the virus but are unable to detect actual viremia. Acute infections can only be detected by HCV antibody assays after a seronegative window [Farci et al., 1991] and antibodies are even absent in immuno-suppressed patients. Due to the high rate of chronicity of hepatitis C infection, elevated ALT levels together with detectable HCV antibodies suggest HCV viremia. However, both are indirect markers.

HCV RNA detection

In the absence of a tissue culture system and antigen assays, detection of the viral RNA genome seems to be the only marker for HCV viremia. Highly sensitive nucleotide amplification methods such as the polymerase chain reaction (PCR), Nucleic Acid System Based Amplification (NASBA) and Ligase Chain Reaction (LCR) allows detection of HCV RNA. Application of these nucleic acid based techniques require conserved viral sequences for development of universal assays. Since the 5'UTR is highly conserved, primers directed to this region were superior when compared to other regions [Bukh et al., 1992a]. HCV RNA

detection is performed to confirm an anti-HCV positive test result for actual viremia, in diagnosis of early HCV infections i.e. during the seronegative window and to monitor the effects of antiviral therapy. Prior to commercial available HCV viremia assays, many hepatitis C research centers have developed an "in-house" HCV RNA PCR assay. In order to evaluate the "in-house" developed assay two HCV proficiency panels have been provided [Zaaijer et al., 1993].

HCV sequence diversity

Mixed populations of closely related HCV RNA sequences, so called "quasi-species", have been observed in the same individual [Oshima et al., 1991; Okada et al., 1992; Tanaka et al., 1992]. Differences among HCV genomes in the same individual can be caused by replication errors in the growing RNA chain. The mutations in the HCV RNA sequence as in any other virus are tolerated if they do not disturb the virus life cycle. In the genetic drift of HCV, rates of nucleotide changes per site per year have been estimated over a relatively short time interval and varied from 1.44×10^{-3} (complete genome) [Okamoto et al., 1992b] to 1.92×10^{-3} (5' half of the genome) [Ogata et al., 1991]. About one third of the nt changes resulted in a different amino acid (aa). The mutation rate in the HCV genome is similar to that of other RNA viruses [Domingo et al., 1988].

The full-length HCV sequence of the chimpanzee isolate as characterized by Houghton and co-workers [Houghton et al., 1990] is known by several names: HCV prototype, HCV-US, HCV-1 and "American" strain. Comparison to full-length HCV sequences from Japanese patients [HCV-J, Kato et al., 1990; HCV-BK, Takamizawa et al., 1991] revealed 78-79% nt sequence homology with HCV-1 [Choo et al., 1991], while the homology among Japanese isolates was 92%. The isolates were therefore classified as the "American" and "Japanese" strain. Later obtained full-length HCV sequences from Japanese patients, HC-J6 and HC-J8 [Okamoto et al., 1991 and 1992a] appeared to be substantially distinct from HCV-1, HCV-J and HCV-BK [Okamoto et al., 1992a]. Similarly, sequence analysis of a part of the NS5 region revealed considerable

sequence variation between HCV isolates, suggesting the existence of at least 2 HCV types in Japan [Enomoto et al., 1990].

The awareness that HCV exists as a number of distinct types has led to sequence analysis of numerous HCV isolates from different geographical origins [Bukh et al., 1992b and 1993; Chan et al., 1992] and the development of fast HCV genotyping assays [Okamoto et al., 1992c; Stuyver et al., 1993].

Epidemiology

The prevalence of HCV infection as estimated by first generation antibody assays ranged from 0.2-1.7% in blood donors from the United States [Weiner et al., 1990], Europe [van der Poel et al., 1991a; Contreras et al., 1991; Kühnl et al., 1989; Janot et al., 1989; Esteban et al., 1990] and Japan [Miyamura et al., 1990]. After the introduction of more specific screening and confirmation assays, the estimated prevalence of HCV among blood donors in Scotland was lowered from 0.5% to 0.09% [Follett et al., 1991; Crawford et al., 1994]. Interestingly, an extraordinarily high rate of 20% anti-HCV positivity was observed in Egyptian volunteer blood donors. This antibody test result was confirmed by HCV RNA analysis [Saeed et al., 1991].

The parenteral route of HCV by transfusion of blood and by use of blood products is well-known. High seroprevalence rates have also been observed in intravenous drug abusers (IVDA), hemophiliacs and hemodialysis patients [Makris et al., 1990; Jeffers et al., 1990]. Transmission by needlestick injury, tattooing and even by a human bite have been reported [Abildgaard et al., 1991; Dusheiko et al., 1990]. HCV transmission among sexual partners was below 3% [Brettler et al., 1992] or even absent [Bresters et al., 1993]. Also, transmission from mother to child is exceptional [Inoue et al., 1991; Reinus et al., 1992; Wejstal et al., 1992; Lam et al., 1993]. However, vertical transmission of HCV occurs frequently in mothers who are HIV coinfected [Thaler et al., 1991; Novati et al., 1992].

Treatment

For treatment of chronic NANBH, corticosteroids did not appear to be beneficial

[Alter et al., 1984]. The availability of recombinant interferon (IFN) has allowed clinical trials of this antiviral and immuno-modulatory agent for treatment of viral infections such as chronic HBV [Sherlock et al., 1985]. A first attempt in treatment of chronic NANBH patients with recombinant IFN alpha-2b was made in 1986 [Hoofnagle et al., 1986]. Normalization in activity of liver disease, as estimated by ALT levels, was obtained in 8 of the 10 patients during treatment. Similar results were obtained by Thompson et al in a pilot study with lymphoblastoid alpha-interferon [Thompson et al., 1987].

These results were promising and have led to larger studies. Three randomized controlled studies confirmed that IFN alpha could reduce the activity (ALT) of liver disease to normal in about 50% of the patients with chronic NANBH [Jacyna et al., 1989; Di Bisceglie et al., 1989; Davis et al., 1989]. However, after cessation of treatment liver disease activity relapses in approximately 50% of the patients with an initial response. In fact only 25% of the patients remained in a biochemical remission after a 6 months IFN course. The effect of IFN alpha on HCV viremia has been addressed in more recent studies [Shindo et al., 1991; Bresters et al., 1992].

Benelux Study Group on treatment of chronic hepatitis C virus

In 1990, a multi center study on treatment of chronically infected HCV patients was initiated in Rotterdam (Prof. Dr. S.W. Schalm). Eighteen clinical centers from Belgium, the Netherlands and Luxembourg (Benelux) participated in a randomized controlled study. At the close of the study 354 patients had enrolled. The patients were either treated with interferon alpha 2B (Intron-A, Schering-Plough) according to a "Standard" schedule, 3MU 3 times a week for 24 weeks, or an "Experimental" schedule comprising a high-dose (6MU 3 times a week) induction phase of 8 weeks followed by a maintenance phase of titrated doses of IFN (3 and 1MU) till biochemical and virological remission has been obtained during the 1MU dose of IFN therapy. The experimental therapy was either also discontinued if ALT levels remained elevated after 12 weeks or HCV RNA was still detected after 1 year of treatment. In this thesis, blood samples from these patients were used for characterization of the hepatitis C virus.

Outline of the thesis

The major objective of this thesis was to characterize hepatitis C virus isolates from chronically infected patients and is discussed in three parts.

The first part focuses on the detection of HCV viremia. Chapter 2 describes the development of a universal HCV RNA PCR assay. The "in-house" HCV RNA assay has been evaluated in 2 Eurohep HCV proficiency panels. This HCV RNA test was developed for diagnostic purposes and in particular for endpoint determination of antiviral therapy with interferon alpha 2B. Chapter 3 describes the application of the HCV RNA assay to investigate the predictive value of HCV RNA detection at an early stage (week 4) during interferon treatment.

The second part of this thesis focuses on the characterization of HCV patient isolates. Classification of HCV isolates by sequence analysis of 5'UTR amplification products is described in Chapter 4. In Chapter 5 a fast 5'UTR genotyping method is evaluated by comparison to sequence data. The correlation between HCV types and antibody profiles to HCV type 1 epitopes is also discussed. Whether sequence variation in the 5'UTR is sufficient to discriminate between the different viral types is described in Chapter 6 by sequence analysis of the core and E1 regions. Chapter 7 compares the classifications of HCV isolates by 2 fast genotyping methods.

The third part of this thesis, in Chapter 8, describes the relationship between the HCV (sub)types and demographical, epidemiological and pathobiological parameters in order to assess the relevance of HCV genotyping.

Finally, the overall results and conclusions of this thesis are discussed and summarized in Chapter 9.

REFERENCES

Abildgaard N, Peterslund NA (1991): Hepatitis C transmitted by tattooing needle. Lancet 338:460.

Alter HJ, Purcell RH, Holland PV, et al., (1978): Transmissible agent in non-A, non-B hepatitis. Lancet 1:459-462.

Alter, H.J., and J.H. Hoofnagle. 1984. Non-A, non-B: observations on the first decade. In: Vyas G.N., Dienstag J.L. Hoofnagle J.H. Eds. Viral Hepatits and liver disease. Orlando, Fla.:Grune & Stratton, 345-355.

Alter HJ, Purcell RH, Shih JW, Melpolder JC, Houghton M, Choo Q-L, Kuo G (1989): Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A non-B hepatitis. New England Journal of Medicine 321:1494-1500.

Bradley DW, Cook EH, Maynard JE McCaustland KE, Ebert JW, Dolana GH, Petzel RA, Kantor RJ, Heilbrunn A, Fields HA, Murphy BL (1979): Experimental infection of chimpanzees with anti-hemophilic (Factor VIII) materials: recovery of virus-like particles associated with non-A, non-B hepatitis. Journal of Medical Virology 3:253-269.

Bradley DW, Maynard JE, Popper H, Ebert JW, Cook EH, Fields HA, Kemler BJ (1981): Persistent non-A, non-B hepatitis in experimentally infected chimpanzees. Journal of Infectious Diseases 143,2:210-218.

Bradley DW, McCaustland KA, Cook EH, Schable CA, Ebert JW, Maynard JE (1985): Post-transfusion non-A, non-B hepatitis in chimpanzees. Physicochemical evidence that the tubule-forming agent is a small, enveloped virus. Gastroenterology 88:773-779.

Bresters D, Mauser-Bunschoten EP, Cuypers HTM, Lelie PN, Han JH, Jansen PLM, Houghton M, Reesink HW (1992): Disappearance of hepatitis C virus RNA in plasma during interferon alpha-2B treatment in hemophilia patients. Scandinavian Journal of Gastroenterology 27:166-168.

Bresters D, Mauser-Bunschoten EP, Reesink HW, Roosendaal G, Van der Poel CL, Chamuleau RAFM, Jansen PLM, Weegink CJ, Cuypers HTM, Lelei PN, van den Berg HM (1993): Sexual transmission of hepatitis C virus. Lancet 342: 210-211.

Brettler DB, Mannucci PM, Gringeri A, Rasko JE, Forsberg AD, Rumi MG, Garcia RJ, Rickard KA, Colombo M (1992): The low risk of hepatitis C virus transmission among sexual partners of hepatits C-infected hemophilic males: an international, multicenter study. Blood 80:540-543.

Brown EA, Zhang H, Ping L-H, Lemon SM (1992): Secondary structure of the 5' nontranslated regions of hepatitis C virus and Pestivirus genomic RNAs. Nucleic Acids Research 20:5041-5045.

Bruix J, Barrera JM, Calvet X, Ercilla G, Costa J, Sanchez-Tapias JM, Ventura M, Vall M, Brugeuera M, Bru C, Castillo R, Rodes J (1989): Prevalence of antibodies to hepatitis C virus in Spanish patients with hepatocellular carcinoma and hepatic cirrhosis. Lancet ii:1004-1006.

Bukh J (1992a): Importance of primers selection for the detection of hepatitis C virus RNA with the polymerase chain reaction assay. Proceedings of the National Academy of Sciences USA 89:187-191.

Bukh J, Purcell RH, Miller RH (1992b): Sequence analysis of the 5' noncoding region of hepatitis C virus. Proceedings of the National Academy of Sciences USA 89:4942-4946.

Bukh J, Purcell RH, and Miller RH. (1993). At least 12 genotypes of hepatitis C virus predicted by sequence analysis of the putative E1 gene of isolates collected worldwide. Proceedings of the National Academy of Sciences USA, 90: 8234-8238.

Chan S-W, McOmish F, Holmes EC, Dow B, Peutherer JF, Follett E, Yap PL, Simmonds P (1992): Analysis of a new hepatitis C virus type and its phylogenetic relationship to existing variants. Journal of General Virology 73:1131-1141.

Choo Q-L, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M (1989): Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 244:359-362.

Choo Q-L, Richman KH, Han JH, Berger K, Lee C, Dong C, Gallegos C et al. (1991): Genetic organization and diversity of the hepatitis C virus. Proceedings of the National Academy of Sciences USA, 88:2451-2455.

Colombo M, Kuo G, Choo Q-L, Donato MF, Del Nino E, Tommasini MA, Dioguardi N, Houghton M (1989): Prevalence of antibodies to hepatitis C virus in Italian patients with hepatocellular carcinoma. Lancet ii:1006-1008.

Contreras M, Barbara JAJ, Anderson CC, Ranasinghe E, Moore C, Brennan MT, Howell DR, Aloysius S, Yardumian A (1991): Low incidence of non-A, non-B post transfusion hepatitis in London confirmed by hepatitis C virus serology. Lancet 337:753-757.

Crawford RJ, Gillon J, Yap PL, Brookes E, McOmish F, Simmonds P, Dow BC, Follett EAC (1994): Prevalence and epidemiological characteristics of hepatitis C in Scottish blood donors. Transfusion Medicine 4:121-124.

Davis GL, Balart LA, Schiff ER, Lindsay K, Bodenheimer HC, Perrillo RP, Carey W, Jacobson IM, Payne J, Dienstag JL, VanThiel DH, Tamburro C, Lefkowitch J, Albrecht J, Meschievitz C, Ortego TJ, Gibas A and The Hepatitis Interventional Therapy Group (1989): Treatment of chronic hepatitis C with recombinant interferon alfa: a multicenter randomized, controlled trial. New England Journal of Medicine 321:1501-1506.

Di Bisceglie AM, Martin P, Kassianides C, Lisker-Melman M, Murray L, Waggonar J, Goodman Z, Banks SM, Hoofnagle J (1989): Recombinant interferon alfa therapy for chronic hepatitis C: a randomized, double-blind, placebo-controlled trial. New England Journal of Medicine 321:1506-1510.

Domingo, E., and J.J. Holland. 1988. In: RNA genetics, Eds. Domingo, E., Holland, J.J. and Ahlquist, P. (CRC, Boca Raton, FL), vol 3, pp.3-36.

Dusheiko GM, Smith M, Scheuer PJ (1990): Hepatitis C virus transmitted by a human bite.

Lancet 336:503-504.

Enomoto N, Takada A, Nakao T, Date T (1990): There are two major type of hepatitis C virus in Japan. Biochemical and Biophysical Research Communications 170, 3:1021-1025.

Esteban JI, González A, Hernández JM, Viladomiu L, Sánchez C, López-Talavera JC, Lucea D, Martin-Vega C, Vidal X, Esteba R, Guardia J (1990): Evaluation of antibodies to hepatitis C virus in a study of transfusion associated hepatitis. New England Journal of Medicine 323:1107-1112.

Farci P, Alter HJ, Wong DC et al. (1991): A long-term study of hepatitis C virus replication in non-A, non-B hepatitis. New England Journal of Medicicne 325:98-104.

Feinstone SM, Kapikian AZ, Purcell RH, et al (1975): Transfusion-associated hepatitis not due to viral hepatitis type A or B. New England Journal of Medicine 292:767-770.

Follett EAC, Dow BC, McOmish F, Yap PL, Hughes W, Mitchell R, Simmonds P (1991): HCV confirmatory testing of blood donors. Lancet 338:1024.

García-Samaniego J, Enríquez A, Soriano V, Baquero M, Munoz F (1993): Third-generation recombinant immunoblot assay to confirm hepatitis C virus-indeterminate serological samples. Vox Sanguinis 64:191-192.

Gerber MA (1993): Relation of hepatitis C virus to hepatocellular carcinoma. Journal of Hepatology 17:S108-S111.

Han JH, Shyamala V, Richman KH, Brauer MJ, Irvine B, Urdea MS, Tekamp-Olsen P et al. (1991): Characterization of the terminal regions of hepatitis C virus RNA: identification of conserved sequences in the 5' untranslated region and poly(A) tails at the 3' end. Proceedings of the National Academy of Sciences USA 88:1711-1715.

Hoofnagle JH, Mullen KD, Jones DB, Rustgi V, Di Biseglie A, Peters M, Waggoner JG, Park Y, Jones EA (1986): Treatment of chronic non-A, non-B hepatitis with recombinant human alpha interferon. A preliminary report. New England Journal of Medicine 315:1575-1578.

Houghton M, Choo Q-L, Kuo G (1990): European patent application appl. no. 88310922.5

Inoue Y, Takeuchi K, Chou WH, Unayama T, Takahashi K, Saito I, Miyamura T (1992): Silent mother-to-child transmission of hepatitis C virus through two generations determined by comparative nucleotide sequence analysis of the viral cDNA. Journal of Infectious Diseases 166:1425-1428.

Jacyna MR, Brooks MG, Loke RTH, Main J, Murray-Lyon IM, Thomas HC (1989): Randomized controlled trial of interferon alfa (lymphoblastoid interferon) in chronic non-A non-B hepatitis. British Medical Journal 298:80-82.

Janot C, Couroucé AM, Maniez M (1989): Antibodies to hepatitis C virus in French blood donors. Lancet ii:796-797.

Jeffers LJ, Perez GO, de Medina MD, Ortiz-Interian CJ, Schiff ER, Reddy KR, Jimenez M, Bourgoignie JJ, Vaamonde CA, Duncan R, Houghton M, Choo Q-L, Kuo G (1990): Hepatitis C infection in two urban hemolysis units. Kidney International 38:320-322.

Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugimura T Shimotohno K (1990): Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proceedings of the National Academy of Sciences USA 87:9524-9528.

Kew MC, Houghton M, Choo QL, Kuo (1990): Hepatitis C virus antibodies in Southern African blacks with hepatocellular carcinoma. Lancet 335:873-874.

Kühnl P, Seidl S, Stangel W, Beyer J, Sibrowski W, Flik J (1989): Antibody to hepatitis C virus in German blood donors. Lancet ii:324.

Kuo G, Choo Q-L, Alter HJ, Gitnick GL, Redeker AG, Purcell RH, Miyamura T, Dienstag JL, Alter MJ, Stevens CE, Tegtmeier GE, Bonino F, Colombo M, Lee W-S, Kuo C, Berger K, Shuster JR, Overby LR, Bradley DW, Houghton M (1989): An assay for circulating antibodies to a major etiologic agent of human non-A, non-B hepatitis. Science 244:362-364.

Lam JPH, McOmish F, Burns SM, Yap PL, Mok JYQ, Simmonds P (1993): Infrequent vertical transmission of hepatitis C virus. Journal of Infectious Diseases 167:572-576.

Makris M, Preston FE, Triger DR, Underwood JCE, Choo Q-L, Kuo G, Houghton M (1990): Hepatitis C antibody and hronic liver disease in haemophilia. Lancet 335:1117-1119.

Miller RH, Purcell RH (1990): Hepatitis C virus shares amino acid sequence similarity with Pestiviruses and Flaviviruses as well as members of two plant virus supergroups. Proceedings of the National Academy of Sciences USA 87:2057-2061.

Miyamura T, Saito I, Katayama T, Kikuchi S, Tateda A, Houghton M, Choo Q-L, Kuo G (1990): Detection of antibody against antigen expressed by molecularly cloned hepatitis C virus cDNA: application to diagnosis and blood screening for posttransfusion hepatitis. Proceedings of the National Academy of Sciences USA 87:983-987.

Nakatsuji Y, Matsumoto A, Tanaka E, Ogata H, Kiyosawa K (1992): Detection of chronic hepatitis C virus infection four diagnostic systems: first-generation and second-generation enzyme-linked immunosorbent assay, second-generation recombinant immunoblot assay and nested PCR. Hepatology 16:300-305.

Novati R, Thiers V, Monforte A, Maisonneuve P, Principi N, Conti M, Lazzarin A, Brechot C (1992): Mother-to-child transmission of hepatitis C virus detected by nested polymerase chain reaction. Journal of Infectious Diseases 165:720-723.

Ogata N, Alter HJ, Miller RH, Purcell RH (1991): Nucleotide sequence and mutation rate of the H strain of hepatitis C virus. Proceedings of the National Academy of Sciences USA 88:3392-3396.

Okada S-I, Akahane Y, Suzuki H, Okamoto H, Mishiro S (1992): The degree of variability in the aminoterminal region of the E2/NS1 protein of hepatitis C virus correlates with responsiveness

to interferon therapy in viremic patients. Hepatology 16:619-624.

Okamoto H, Okada S, Sugiyama Y, Kurai K, Lizuka H, Machida A, Miyakawa Y, Mayumi M (1991): Nucleotide sequence of the genomic RNA of hepatitis C virus isolated from a human carrier: comparison with reported isolates for conserved and divergent regions. Journal of General Virology 72:2697-2704.

Okamoto H, Kurai K, Okada S-I, Yamamoto K, Lizuka H, Tanaka T, Fukuda S, Tsuda F, Mishiro S (1992a): Full-length sequence of a hepatitis C virus genome having poor homology to reported isolates: comparative study of four distinct genotypes. Virology 188:331-341.

Okamoto H, Kojima M, Okada S-I, Yoshizawa H, Lizuka H, Tanaka T, Muchmore EE, Peterson DA, Ito Y, Mishiro S (1992b): Genetic drift of hepatitis C virus during an 8.2-year infection in a chimpanzee: variability and stability. Virology 190:894-899.

Okamoto H, Sugiyama Y, Okada S, Kurai K, Akahane Y, Sugai Y, Tanaka T, Sato K, Tsuda F, Miyakawa Y, Mayumi M (1992c): Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. Journal of General Virology 73:673-679.

Oshima M, Tsuchiya M, Yagasaki M, Orita T, Hasegawa M, Tomonoh K, Kojima T, Hirata Y, Yamamoto O, Sho Y, Maeda E, Arima T (1991): cDNA clones of Japanese hepatitis C virus genomes derived from a single patient show sequence heterogeneity. Journal of General Virology 72:2805-2809.

Prince, AM, Grady GF, Hazzi C et al (1974): Long-incubation post-transfusion hepatitis without serological evidence of exposure to hepatitis B virus. Lancet 1:241.

Purcell RH (1994): Hepatitis C virus: Historical perspective and current concepts. Federation of European Microbiological Societies 14:181-192.

Reinus JF, Leikin EL, Alter HJ, Cheung L, Shindo M, Jett B, Piazza S, Shih JW-K (1992): Failure to detect vertical transmission of hepatitis C. Ann. Int. Med. 117:881-886.

Reyes, GR, Purdy MA, Kim JP, Luk, K-C, Young LM, Fry KE, Bradley DW (1990): Isolation of a cDNA clone from the virus responsible for enterically transmitted non-A, non-B hepatitis. Science 247:1335-1339.

Rizetto M (1983): The delta agent. Hepatology 3:729-737.

Saeed AA, Al-Admawi AM, Al-Rasheed A, Fairclough D, Bacchus R, Ring C, Garson JA (1991): Hepatitis C virus infection in Egyptian volunteer blood donors in Riyadh. Lancet 338:459-460.

Saito I, Miyamura T, Ohbayashi A, Harada H, Katayama T, Kikuchi S, Watanabe Y, Koi S, Onji M, Ohta Y, Choo QL, Houghton M, and Kuo G. (1990) Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. Proceedings of the National Academy of Sciences USA 87:6547-6549.

Sherlock, S., and H.C. Thomas. 1985. Treatment of chronic hepatitis due to hepatitis B virus.

Lancet 2:1343-1346.

Shindo M, DiBisegli A, Cheung L, Shih WK, Christiano K, Feinston S, Hoofnagle J (1991): Decrease in serum hepatitis C viral RNA during alpha-interferon therapy for chronic hepatitis C. Annals of Internal Medicine 115:700-704.

Stuyver L, Rossau R, Wyseur A, Duhamel M, Vanderbroght B, Van Heuverswyn H, Maertens G (1993): Typing of HCV isolates and characterization of new (sub)types using a line probe assay. Journal of General Virology, 74, 1093-1102.

Takamizawa A, Mori C, Fuke I, Manabe S, Murakami S, Fujita J, Onichi E, et al (1991): Structure and organization of the hepatitis C virus genome isolated from human carriers. Journal of Virology 65:1105-1113.

Tanaka T, Kato N, Nakagawa M, Oosuyama Y, Cho M-J, Nakazawa T, Hijikata M, Ishimura Y, Shimotohno K (1992): Molecular cloning of hepatitis C virus genome from a single Japanes carrier: sequence variation within the same individual and among infected individuals. Virus Research 23,39-53.

Thaler MM, Park C-K, Landers DV, Wara DW, Houghton M, Veereman-Wauters G, Sweet RL, Han JH (1991): Vertical transmission of hepatitis C virus. lancet 338:17-18.

Thompson BJ, Doran M, Lever AML, Webster ADB (1987): Alpha-interferon therapy for non-A, non-B hepatitis treatment transmitted by gammaglobulin replacement therapy. Lancet 539-541.

Tiollais P, Pourcel C, Dejean A (1985): The hepatitis B virus. Nature 317: 489-495.

Tsukiyama-Kohara K, Liauka N, Kohara M, Nomoto A (1992): Internal ribosomal entry site within hepatitis C virus RNA. Journal of Virology 66:1476-1483.

van der Poel CL, Reesink HW, Schaasberg W, Leentvaar-Kuypers A, Bakker E, Exel-Oehlers PJ, Lelie PN (1990): Infectivity of blood seropositive for hepatitis C virus antibodies. Lancet 335:753-757.

van der Poel CL, Reesink HW, Mauser-Bunschoten EP, Kaufmann RH, Leentvaart-Kuypers A, Chamuleau RAFM, Schaasberg W, Bakker E, Exel-Oehlers PJ, Theobalds I, van Boven JJP, Cameron A, Lelie PN (1991a): Prevalence of anti-HCV antibodies confirmed by recombinant immunoblot in different population subsets in the Netherlands. Vox Sanguinis 61:30-36.

van der Poel CL, Cuypers HTM, Reesink HW, Weiner AJ, Quan, Di Nello R, van Boven JJp, Winkel I, Mulder-Folkerts, Exel-Oehlers PJ, Schaasberg W, Leentvaart-Kuypers A, Polito A, Houghton M, Lelie PN (1991b): Confirmation of hepatitis C virus infection by new four-antigen recombinant immunoblot assay. Lancet 337:317-319.

Weiner AJ, Kuo G, Bradley DW, Bonino F, Saracco G, Lee C, Rosenblatt J, Choo QL, Hougthon M (1990): Detection of hepatitis C viral sequences in non-A, non-B hepatitis. Lancet 335:1-3.

Weiner AJ, Truett MA, Rosenblatt J, Han J, Quan S, Polito A, Kuo G, Choo Q-L, Houghton M, Agius C, Page E, Nelles MJ (1990): HCV testing in low-risk population. Lancet 336:695.

Wejstal R, Widell A, Mansson A-S, Hermodsson S, Norkrans G (1992) Mother-to-infant transmission of hepatitis C virus. Ann. Int. Med. 177:887-890.

Zaaijer HL, Cuypers HTM, Reesink HW, Winkel IN, Gerken G, Lelie PN (1993): Reliability of polymerase chain reaction for detection of hepatitis C virus. Lancet 341:722-724.

CHAPTER 2

Evaluation of several 5'UTR primer sets for detection of hepatitis C virus RNA by single and nested PCR

G.E.M. Kleter

ABSTRACT

An "in-house" developed HCV RNA assay was evaluated in two quality control studies with 38 and 97 participants which supplied respectively 31 and 136 data sets for evaluation. The two HCV RNA panels each consisted of 10 undiluted samples and two dilution series. These samples were analyzed by single round and nested PCR with four different primer sets, respectively NC, NCC, NC3 and HC3/4, and two probes, HCV17 and NC-71, based on sequences of the 5'untranslated region. Obtained PCR data were confirmed by repeating the experiment. The NC primer set was not able to detect HCV RNA at all in one of the dilution series. This lack in HCV RNA detection was probably caused by sequence variation in the target region of the NC primerset. The primer sets NCC and NC3 were almost similar in sensitivity and HCV specific in the analysis of both panels. First round PCR product analysis by nested PCR with inner primer set HC3/4 did not improve the sensitivity when compared to Southern blot hybridization with probe NC-71. In comparison to the reference laboratory with the highest sensitivity and participants with a higher sensitivity as we, HCV RNA could not be detected by us in one of the 20 undiluted samples, suggesting that it was a borderline sample. In conclusion, the "in-house" developed HCV RNA PCR assay meets the standards of specificity and sensitivity of the reference laboratories.

INTRODUCTION

Hepatitis C Virus (HCV) is the causative agent of parenterally transmitted non-A, non-B hepatitis (NANBH) [Choo et al., 1989], and belongs to the family of Flaviviridae [Miller et al., 1990]. The viral RNA genome encodes 3 structural antigens (core, E1 and E2/NS1) and 4 non-structural antigens (NS2 to NS5).

One of the first assays for detection of hepatitis C viral sequences in NANBH patients was reported in 1990 by using primers in the NS3/NS4 region [Weiner et al., 1990]. In the last two years several papers have discussed

technical aspects of HCV RNA detection by PCR. Various parts of the procedures like method of isolation, handling and storage of materials, choice of primers and PCR product analysis have been investigated extensively. A summary of these technical reports is given below.

HCV RNA can be efficiently isolated from blood or liver tissue by lysis with the chaotropic detergent guanidinium thiocyanate [Chomczynski et al., 1987] and by proteinase K digestion [Houghton et al., 1990]. Almost similar results were obtained by these two methods [Wolff et al., 1992; Tilston et al., 1993; Castillo et al., 1992], although chemical lysis is preferable above enzymatical lysis, due to a more rapid denaturation of RNases.

False-negative results can be obtained by suboptimal specimen handling i.e. repeatedly freezing and thawing of samples. Also storage at ambient temperature and 4°C resulted in a reduction of the yield of PCR product [Busch et al., 1992; Cuypers et al., 1992]. Blood samples are the most convenient specimens for HCV RNA detection, although it has been reported that heparinized plasma samples are not suitable, due to the inhibitory effect of heparin on Taq DNA polymerase [Willems et al., 1993]. For proper analysis of these samples the isolation procedure can be extended by a heparinase treatment or alternatively HCV RNA can be isolated by capture onto paramagnetic beads [van Doorn et al., 1994].

Primers are crucial for efficient detection of HCV RNA since they determine specificity as well as sensitivity. Analysis of the NS4 region with five primer sets revealed a lack of sensitivity for HCV RNA detection [Cristiano et al., 1991]. Highest specificity and sensitivity was obtained by primer sets directed to 5'UTR when compared to the core, NS2, NS3, NS4 and NS5 regions [Garson et al., 1990 and 1991; Inchauspe et al., 1991; Bukh et al., 1992a; Cuypers et al., 1992; Castillo et al., 1992]. Currently, at least 9 major HCV types exist [Bukh et al., 1993; Simmonds et al., 1993a; Tokita et al., 1994]. Within the 5'UTR approximately 90% sequence homology is observed among HCV types [Chan et al., 1993]. The conservation of this region is probably caused by functional restrictions [Brown et al., 1992; Tsukiyama-Kohara et al., 1992; Yoo et al., 1992; Wang et al., 1993] and sequence variation was mainly observed

in two sequence motifs [Bukh et al., 1992b; Simmonds et al., 1993b; Kleter et al., 1994]. Therefore, 5'UTR appears to be the best region for detection of HCV viremia.

Analysis of PCR products can be performed by hybridization with a probe or by subsequent nested PCR with an inner primer set. Nested PCR has higher specificity and probably a higher sensitivity but is more prone to contamination than hybridization. Comparison of single round and nested PCR by analysis of a cDNA dilution series resulted in similar sensitivity and specificity [Gretch et al., 1993].

The "in-house" developed HCV RNA PCR assay as described in this chapter was the basis of all studies in this thesis. Over time the "in-house" assay has been adapted by changing primers and probes due to obtained knowledge on HCV RNA sequences and HCV RNA detection results on several HCV samples for first diagnosis. Here, the assay was evaluated in two quality control studies organized in 1992 and 1993 by the Central Laboratory for Blood transfusion (CLB, Amsterdam the Netherlands) under auspices of the "Eurohep" group (i.e. an European expert group on viral hepatitis). Blood samples were analyzed by single round and nested PCR with several 5'UTR primer sets to determine their specificity and sensitivity for detection of HCV RNA.

MATERIALS AND METHODS

Plasma samples

Coded plasma samples for the HCV RNA quality control panels from study 1992 and 1993 were provided by the Central Laboratory of The Netherlands Red Cross Blood Transfusion Service (Amsterdam, the Netherlands).

The 1992 HCV RNA quality control panel ("1992 panel") consisted of 22 EDTA-plasma samples, including 10 undiluted blood donor samples (6 without HCV RNA and 4 with different quantities of HCV RNA). The remaining 12 samples were divided into two tenfold dilution series of 6 samples each (10^{-2} to 10^{-7}); one from an infected donor with HCV type 1 and the other from a

chimpanzee infected with a known infectious dose of the HCV-H strain. This coded test panel was sent to 38 hepatitis C research laboratories in Europe (30), the USA (7) and Japan (1); 7 laboratories did not supply results and 31 laboratories returned evaluable data sets [Zaaijer et al., 1993].

The 1993 HCV RNA quality control panel ("1993 panel") consisted of 26 EDTA-plasma samples, including 10 undiluted samples (6 without HCV RNA, 3 were of HCV type and in 1 sample a discordant test result was obtained by the 2 reference laboratories). The remaining 16 samples were divided in two dilution series, containing HCV type 1 and type 3, respectively. This coded test panel was sent to 97 hepatitis C research laboratories around the world; 86 laboratories participated actually and returned 136 evaluable data sets [Cuypers et al., 1994].

Primers and Oligonucleotide probes

Known HCV CDNA sequences [Kato et al., 1990; Takamizawa et al., 1991; Okamoto et al., 1990, 1991 and 1992; Choo et al., 1991] were used for selection of the primers and probes. The sequences of primers and probes are shown in Table 1. Relative positions of applied primer sets and probes are shown in Figure 1. Primers and probes were synthesized on an Applied Biosystems 381 A DNA synthesizer using the ß-cyano-ethylphosphoramidite method.

HCV RNA isolation

HCV RNA was extracted from plasma samples by a modification of the guanidinium method as described by Chomczynski [Chomczynski et al., 1987]. In detail, 100 µl plasma was dissolved in 500 µl of a solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% (wt/vol) sarcosyl, 0.1 M ß-mercaptoethanol and 20 µg poly A. This mixture was extracted twice with an equal volume of phenol/chloroform (1:1/v:v) and once with chloroform/isoamyl-alcohol (49:1) by shaking 10 min and subsequently centrifuged for 20 min in the first extraction, and 5 min for the remaining. The aqueous phase was precipitated with one volume of isopropanol for 1 hour at

Table 1. HCV oligonucleotide sequences

name	position	polarity	sequence (5'-3')
HCV18	-324 to -305	+	GGCGACACTCCACCATAGAT
HCV35	-318 to -296	+	TTGGCGGCCGCACTCCACCA TGAATCACTCCCC
NCR3	-314 to -288	+	GGGGCGGCCGCCACCATRRA TCACTCCCCTGTGAGG
HC3	-264 to -239	+	TCTAGCCATGGCGTTAGTRYGAGTGT
HCV17	-86 to -67	+	GAGTAGTGTTGGGTCGCGAA
NC-71	-71 to -52	+	GCGAAAGGCCTTGTGGTACT
HC4	-29 to -54	-	CACTCGCAAGCACCCTATCAGGCAGT
HCV19	-1 to -20	-	GTGCACGGTCTACGAGACCT

+ indicates sense, - indicates antisense orientation. Underlined sequences are not complementary to the HCV sequence. R = A or G; Y = T or C.

-70°C. After 20 min centrifugation at 4°C the RNA pellet was resuspended in 80% ethanol and centrifuged for 15 min. The pellet was vacuum dried and dissolved in 30 μl diethylpyrocarbonate-treated H_2O.

RNA PCR

For cDNA synthesis 20 pmol of an antisense HCV primer (Table 1) was added to 10 μl HCV RNA solution. The mixture was heated at 80°C for 2 min and immediately cooled on ice to allow annealing of the cDNA primer. cDNA synthesis was performed in a final volume of 25 μl after adjustment of the mixture to contain 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM of dNTP, 30 U RNasin (Promega, Madison, Wis.) and 200 U Moloney murine leukemia virus reverse transcriptase (GIBCO-Bethesda Research Laboratories, Gaithersburg, Md.) and incubated at 42°C for 30 min.

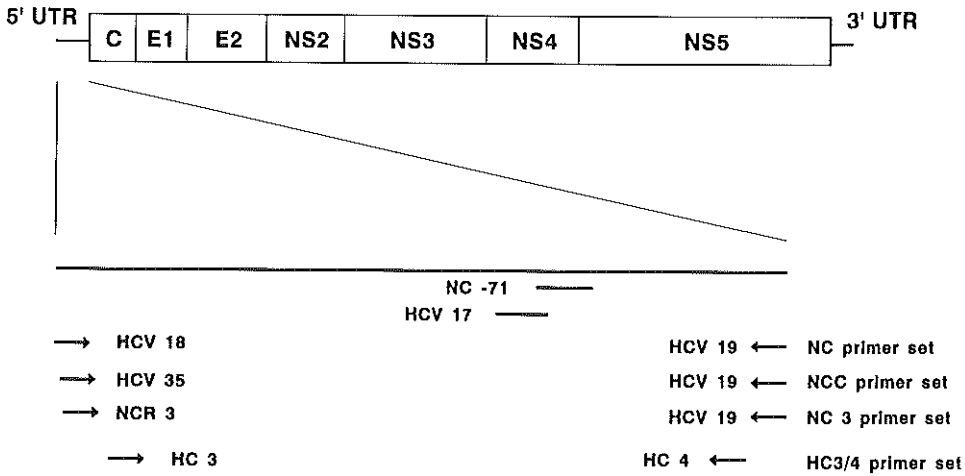


Figure 1. Relative position of HCV primers and probes

Amplification of HCV CDNA sequences was in accordance with the PCR method originally described by Saiki [Saiki et al., 1988]. PCR was performed in a final volume of 100 μl. Taq buffer, dNTP's, 20 pmol of the sense primer (Table 1) and 1 U of Taq DNA polymerase (Promega, Leiden, the Netherlands) were added to the cDNA reaction after an initial 5 min denaturation at 95°C. The PCR solution was covered with two drops of mineral oil (Sigma, St. Louis, Missouri), to prevent evaporation, and subjected to 40 cycles of amplification using a PCR thermocycler (Biomed, Bitfurth, FRG). Each reaction cycle consisted of a DNA denaturation step at 94°C for 1 min, a primer annealing step at 52°C for 2 min and a primer extension step at 72°C for 2 min.

PCR product analysis

After amplification 20 μl of the first round product was electrophoresed on a 2% agarose gel. For Southern blot analysis the gel was denatured for 15 min in 0.5 M NaOH, 1.5 M NaCl and neutralized for 15 min in 3 M Na-Acetate pH 4.8. The

amplification products were transferred to a nylon membrane (Hybond N, Amersham, Buckinghamshire, United Kingdom) by blotting in 10xSSC (1xSSC is 15 mM sodium citrate, 150 mM NaCl). The blots were baked for 2 hours at 80°C. The filters were hybridized with an internal probe 5'-end labelled with ^{32}P (Sambrook et al., 1989). Hybridization was performed in 5xSSC, 5xDenhardt's (0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinyl pyrrolidone), 0.5% SDS, 75 mM EDTA and 100 μg/ml herring sperm DNA with 10^6 cpm of probe per ml for 16 hours at 37°C in a shaking water bath. After hybridization filters were rinsed briefly in 6xSSC and subsequently washed twice in 2xSSC, 0.1% SDS for 15 min at 37°C and once in 1xSSC, 0.1% SDS for 15 min at 45°C. The filters were autoradiographed between intensifying screens at -70°C on Fuji RX 100 X-ray films.

For analysis by nested PCR (40 cycles of 1 min at 94°C, 2 min at 55°C, 2 min at 72°C) 1 μl of the first round product was transferred to a solution containing: 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM of dNTP, 1 U Taq DNA polymerase and 10 pmol of each inner primer (Table 1). Finally, 20 μl of the nested PCR product was visualized by ethidium bromide staining after 2% agarose gel electrophoresis.

PCR control

In each experiment, along with a maximum number of 15 test samples, 2 water samples and 1 HCV RNA positive sample were included for reproducibility of HCV RNA extraction, reverse transcription and amplification. The sensitivity of each assay is controlled by three members of a tenfold dilution series of the positive control. All samples were tested in at least two independent experiments, starting from HCV RNA isolation. To prevent the possibility of cross contamination the process steps, plasma preparation, HCV RNA isolation and cDNA synthesis, PCR and product analysis, were performed in different rooms. Additionally, sterile filter tips and disposable pastettes were used as recommended by Kwok and Higuchi [Kwok et al., 1989].

Strategy and interpretation of PCR results

The plasma samples were analyzed in two independent experiments. Confirmed results of the tested sample were accepted as valid and formed the final test result. If a positive result was obtained in two experiments, the interpretation was HCV RNA positive. Samples in which no HCV RNA could be detected after two experiments were considered as "HCV RNA *undetectable*" rather than negative, because of a limited detection of nucleic acids obtained from clinical specimens. In case of a discordant result, the sample was analyzed again in two independent experiments. If the result was still discordant then the sample was classified as indeterminate.

RESULTS

HCV quality control panel 1992

In this coded panel HCV RNA was detected by single PCR with 5'UTR primersets NC, NCC and NC3 and the amplification products were analyzed by Southern blot hybridization with probe HCV17 (Figure 1). Identical test results were obtained in the 10 undiluted plasma samples in 2 experiments. Besides the 6 negative and 3 "strongly" positive samples, the "weakly" positive sample was detected by all three primer sets. In the coded samples of the "donor" and "chimpanzee" dilution series, a distinct end point in detection of HCV RNA was obtained by the three primer sets (Table 2). The NC3 primer set was slightly more sensitive than the NCC primer set and the NC primer set was not able to detect any HCV RNA in the "donor" dilution series (Table 2).

HCV quality control panel 1993

The coded plasma samples of this test panel were analyzed by single round PCR as well as nested PCR. First round PCR was performed with the primer sets NCC and NC3 and obtained PCR products were subsequently analyzed by either Southern blot hybridization with probe NC-71 or nested PCR with primer set HC3/4 (Figure 1). After two independent experiments, discordant test results

were obtained in 7 of the 26 samples. After repeating the procedure (see strategy) the interpretation in these 7 cases was negative in 5 and positive in 2. By applying the four described combinations of primers for the detection of HCV RNA (Figure 1), identical test results were obtained in all 10 undiluted plasma samples. In comparison to test results obtained by both reference laboratories, discrepancy was observed in one sample. HCV RNA was undetectable by us as well as by reference laboratory number 2. Reference laboratory number 1 classified this sample as "weakly" positive [Cuypers et al., 1994]. The HCV type of this isolate is yet not known while the other positive samples were identified as HCV type 1.

TABLE 2. Detection of HCV RNA in two dilution series from the 1992 HCV quality control panel.

Serie	Dilution	primer set Ref[a]	NC3	NCC	NC
Donor	10^{-2}	+	+	+	—
	10^{-3}	+	+	+	—
	10^{-4}	+	+	+	—
	10^{-5}	—	+	—	—
	10^{-6}	—	—	—	—
	10^{-7}	—	—	—	—
Chimp.	10^{-2}	+	+	+	+
	10^{-3}	+	+	+	+
	10^{-4}	+	+	+	±
	10^{-5}	—	—	—	—
	10^{-6}	—	—	—	—
	10^{-7}	—	—	—	—

+ HCV RNA positive; ± indeterminate test result; — HCV RNA undetectable

[a] PCR results by the reference laboratory

After breaking the code, it appeared that in the two dilution series a clear end point in detection of HCV RNA was obtained (Table 3). The sensitivity for detection of HCV RNA was almost similar by the four combinations of primers. In detection of the HCV RNA in the type 3 dilution series, the combination of

TABLE 3. HCV RNA detection in two dilution series from the 1993 HCV quality control study by single and nested PCR with different 5'UTR primer sets

			primer sets			
	Reference		single PCR		nested PCR	
Dilution series	1	2	NC3	NCC	NC3 and HC3/4	NCC and HC3/4
HCV type 1						
100	+	+	+	+	+	+
1,000	+	+	+	+	+	+
4,000	+	+	+	+	+	+
16,000	+	—	—	—	—	—
64,000	±	—	—	—	—	—
256,000	—	—	—	—	—	—
1,024,000	—	—	—	—	—	—
4,096,000	—	—	—	—	—	—
HCV type 3						
10	+	+	+	+	+	+
100	+	+	+	+	+	+
1,000	+	+	+	+	+	+
4,000	+	—	—	—	+	—
16,000	±	—	—	—	—	—
64,000	—	—	—	—	—	—
256,000	—	—	—	—	—	—
1,024,000	—	—	—	—	—	—

+ HCV RNA positive; ± indeterminate test result; — HCV RNA undetectable

nested PCR by outer primer set NC3 and inner primer set HC3/4 was 4 times more sensitive than the other three approaches. In both dilution series 2 samples had to be analyzed in four experiments instead of two. These samples contained HCV RNA levels close to the detection limit of the applied primer sets (Table 3; type 1, dilution 1/4,000 and 1/16,000; type 3, dilution 1/1,000 and 1/4,000).

DISCUSSION

The specificity and sensitivity of the "in-house" developed HCV RNA assay was determined by analysis of two coded hepatitis C quality control panels. A total of four 5'UTR primer sets and two probes were used in the evaluation of HCV RNA detection by single round and nested PCR.

HCV quality control panel 1992

Analysis of the "1992 panel" revealed that the NC primer set was not universal for detection of HCV viremia. HCV RNA isolated in the "donor" dilution series, characterized as type 1 by the reference laboratory, could not be detected. This lack in detection by the NC primer set was caused by mismatching of primer HCV18. Comparison of several reported 5'UTR HCV sequences, revealed sequence heterogeneity in the target region near the 3' end of HCV18 [Kato et al., 1990; Takamizawa et al., 1991; Okamoto et al., 1990, 1991 and 1992; Choo et al., 1991]. Similarly, Probe HCV17 contained two mismatches when compared to HCV type 2 sequences (positions -80 and -72). But still, all known HCV types could be detected by probe HCV17 after washing at low stringency (data not shown). In contrast, the primer sets NC3 and NCC were HCV specific and highly sensitive in HCV RNA detection when compared to the reference laboratory. The data obtained in both dilution series showed a clear end point in the detection of HCV RNA. This distinct point of detectable HCV RNA is an indication for a good performance of the PCR assay.

HCV quality control panel 1993

Analysis of the "1992 panel" revealed a slight difference in sensitivity between the NCC and NC3 primer set. Therefore, both primer sets were used in the analysis of the "1993 panel". Obtained first round PCR products were analyzed by Southern blot hybridization with probe NC-71 and nested PCR which is potentially more sensitive as single PCR.

In comparison of HCV RNA results in the 10 undiluted plasma samples discrepancy was observed in one sample. By the used four primer sets a discordant test result was initially obtained by the NCC primer set in this particular sample. After repeating the procedure this sample was also classified as HCV RNA undetectable by the NCC primer set. The discrepant result could be caused by either specificity or sensitivity of the HCV RNA assays. The possible lack of specificity could be caused by crucial mutation(s) in this particular HCV isolate at the primer target region to prevent proper amplification by the NC3 and NCC primer sets. However, this explanation is not likely because the primer sets NC3 and NCC are able to detect HCV types 1 to 5 [Kleter et al., 1994]. The most likely explanation for the discrepancy is the slight difference in sensitivity between our HCV RNA assay and that of reference laboratory number 1 (Table 3). The detection level of the primer sets NCC and NC3 was 500-1000 HCV equivalents per ml as estimated by the branched DNA assay (Quantiplex, Chiron Emeryville, CA) in dilution series. Moreover, in 30 (22%) of the 136 received data sets HCV RNA was detected, suggesting that it was a borderline sample [Cuypers et al., 1994].

When analyzing the two dilution series, a minimal difference in sensitivity between single round PCR and nested PCR was obtained (Table 3). These results revealed that the performance of nested PCR, which is highly prone to contamination, is not really necessary to improve the sensitivity of HCV RNA detection. It should be noted that the 4 to 16 times higher degree in sensitivity obtained by reference number 1 was achieved by single PCR at the 5'UTR followed by liquid hybridization.

Interpretation of test results

The overall results of the 1992 HCV RNA quality control study revealed that only 5 (16%) of the 31 participating centers were able to produce PCR data without false-positivity and a high sensitivity. The results of the "1992" study and also that of other quality control studies revealed that false-positive results were the main problem in the application of PCR technology [Zaaijer et al., 1993; Quint et al., 1995; Bootman et al., 1992; Kuypers et al., 1993].

As indicated in this study and by others, results of enzymatic amplification methods should be confirmed by two independent experiments or performing duplicates to exclude false-positivity and/or false-negativity [Gerken et al., 1992; Mahony et al., 1994; Kitchen et al., 1993]. Samples with a discordant result are a matter of concern. The discrepancies can be caused by either contamination and the sensitivity of the assay. In the 1993 panel 7 samples had to be assayed again. After breaking the code it appeared that the amount of HCV RNA in 4 of these samples was close to the detection limit of the applied primer sets. The performed strategy for interpretation of test results was useful in the analysis of both quality control panels. *No* false-positive final test results were produced. Therefore, one could consider application of our strategy for every HCV RNA test.

When starting this thesis HCV RNA detection was performed with the NC primer set and a primer set directed to the NS5 region (Chapter 3). In the analysis of the "1992 panel" and isolates obtained from chronically infected non-A, non-B hepatitis patients it appeared that these two primer sets lacked sensitivity. This sensitivity problem was mainly caused by sequence variation and especially observed after introduction of second generation antibody screening assays. In chapter 4 to 7 HCV RNA isolates were therefore analyzed by the NCC primer set.

In conclusion, evaluation of HCV RNA detection in the two analyzed coded quality control panels revealed that the HCV RNA data obtained by our technology and strategy were of high sensitivity and specificity.

REFERENCES

Bootman JS, Kitchin PA (1992): An international collaborate study to assess a set of 2 reference reagents for HIV-1 PCR. Journal of Virological Methods 37:23-42.

Brown EA, Zhang H, Ping L-H, Lemon SM (1992). Secundary structure of the 5' nontranslated regions of hepatitis C virus and pestivirus genomic RNAs. Nucleic Acids Research. 20:5041-5045.

Bukh J, Purcell RH, Miller RH (1992a): Importance of primer selection for the detection of hepatitis C virus RNA with the polymerase chain reaction assay. Proceedings of the National Academy of Sciences USA 89:187-191.

Bukh J, Purcell RH, Miller RH (1992b): Sequence analysis of the 5' noncoding region of hepatitis C virus. Proceedings of the National Academy of Sciences USA 89:4942-4946.

Bukh J, Purcell RH, Miller RH (1993): At least 12 genotypes of hepatitis C virus predicted by sequence analysis of the putative E1 gene of isolates collected worldwide. Proceedings of the National Academy of Sciences USA 90: 8234-8238.

Busch MP, Wilber JC, Johnson P, Tobler L, Evans CS (1992): Impact of specimen handling and storage on detection of hepatits C virus RNA. Transfusion 32:420-425.

Castillo I, Bartolome J, Quiroga JA, Carreno V (1992): Comparison of several PCR procedures for detection of serum HCV-RNA using different regions of the HCV genome. Journal of Virological Methods 38:71-80.

Chan S-W, McOmish F, Holmes EC, Dow B, Peutherer JF, Follett E, Yap PL, Simmonds P (1992): Analysis of a new hepatitis C virus type and its phylogenetic relationship to existing variants. Journal of General Virology 73:1131-1141.

Chomczynski P, Sacchi N (1987): Single step method of RNA isolation by guanidinium thiocyanate-phenol-chloroform extraction. Analytical Biochemistry 162:152-159.

Choo Q-L, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M (1989): Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 244:359-362.

Choo Q-L, Richman KH, Han JH, Berger K, Lee C, Dong C, Gallegos C et al. (1991): Genetic organization and diversity of the hepatitis C virus. Proceedings of the National Academy of Sciences USA, 88:2451-2455.

Cristiano K, Di Bisceglie AM, Hoofnagle JH, Feinstone S (1991): Hepatitis C viral RNA in serum of patients with chronic non-A, non-B hepatitis: detection by the polymerase chain reaction using multiple primersets. Hepatology 14:51-55.

Cuypers HTM, Bresters D, Winkel IN, Reesink HW, Weiner AJ, Houghton M, van der Poel CL, Lelie PN (1992): Storage conditions of blood samples and primer selection affect the yield of cDNA polymerase chain reaction products of hepatitis C virus. Journal of Clinical Microbiology

30:3220-3224.

Cuypers HTM, Damen M, Zaayer HL, Reesink HW, Niesters B, Lelie PN (1994): Quality control of HCV detection methods: interim analysis of the second Eurohep HCV-RNA proficiency panel. EASL meeting, Athens, September 7-10, 1994.

Garson JA, Ring C, Tuke P, Tedder RS (1990): Enhanced detection by PCR of hepatitis C virus RNA. Lancet 336:878-879

Garson JA, Ring CJA, Tuke PW (1991): Improvement of HCV genome detection with "short" PCR products. Lancet 338:1466-1467.

Gretch DR, Wilson JJ, Carithers RL, dela Rosa C, Han JH, Corey L (1993): Detection of hepatitis C virus RNA: Comparison of one-stage polymerase chain reaction (PCR) with nested-set PCR. Journal of Clinical Microbiology 31:289-291.

Gerken G, Gerlich WH, Brechot C, Thomas HC, Bonino F, de Moura C, zum Busschenfelde K-H (1992): Biological standards for hepatits B virus assays. Journal of Hepatology 15:251-255.

Houghton M, Choo Q-L, Kuo G (1990): European patent application 88,310,922.5.

Inchauspe I, Abe K, Zebedee S, Nasoff M, Prince AM (1991): Use of conserved sequences from the hepatitis C virus for the detection of viral RNA in infected sera by polymerase chain reaction. Hepatology 14:595-600.

Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugimura T Shimotohno K (1990): Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proceedings of the National Academy of Sciences USA 87:9524-9528.

Kitchin PA, Bootman JS (1993): Quality control of the polymerase chain reaction. Reviews in Medical Virology 3:107-114.

Kleter GEM, van Doorn L-J, Brouwer JT, Schalm SW, Heijtink RA, Quint WGV (1994): Sequence analysis of the 5'untranslated region in isolates of at least four genotypes of hepatitis C virus in The Netherlands. Journal of Clinical Microbiology 32:306-310.

Kuypers JM, Critchlow CW, Gravitt PE, Vernon DA, Sayer JB, Manos MM, Kiviat NB (1993) Comparison of dot filter hybridization, Southern transfer hybridization, and the polymerase chain reaction amplification for diagnosis of anal human papillomavirus infection. Journal of Clinical Microbiology 31:1003-1006.

Kwok S, Higuchi S (1989): Avoiding false positives with PCR. Nature 339:237-238.

Mahony JB, Luinstra KE, Waner J, McNab G, Hobranzska H, Gregson D, Sellors JW, Chernesky MA (1994): Interlaboratory agreement study of a double set of PCR plasmid primers for detection of chlamidia trachomatis in a variety of genitourrinary specimens. Journal of Clinical Microbiology 32:87-91.

Miller RH, Purcell RH (1990): Hepatitis C virus shares amino acid sequence similarity with

Pestiviruses and Flaviviruses as well as members of two plant virus supergroups. Proceedings of the National Academy of Sciences USA 87:2057-2061

Okamoto H, Okada S, Sugiyama Y, Yotsumoto S, Tanaka T, Yoshizawa H, Tsuda F, Miyakawa Y, Mayumi M (1990): The 5' terminal sequence of the hepatitis C virus genome. Japanese Journal of Experimental Medicine 60:167-177.

Okamoto H, Okada S, Sugiyami Y, Kurai K, Lizuka H, Machida A, Miyakawa Y, Mayumi M (1991): Nucleotide sequence of the genomic RNA of hepatitis C virus isolated from a human carrier: Comparison with reported isolates for conserved and divergent regions. Journal of General Virology 72:2697-2704.

Okamoto H, Kurai K, Okada S-I, Yamamoto K, Lizuka H, Tanaka T, Fukuda S, Tsuda F, Mishiro S (1992): Full-length sequence of a hepatitis C virus genome having poor homology to reported isolates: Comparative study of four distinct genotypes. Virology 188:331-341.

Quint WGV, Heijtink RA, Schrim J, Gerlich WH, Niesters HGM (1994): Reliability of hepatits B virus DNA detection. Journal of Clinical Microbiology 33:225-228.

Saiki RK, Gelfland DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988): Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487-491.

Sambrook J, Fritsch EF, Maniatis T (1989): Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Simmonds P, Holmes EC, Cha T-C, Chan S-W, McOmish F, Irvine B, Beall E, Yap PL, Kolberg J, Urdea MS (1993a): 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 74:2391-2399.

Simmonds P, McOmish F, Yap PL, Chan S-W, Lin CK, Dusheiko G, Saeed AA, Holmes EC (1993b): Sequence variability in the 5' non-coding region of hepatitis C virus: identification of a new virus type and restrictions on sequence diversity. Journal of General Virology 74:661-668.

Takamizawa A, Mori C, Fuke I, Manabe S, Murakami S, Fujita J, Onichi E, et al (1991): Structure and organization of the hepatitis C virus genome isolated from human carriers. Journal of Virology 65:1105-1113.

Tilston P, Corbit G (1993): Detection of hepatits C virus RNA in serum, by combing reverse transcription and polymerase chain reaction in one tube. Journal of Virological Methods 44:57-66.

Tokita H, Okamoto H, Tsuda, F, Song P, Nakata S, Chosa T, Iizuka H, Mishiro S, Miyakawa Y, Mayumi M (1994): Hepatitis C virus variants from Vietnam are classifiable into the seventh, eighth and ninth major genetic groups. Proceedings of the National Academy of Sciences of the USA 91:11022-11026.

Tsukiyama-Kohara K, Liauka N, Kohara M, Nomoto A (1992): Internal ribosomal entry site within

hepatitis C virus RNA. Journal of Virology 66:1476-1483.

van Doorn L-J, Shyamala V, Han JH, Kleter GEM (1994): HCV RNA detection in heparinized blood by direct genomic RNA capture onto paramagnetic particles. J. Virol. Methods 48:339-341.

Wang C, Sarnow P, Siddiqui A (1993): Translation of human hepatits C virus RNA in cultured cells mediated by an internal ribosome-binding mechanism. Journal of Virology 67:3338-3344.

Weiner AJ, Kuo G, Bradley DW, Bonino F, Choo Q-L, Houghton M (1990): Detection of hepatitis C viral sequences in non-A, non-B hepatits. Lancet 335:1-3.

Willems M, Moshage H, Nevens F, Fevery J, Yap SH (1993): Plasma collected from heparinized blood is not suitable for HCV-RNA detection by conventional RT-PCR assay. Journal of Virological Methods 42:127-130.

Wolff C, Schluter K, Prohaska W, Kleesiek K (1992): Improved detection of hepatits C virus RNA by reverse transcription and polymerase chain reaction. European Journal of Clinical Chemistry and Clinical Biochemistry 30:717-727.

Yoo BJ, Spaete RR, Geballe AP, Selby M, Houghton M, Han JH (1992): 5' end-dependent translation initiation of hepatitis C virus RNA and the presence of putative positive and negative translational control elements within the 5' untranslated region. Virology 191:889-899.

Zaaijer HL, Cuypers HTM, Reesink HW, Winkel IN, Gerken G, Lelie PN (1993): Reliability of polymerase chain reaction for detection of hepatitis C virus. Lancet 341:722-724.

CHAPTER 3

Detection of hepatitis C virus RNA in patients with chronic hepatitis C virus infections during and after therapy with alpha interferon

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ABSTRACT

In 24 patients with hepatitis C virus (HCV) infection who participated in a randomized trial with alpha 2B interferon, HCV RNA analysis by the polymerase chain reaction with two separate primer sets was performed at weeks 0, 4, 24, and during a follow-up period of 6 to 9 months. Prior to therapy all patients were HCV RNA positive. During therapy HCV RNA decreased to an undetectable level (< 1 chimpanzee infectious dose per ml) in nine patients at week 4. After week 4, no additional cases of HCV RNA disappearance (< 1 chimpanzee infectious dose per ml) were observed. During follow-up, HCV RNA could not be detected in four of the six patients with a sustained alanine aminotransferase response. These results suggest the probable predictive value of HCV RNA levels for detecting the failure of therapy in an early stage of treatment.

INTRODUCTION

In recent years, several randomized controlled studies were performed with alpha interferon (IFN) for treatment of non-A, non-B hepatitis (NANBH) [Davis et al., 1989; Di Bisceglie et al., 1989; Jacyna et al., 1989; Marcellin et al., 1991; Sáez-Royuela et al., 1991; Shindo et al., 1991]. The effect of interferon was evaluated by measuring the alanine aminotransferase (ALT) level in serum, which reflects the activity of liver disease.

Recently, the etiological agent for NANBH has been identified [Choo et al., 1989; Hosoda et al., 1992]. The causative agent is now known as Hepatitis C Virus (HCV). The HCV genome is a positive stranded RNA molecule of about 9,400 nucleotides. Sequence homology between the known HCV strains is about 80% [Chan et al., 1992; Choo et al., 1991; Kato et al., 1990; Okamoto et al., 1991; Takamizawa et al., 1991].

In the study described here, 24 patients were investigated for the presence of HCV RNA during and after IFN therapy, to determine directly the effect of IFN treatment on viremia. HCV RNA analysis by the polymerase chain

reaction was performed using primer sets from the highly conserved non-coding (NC) region and a conserved sequence from non-structural region 5 (NS5).

MATERIALS AND METHODS

Patients between 18 and 70 years of age with elevated ALT levels ($\geq$2 times the upper limit of normal), a biopsy-proven chronic NANBH, antibodies to HCV determined by a second-generation enzyme immunosorbent assay (Abbott, North Chicago, Ill.) and a confirmatory assay RIBA IV (Ortho, Raritan, N.J.), and no recent history of hepatitis A virus, hepatitis B virus, cytomegalovirus, or Epstein-Barr virus were included in the study. All patients gave informed consent prior to participation in the study. Patients were treated in a randomized controlled trial with either a standard scheme (12 patients) consisting of 3 MU of recombinant IFN α2B (Intron A, Schering Plough, Kenilworth, N.J.) three times a week for 24 weeks or an experimental scheme (12 patients). In the experimental scheme, therapy started with 6 MU of recombinant IFN α2B three times a week for at least 8 weeks. Therapy was stopped at week 12 if ALT levels remained elevated. If the ALT level normalized, therapy was continued with 3 MU of recombinant IFN α2B three times a week for 8 weeks; this was followed by treatment with 1 MU of recombinant IFN α2B three times a week until a normal ALT was accompanied by an undetectable HCV RNA level for a period of one month.

Blood samples were taken prior to treatment and at least every fourth week during treatment and follow-up. For HCV RNA detection, EDTA-blood was collected and plasma was prepared within 2 hours after sampling; aliquots were quickly frozen in liquid nitrogen and stored at -70°C.

HCV RNA was extracted from 100 μl plasma by a modification of the guanidinium thiocyanate method as described by Chomczynski and Sacchi [1987]. cDNA synthesis was performed with 200 U of Moloney murine leukemia virus reverse transcriptase (GIBCO-Bethesda Research Laboratories, Gaithersburg, Md.) and 20 pmol of antisense primer in a 25 μl reaction volume.

Antisense primers were chosen as follows (positions according to Okamoto et al. [1991]): for the NC region, residues 323 to 304 (GTGCACGGTCTACGAGACCT, HCV19); for the NS5 region residues 7020 to 7001 (AGGAGGTTGGCCTCTATGAG). For amplification of HCV cDNA (40 cycles of 1 min at 94°C, 2 min at 48°C, 3 min at 74°C) 1 U of Taq DNA polymerase (Promega, Madison, Wis.), reaction buffer and 20 pmol of sense primer were added to a final volume of 100 μl. Sense primers were chosen as follows: for NC region residues 1 to 20 (GGCGACACTCCACCATAGAT, HCV18); for NS5, CCCTCCCATATAACAGCAGA, residues 6857 to 6877). Twenty microliter of the amplification product were analyzed by Southern blot hybridization. The probe for the NC region (GAGTAGTGTTGGGTCGCGAA, residues 239 to 258, HCV17) detects a product of 324 bp. For detection of the NS5 product (164 bp) two probes were used: probe A (GGGTCTCCCCCCTCCTTGGCCAGCTCTTCAGCTA, 6902 to 6931) is identical to HCV-J [Kato et al., 1990] and HCV-BK [Takamizawa et al., 1991], and probe B (CATGACTCCCCTGATGCTGA, 6980 to 6999) is identical to HCV-1 [Choo et al., 1991]. In all cases HCV RNA was sought with both primer sets. Known HCV cDNA sequences (Choo et al., 1991; Kato et al., 1990; Maéno et al., 1990; Okamoto et al., 1990; Takamizawa et al., 1991] were used for selection of the primers and probes. The detection limit of our assay is 1 chimpanzee infectious dose per ml, as estimated with challenge plasma in the Eurohep HCV RNA proficiency panel (provided by dr P.N. Lelie, CLB, Amsterdam, the Netherlands). Both primer sets showed the same sensitivity. Distilled water, anti-HCV negative and positive plasma as negative and positive controls, respectively, were used in each experiment. All results were confirmed by repeat testing.

RESULTS AND DISCUSSION

Prior to IFN treatment, all patients were HCV RNA positive as determined with the NC primer set. In one patient, HCV RNA was not detected by the NS5 primer

TABLE 1. Biochemical response and HCV RNA results in 24 patients during and after IFN therapy

ALT response	No. of patients	No. (%) of patients negative for HCV RNA			
		0 wk	4 wk	24 wk	Follow-up[a]
Sustained[b]	6	0	5 (83)	5 (83)	4 (67)
Transient[c]	2	0	2 (100)	1 (50)	0
None	16	0	2 (13)	1 (6)	0
Total	24	0	9 (37)	7 (29)	4 (16)

[a] Follow-up was at 6 to 9 months after the end of therapy.
[b] Decrease in ALT levels during treatment and normal ALT levels during Follow-up.
[c] Normalization in ALT levels during treatment and elevation in ALT levels during follow-up.

set. Four patients, which were treated by the experimental scheme, stopped therapy before week 24. Two of them stopped therapy after 12 weeks and one stopped therapy after 20 weeks because ALT remained elevated. One patient stopped therapy at week 20 because of non-compliance.

After 4 weeks of treatment, 9 of the 24 patients (5 in the standard scheme and in the experimental 4 scheme) showed a decrease in HCV RNA and HCV RNA became undetectable by both primer sets. At the end of therapy (week 24), HCV RNA reappeared in two patients (standard scheme) and after 6 to 9 months of follow-up in another three patients (one of the standard scheme, two on the experimental scheme). A normal ALT (< 30 U/l) was observed at week 4, 24 and the follow-up period in respectively 8 (33%), 8 (33%) and 6 (25%) patients, respectively. A simultaneous absence of HCV RNA and a normal ALT level in the follow-up study was limited to four patients (two on the standard scheme, two on the experimental scheme) (Table 1). The rapid decline

in the amount of HCV RNA in responders between weeks 0 and 4 and the lack of an increase in the number of patients with HCV RNA present at a level of less than 1 chimpanzee infectious dose per ml after 4 weeks of treatment are of considerable interest; if confirmed, these findings may have implications for the duration of IFN therapy.

Similar results on the transient nature of HCV RNA disappearance, discrepancies between normalization of ALT levels and the presence of HCV RNA [Bresters et al., 1992; Brillanti et al., 1991], and relapse within 6 months after termination of therapy were also observed by other investigators who used primers directed to the NC or NS4 regions [Chayama et al., 1991; Shindo et al., 1991].

The use of 2 primer sets, NC and NS5, resulted in differences between the outcomes in plasma samples from five patients in our study. The HCV RNA was consistently undetectable with the NS5 primer set in one patient (non-responder; standard scheme). In three other patients (non-responders; two on the standard scheme, one on the experimental scheme), HCV RNA was not detected with the NS5 primer set at week 4 but was detected at week 24 or 48. During therapy, a change in HCV RNA positivity depending on the primer set was observed in one patient (non-responder; experimental scheme).

The NC primer set was very suitable for monitoring HCV RNA levels during IFN therapy [Choo et al., 1991; Han et al., 1991; Kato et al., 1990; Okamoto et al., 1991; Takamizawa et al., 1991; Takeuchi et al., 1990]. The discongruent results for the two sets of primers at the beginning and during therapy could not be the result of a reduced sensitivity of the assay [Cristiano et al., 1991]. In fact, these results are suggestive for the presence of HCV variants in these patients, and the individual sensitivities of these variants for interferon therapy are under investigation.

In conclusion, results of the present study suggest that monitoring of HCV RNA levels in the first months of IFN therapy may be of prognostic value for failure of therapy aimed at eradicating HCV RNA (in the present study four patients [16%]). However, the normalization of ALT levels alone, as observed in an additional two patients, could be beneficial if sustained for long periods of time.

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REFERENCES

Bresters D, Mauser-Bunschoten EP, Cuypers HTM, Lelie PN, Han JH, Jansen PLM, Houghton M, Reesink HW (1992): Disappearance of hepatitis C virus RNA in plasma during interferon alpha-2B treatment in hemophilia patients. Scandinavian Journal of Gastroenterology 27:166-168.

Brillanti S, Garson JA, Tuke PW, Ring C, Briggs M, Masci C, Miglioi M, Barbara L, Tedder RS (1991): Effect of α-interferon therapy on hepatitis C viraemia in community-aquired chronic non-A, non-B hepatitis: a quantitative polymerase chain reaction study. Journal Medical Virology 34:136-141.

Chan SW, McOmish F, Holmes EC, Dow B, Peutherer JF, Yap PL, Simmonds P (1992): Analysis of a new hepatitis C virus type and its phylogenetic relationship to existing variants. Journal of General Virology 73:1131-1341.

Chayama K, Saitoh S, Arase Y, Ikeda K, Matsumoto T, Sakai Y, Kobayashi M, Unikami M, Morinaga T, Kumada H (1991): Effect of interferon administration on serum hepatitis C virus RNA in patients with chronic hepatitis. Hepatology 13:1040-1043.

Chomczynski P, Sacchi N (1987): Single step method of RNA isolation by guanidinium thiocyanate-phenol-chloroform extraction. Analitical Biochemistry 162:156-159.

Choo Q-L, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M (1989): Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 244:359-362.

Choo Q-L, Richman KH, Han JH, Berger K, Lee C, Dong C, Gallegos C, Coit D, Medina-Selby A, Barr PJ, Weiner AJ, Bradley DW, Kuo G, Houghton M (1991): Genetic organization and diversity of the hepatitis C Virus. Proceedings of the National Academy of Sciences USA 88:2451-2455.

Cristiano K, Di Bisceglie AM, Hoofnagle JH, Feinstone S (1991): Hepatitis C viral RNA in serum of patients with chronic non-A, non-B hepatitis: detection by the polymerase chain reaction using multiple primer sets. Hepatology 14:51-55.

Davis GL, Balart LA, Schiff ER, Lindsay K, Bodenheimer HC, Perrillo RP, Carey W, Jacobson IM, Payne J, Dienstag JL, VanThiel DH, Tamburro C, Lefkowitch J, Albrecht J, Meschievitz C, Ortego TJ, Gibas A, and The Hepatitis Interventional Therapy Group (1989): Treatment of

chronic hepatitis C with recombinant interferon alfa: a multicenter randomized, controlled trial. New England Journal of Medicine 321:1501-1506.

Di Bisceglie AM, Martin P, Kassianides C, Lisker-Melman M, Murray L, Waggonar J, Goodman Z, Banks SM, Hoofnagle J (1989): Recombinant interferon alfa therapy for chronic hepatitis C: a randomized, double-blind, placebo-controlled trial. New England Journal of Medicine 321:1506-1510.

Han JH, Shyamala V, Richman KH, Bauer MJ, Irvine B, Urdea MS, Tekamp-Olson P, Kuo G, Choo Q-L, Houghton M (1991): Characterization of the terminal regions of the hepatitis C viral RNA: Identification of conserved sequences in the 5' untranslated region and poly (A) tails at the 3' end. Proceedings of the National Academy of Sciences USA 88:1711-1715.

Hosoda K, Omata M, Yokosuka O, Kato N, Ohto M (1992): Non-A, Non-B chronic hepatitis is chronic hepatitis C: A sensitive assay for detection of hepatitis C virus RNA in the liver. Hepatology 15:777-781.

Jacyna MR, Brooks MG, Loke RTH, Main J, Murray-Lyon IM, Thomas HC (1989): Randomized controlled trial of interferon alfa (lymphoblastoid interferon) in chronic non-A non-B hepatitis. British Medical Journal 298:80-82.

Kato N, Hijikata M, Ootsumaya Y, Nakagawa M, Ohkoshi S, Sugimura T, Shimotohno K (1990): Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proceedings of the National Academy of Sciences USA 87:9524-9528.

Maéno M, Kaminaka K, Sugimoto H, Esumi M, Hayashi N, Komatsu K, Abe K, Sekiguchi S, Yano M, Mizuno K, Shikata T (1990): A cDNA clone closely associated with non-A, non-B hepatitis. Nucleic Acids Research 18:2685-2689.

Marcellin P, Boyer N, Giostra E, Degott C, Couroucé A-M, Degos F, Coppere H, Cales P, Couzigou P, Benhamou JP (1991): Recombinant human α-interferon in patients with chronic non-A, non-B hepatitis: a multicenter randomized controlled trial from France. Hepatology 13:393-397.

Okamoto H, Okada S, Sugiyama Y, Kurai K, Iizuka H, Machida A, Miyakawa Y, Mayumi M (1991): Nucleotide sequence of the genomic RNA of hepatitis C virus isolated from a human carrier: comparison with reported isolates for conserved and divergent regions. Journal of General Virology 72:2697-2704.

Okamoto H, Okada S, Sugiyama Y, Yotsumoto S, Tanaka T, Yoshizawa H, Miyakawa Y, Mayumi M (1990): The 5'-terminal sequence of the hepatitis C virus genome. Japanese Journal of Experimental Medicine 60:167-177.

Sáez-Royuela F, Porres JC, Moreno A, Castillo I, Martinez G, Galiana F, Carreno V (1991): High doses of recombinant α-interferon or γ-interferon for chronic hepatitis C: a randomized, controlled trial. Hepatology 13:327-331.

Shindo M, Di Bisceglie AM, Cheung L, Shih JW, Cristiano K, Feinstone SM, Hoofnagle JH (1991): Decrease in serum hepatitis C viral RNA during alpha- interferon therapy for chronic

hepatitis C. Annals of Internal Medicine 115:700-704.

Takamizawa A, Mori C, Fuke I, Manabe S, Murakami S, Fujita J, E.Onishi, Andoh T, Yoshida I, Okayama H (1991): Structure and organization of the hepatitis C virus genome isolated from human carriers. Journal of Virology 65:1105-1113.

Takeuchi K, Kubo Y, Boonmar S, Watanabe Y, Katayama T, Choo Q-L, Houghton M, Saito I, Miyamura T (1990): The putative nucleocapsid and envelope protein genes of the hepatitis C virus determined by comparison of the nucleotide sequences of two isolates derived from an experimentally infected chimpanzee and healthy human carriers. Journal of General Virology 71:3027-3033.

CHAPTER 4

Sequence analysis of the 5′ untranslated region in isolates of at least 4 genotypes of hepatitis C virus in the Netherlands

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ABSTRACT

The RNAs of hepatitis C virus (HCV) from 62 patients with chronic HCV infection were analyzed by direct sequencing of the 5' untranslated region. Two important sequence motifs were recognized: one between position -170 and -155 and the other between -132 and -117. These motifs are partly complementary. All three previously published genotypes were observed; 34 (55%) isolates were classified as type 1 (including prototype from the [United States] and HCV-BK [from Japan] sequences), 11 (18%) were classified as type 2 (including HC-J6 and HC-J8), and 12 (19%) were classified as type 3 (including EB1); one patient was infected with genotypes 1 and 2. Four (6%) isolates showed aberrant sequences and were therefore provisionally classified as type 4. These results indicate the significance of sequence variation among the 5'untranslated regions of different HCV genotypes and indicate that this region could possibly be used for consistent genotyping of HCV isolates.

INTRODUCTION

Since the discovery of hepatitis C virus (HCV), a flavi-like virus with a positive-sense, single stranded RNA genome of approximately 9.400 nucleotides [Choo et al., 1989], several full length sequences have been obtained from various isolates [Choo et al., 1991; Kato et al., 1990; Okamoto et al., 1991; Okamoto et al., 1992a; Takamizawa et al., 1991] and investigators have proposed [Cha et al., 1992; Chan et al., 1992; Enomoto et al., 1990; Houghton et al., 1991; Mori et al., 1992; Okamoto et al., 1992a] that HCV isolates can be classified into different types and subtypes. On the basis of all available sequence information, Chan et al. [1992] distinguished three HCV types. Type 1 isolates

include the prototype strain HCV-1 [Choo et al., 1991], and strains HCV-H [Ogata et al., 1991], HCV-K1 [Enomoto et al., 1990], HCV-J [Kato et al., 1990] and HCV-BK [Takamizawa et al., 1991]; type 2 includes strains HCV-K2 [Enomoto et al., 1990], HC-J6 [Okamoto et al., 1991] and HC-J8 [Okamoto et al., 1992a]; type 3 includes strains HCV E-b1 [Chan et al., 1992], HCV-T [Mori et al., 1992] and HCV1196 [Lee et al., 1992].

HCV genotyping is of interest in viral transmission studies and HCV epidemiology. Furthermore, the success of interferon treatment may be type or subtype related [Pozatto et al., 1991; Yoshioka et al., 1992].

There are several reasons to choose the 5′ untranslated region (5′ UTR) for HCV genotyping. (i) Analysis of a large number of HCV isolates resulted in similar phylogenetic trees for the 5′ UTR, the core, NS3, and NS5 regions [Chan et al., 1992]; (ii) the observed mutation rate of the 5′ UTR is extremely low [Ogata et al., 1991; Okamoto et al., 1992b]; (iii) sequence variation within the conserved 5′ UTR is mainly limited to specific regions; and (iv) the putative secondary structure of the 5′ UTR, as established from biochemical and phylogenetic data [Brown et al., 1992], suggests functional conservation of this region.

In the study described here, the RNAs of 62 HCV isolates from patients in the Netherlands with chronic HCV infections were investigated to determine whether HCV genotyping by sequence analysis of PCR products derived from the 5′UTR could be performed.

MATERIALS AND METHODS

Patients

Sixty-two patients from the Netherlands between the ages of 26 and 74 years

with elevated alanine aminotransferase (ALT) levels, a biopsy-proven chronic non-A, non-B hepatitis, antibodies to HCV, and no recent history of infection with hepatitis B virus, hepatitis A virus, cytomegalovirus or Epstein-Barr virus were analyzed.

Anti-HCV

Antibodies to HCV were tested by a second generation enzyme immunoassay (Abbott, North Chicago, Ill.) and were confirmed by the recombinant immunoblot assay (RIBA-4, Ortho Diagnostics, Raritan, N.J.) according to the instructions of the manufacturer.

Blood plasma

For HCV RNA detection, EDTA-blood was collected by venipuncture, and plasma was prepared within 2 hours after sampling. One-milliliter aliquots were quickly frozen in liquid nitrogen and stored at -70°C until use.

HCV RNA PCR

HCV RNA was isolated from 100μl of plasma by a modified version of the guanidinium method described previously [Chomczynski & Sacchi, 1987]. cDNA synthesis was performed on one-third of the isolated RNA in a 25 μl reaction volume [Kleter et al., 1993] using 20 pmol antisense primer HCV19 (GTGCACG-GTCTACGAGACCT; positions -1 to -20), 200 U of Moloney murine leukemia virus reverse transcriptase (Gibco-Bethesda Research Laboratories, Gaithersburg, Md.), 30 U RNasin (Promega, Madison, Wis.), and 0.5 mM (each) deoxyribonucleotide (Boehringer, Mannheim, Germany) at 42°C for 30 min after brief denaturation at 80°C. PCR was performed (40 cycles of 1 min at 94°C, 2 min at 48°C, and 3 min at 74°C) with antisense primer HCV19 and the sense primer HCV18 (GGCGACACTCCACCATAGAT; positions -304 to -324) or the

sense primer HCV35 (TTGGCGGCCGCACTCCACCATGAATCACTCCCC; positions -296 to -318; underlined sequences are not complementary to the HCV sequence). For diagnosis, the first round PCR products were analyzed by Southern blot hybridization with probe HCV17 (GAGTAGTGTTGGGTCGCGAA; position -86 to -67); this was followed by washing at low stringency.

Direct sequencing of PCR products

For direct sequencing [Hultman et al., 1991], a second round of PCR (40 cycles of 1 min at 94°C, 2 min at 48°C, and 3 min at 74°C) was performed with the sense primer NCR3 (GGGGCGGCCGCCACCATRRATCACTCCCCTGTGAGG; positions -288 to -314) and the antisense primer LD58 (5′ bio-GGCCGGGGCGGCCGCCAAGCACCCTATCAGGCAGTACCACAAGGC; positions -37 to -64). LD58 is biotinylated at the 5′ end. Biotinylated PCR products (estimated by agarose gelelectrophoresis at approximately 100 ng) were captured onto streptavidin-coated paramagnetic particles (Dynabeads M-280, Dynal, Oslo, Norway). Single-stranded DNA was prepared by denaturation of the captured amplification product by alkaline treatment according to the instructions of Dynal. Separate strands were sequenced by using the T7 DNA sequencing kit (Pharmacia, Uppsala, Sweden) and [^{32}P]dATP (Amersham, Buckinghamshire, United Kingdom). NCR3 served as a sense primer on the minus strand captured on the beads, and NCR4 (CACTCTCGAGCACCCTATCAGGCAGTACC; positions -57 to -29) was used as the antisense primer on the plus strand in the supernatant. DNA sequences were read manually from autoradiographs and were analyzed with the PC/Gene computerprogram (Intelligenetics Inc., Mountain View, Calif.).

Nucleotide sequence accession number

EMBL data library accession numbers X58937 to X58953.

RESULTS

Sixty-two patients with chronic HCV infection were anti-HCV positive by enzyme immunoassay which was confirmed by RIBA-4, and were HCV RNA positive by reverse transcription-PCR aimed at the 5' UTR. The sense primer HCV18, which was based on the first published HCV sequences [Garson et al., 1990; Okamoto et al., 1990], was initially used for the diagnosis of HCV viremia by PCR. In the case of low yields of PCR products, sense primer HCV35 improved the PCR results considerably (data not shown), and in these cases, PCR products obtained by HCV35 were used for sequence analysis.

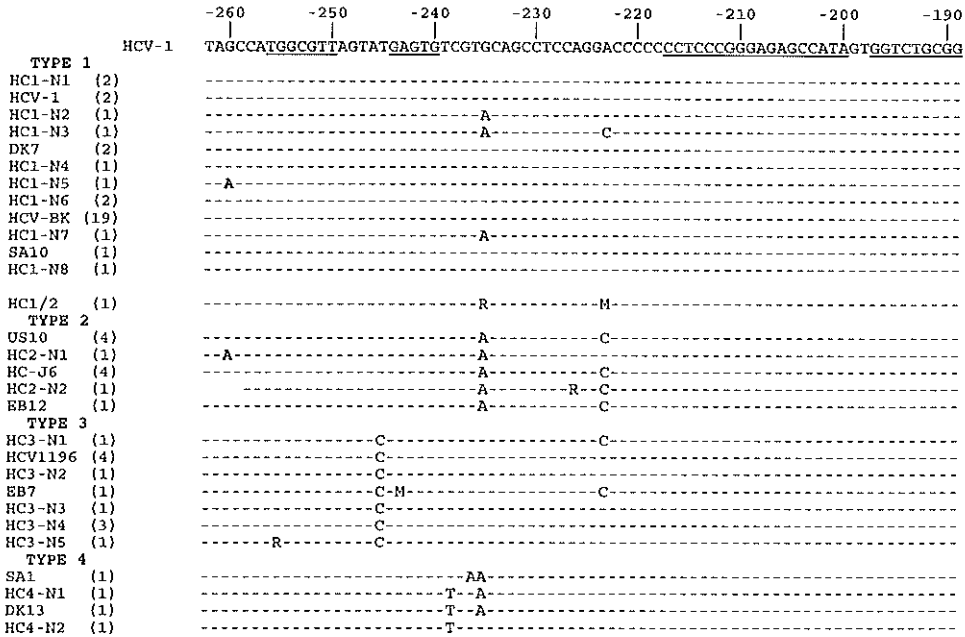
Nested PCR products were sequenced directly, and the results are represented in Fig. 1. If identical sequences were obtained from a number of isolates, only one representative sequence is shown. Several sequences have been reported previously and are identified by their original names. Limited sequence variations were observed essentially in two motifs: motif 1 is located between nucleotides (nt) -170 and -155, and motif 2 is located between nt -132 and -117 (Fig. 1). On the basis these motifs, 58 of 62 isolates could be classified into the three HCV types as proposed previously. Thirty-four (55%) isolates were classified as type 1; this included the prototype sequence HCV-1. Isolate HC1-N8 showed a single nucleotide insertion at position -138. In one patient (isolate HC1/2), a double infection with types 1 and 2 was observed, as deduced from bands with identical mobilities in two different lanes on the gel (Fig. 2). Type 2 sequences, including the sequence of HC-J6, were observed in 11 (18%) patients. These isolates are also recognized by the presence of a T at position -72 and a C at position -80. HC2-N2 contains a mutation at nt -127 in motif 2. Within type 2, sequence heterogeneity was observed at position -119, showing either a T or C. Type 3 sequences were detected in 12 (19%) patients. In addition to the type-specific sequence motifs, type 3 isolates could also be

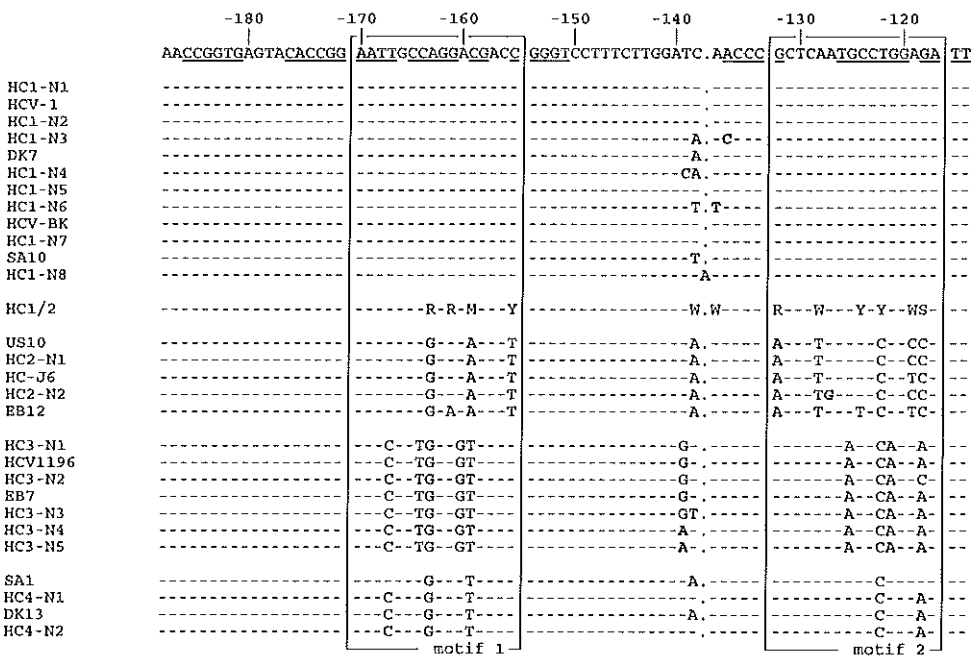
identified by a TCA sequence at positions -93 to -95. HC3-N2 contained a point mutation at position -118 in motif 2. Four (6%) isolates were provisionally classified as type 4. These isolates showed additional sequence heterogeneity between positions -238 and -235 in comparison with the sequences of strains of types 1, 2, and 3.

Table 1. Mean nucleotide variation among four HCV types in the entire 197-bp 5' UTR fragment and motifs 1 and 2.

Fragment and type	No. of isolates*	% nucleotide variation			
		1	2	3	4
197-bp 5'UTR					
1	34	2.5			
2	11	7.1	2.0		
3	12	7.1	11.2	1.0	
4	4	4.1	6.1	6.1	2.0
Motifs 1 and 2					
1	34	0			
2	11	28.0	2.5		
3	12	28.0	35.5	0.3	
4	4	12.5	23.5	15.8	1.5

* Sixty-one HCV isolates were analyzed for sequence variation in 197-bp fragments from the 5' UTR (nt -262 to -66).


-260 -250 -240 -230 -220 -210 -200 -190
HCV-1 TAGCCATGGCGTTAGTATGAGTGTCGTGCAGCCTCCAGGACCCCCCCTCCCGGGAGAGCCATAGTGGTCTGCGG
TYPE 1
HC1-N1 (2)
HCV-1 (2)
HC1-N2 (1)
HC1-N3 (1)
DK7 (2)
HC1-N4 (1)
HC1-N5 (1)
HC1-N6 (2)
HCV-BK (19)
HC1-N7 (1)
SA10 (1)
HC1-N8 (1)
HC1/2 (1)
TYPE 2
US10 (4)
HC2-N1 (1)
HC-J6 (4)
HC2-N2 (1)
EB12 (1)
TYPE 3
HC3-N1 (1)
HCV1196 (4)
HC3-N2 (1)
EB7 (1)
HC3-N3 (1)
HC3-N4 (3)
HC3-N5 (1)
TYPE 4
SA1 (1)
HC4-N1 (1)
DK13 (1)
HC4-N2 (1)


-180 -170 -160 -150 -140 -130 -120
AACCGGTGAGTACACCGG AATTGCCAGGACGACC GGGTCCTTTCTTGGATC.AACCC GCTCAATGCCTGGAGA TT
motif 1
motif 2

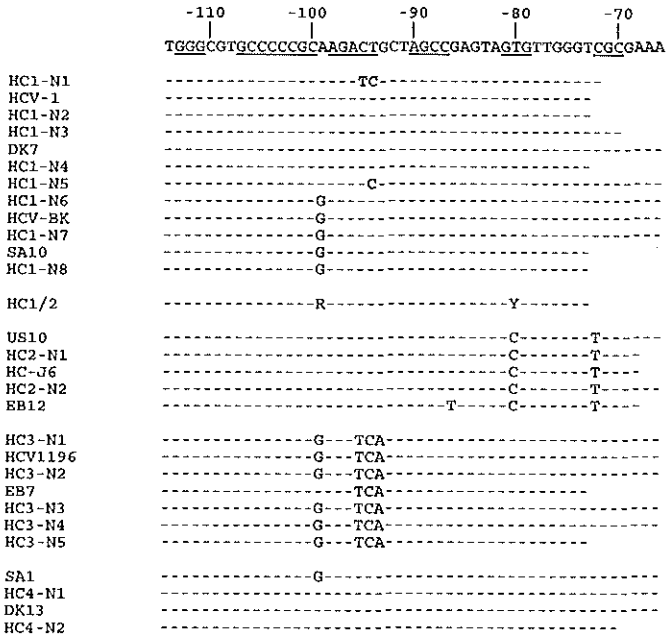


Figure 1. Alignment of 5′ UTR sequences from 62 patients. Sequences between position -262 and -66 were classified into types 1, 2, 3 (as proposed by Chan [1992]), and 4. Previously published sequences are identified by their original names: HCV-1 [Choo et al., 1991]; DK7, SA10, US10, SA1, and DK13 [Bukh et al., 1992]; HCV-BK [Takamizawa et al., 1991]; HC-J6 [Okamoto et al., 1992a]; EB-12 and EB-7 [Chan et al., 1992] and HCV1196 [Lee et al., 1992]. Numbers in parentheses indicate the number of isolates with that sequence. Hyphens indicate presence of nucleotides identical to those of the prototype strains. Characters in boldface type are newly obtained point mutations. Sequence motifs 1 and 2 are boxed. Underlined nucleotides in the HCV-1 sequence are involved in the putative double-stranded RNA stem structure [Brown et al., 1992]. Abbreviations: M (A or C); R (A or G); Y (T or C); W (A or T) and S (G or C).

Comparison between the sequences obtained in the present study (nt -262 to -66) and all previously reported 5' UTR sequences [Bukh et al., 1992; Cha et al., 1992; Chan et al., 1992;Han et al., 1991], revealed a number of new 5' UTR mutations within all HCV types (Fig. 1).

The overall sequence heterogeneity among the four HCV types in the present study ranged from 4% between types 1 and 4 to 11% between types 2 and 3, whereas heterogeniety within each type was less than 2.5% (Table 1). Mutations were not randomly distributed along the 5' UTR but were clustered in motifs 1 and 2. Comparison of these motifs among the four types revealed strong conservation within each type and showed significant differences between the types (Table 1). Further analysis of the sequence variation in motifs 1 and 2 revealed the presence of covariants, i.e., compensatory mutations in each motif, maintaining the postulated secundary structure of the 5' UTR genomic RNA [Brown et al., 1992]. The *P* values for the occurence of one, two, or three covariant mutations in motifs 1 and 2 to have arisen by chance were very low ($P = 0.06$, $P = 0.004$, $P = 0.0002$, respectively). Covariance occurred at positions -164, -163, -161, and -155 in motif 1, and positions -132, -124, -122, and -121 in motif 2. This covariance was consistenly observed in all 62 sequences, indicating the importance of this phenomenon.

DISCUSSION

Sequence analysis of the 5' UTR of HCV isolates from 62 patients with chronic HCV infection allowed consistent and efficient genotyping. Fifty-eight (94%) isolates could be classified into the 3 different types proposed by Chan et al. [1992]. Identification of the HCV types is essentially based on the sequence variation in the defined motifs 1 and 2.

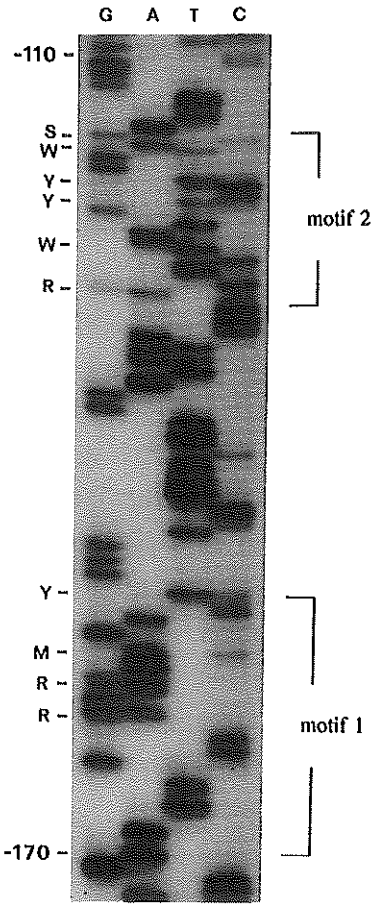


Figure 2. Direct sequence analysis of the 5' UTR from isolate HC1/2 with sense primer NCR3. Abbreviations: S (G or C); W (A or T); Y (T or C); R (A or G) and M (A or C).

All three types reported so far were observed in the HCV-infected patient population in the Netherlands. The coexistence of HCV types in several geographic regions has been indicated earlier Cah et al., 1992; Chan et al., 1992]. However, little is known about the distribution of HCV types in Europe. The ma-

jority of published isolates belong to type 1 [Cuypers et al., 1991; Kremsdorf et al., 1991]. Types 2 and 3 were detected in European isolates only recently [Bukh et al., 1992; Chan et al., 1992].

Isolates belonging to a new HCV type, tentatively designated type 4, were observed in the Netherlands. Similar sequences were first found in isolates from South Africa [Cha et al., 1992] and Denmark [Bukh et al., 1992]. To justify classification of these isolates as a new type 4, sequence analysis of the coding regions is necessary. Preliminary results (data not shown) indicate significant nucleotide sequence differences between the core region from type 4 isolates and the corresponding sequences from type 1, 2, and 3 isolates. One sequence (SA1), classified here as type 4, showed minor differences with the other type 4 sequences, and might be classified as a separate type, HCV type 5 [P. Simmonds, unpublished data]. All types probably have a worldwide distribution, but the relative abundances per geographic region may differ considerably.

A double infection involving types 1 and 2 was found in one patient. This was in accordance with the reported frequency of double infections which has been observed by others [Okamoto et al., 1992c; Yoshioka et al., 1992] by using type-specific primers or probes.

New 5'UTR sequences were observed in 21 of the 62 isolates analyzed. The distribution of the sequence variation was not random. Recently, the putative secundary structure and possible functional elements of the 5' UTR of the HCV genome were postulated [Brown et al., 1992; Tsukiyama-Kohara et al., 1992; Yoo et al., 1992]. The defined sequence motifs 1 and 2 show partial complementarity and are able to form a stable stem-loop structure; e.g., nt A at position -170 is complementary to nt T at position -115 and the nt C (or T for type 2) at position -155 is complementary to nt G (or A for type 2) at position -132. Covariance in motifs 1 and 2 consistently preserves the secundary structure. Therefore, mutations are more likely to be tolerated in single stranded

regions. This is confirmed by the relatively high mutation rate in the single-stranded RNA loop between positions -136 and -151. Most of the point mutations are located outside the defined sequence motifs and do not affect classification. Only two isolates showed single point mutations within the two motifs: HC2-N2 contains a G at position -127, and HC3-N2 contains a C at position -118. Despite these variations, classification of HC2-N2 and HC3-N2 as types 2 and 3, respectively, was obvious.

The overall sequence variation between the 197 bp 5' UTR fragments was not statistically significant, because only 6 to 21 mutations were observed among the four types. Since the mutations were not randomly distributed along the 5' UTR, it is impossible to apply regular statistical methods to this problem, because these require random distribution of events. The secundary structure, i.e., the stem-loop structure formed by partial complementarity of motifs 1 and 2, must be maintained (with a sufficiently low free energy) to function properly as an internal ribosomal entry site [Tsukiyama-Kohara et al., 1992]. Therefore, mutations in these motifs have functional restrictions. Comparison of the 5' UTR sequences described in this report reveals the existence of covariants. These complementary mutations in motifs 1 and 2 indeed completely conserve the secundary structure, indicating the significance of covariance. On the basis of these findings, classification of HCV isolates should preferably be based on covariance in motifs 1 and 2.

In conclusion, consistent genotyping based on 5' UTR sequence analysis is possible and may complement studies on antiviral treatment and the transmission of HCV.

ACKNOWLEDGMENTS

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REFERENCES

Brown EA, Zhang H, Ping L-H, Lemon SM (1992): Secundary structure of the 5' nontranslated regions of hepatitis C virus and pestivirus genomic RNAs. Nucleic Acids Research 20:5041-5045.

Bukh J, Purcell RH, Miller RH (1992): Sequence analysis of the 5' noncoding region of hepatitis C virus. Proceedings of the National Academy of Science USA 89:4942-4946.

Cha T-A, Beall E, Irvine B, Kolberg J, Chien D, Kuo G, Urdea MS (1992): At least five related, but distinct, hepatitis C viral genotypes exist. Proceedings of the National Academy of Science USA 89:7144-7148.

Chan S-W, McOmish F, Holmes EC, Dow B, Peutherer JF, Follett E, Yap PL, Simmonds P (1992): Analysis of a new hepatitis C virus type and its phylogenetic relationship to existing variants. Journal of General Virology 73:1131-1141.

Chomczynski P, Sacchi N (1987): Single step method of RNA isolation by guanidinium thiocyanate-phenol-chloroform extraction. Analitical Biochemistry 162:152-159.

Choo Q-L, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M (1989): Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 244:359-362.

Choo Q-L, Richman KH, Han JH, Berger K, Lee C, Dong C, Gallegos C, Coit D, Medina-Selby A, Barr PJ, Weiner AJ, Bradley DW, Kuo G, Houghton M (1991): Genetic Organization of the hepatitis C virus. Proceedings of the National Academy of Science USA 88:2451-2455.

Cuypers HTM, Winkel IN, van der Poel CL, Reesink HW, Lelie PN, Houghton M, Weiner A (1991): Analysis of genomic variability of hepatitis C virus. Journal of Hepatology. 13 (suppl.4):-S15-S19.

Enomoto N, Takada A, Nakao T, Date T (1990): There are two major types of hepatitis C virus in Japan. Biochem. and Biophys. Res. Comm. 170:1021-1025.

Garson JA, Ring C, Tuke P, Tedder RS (1990): Enhanced detection by PCR of hepatitis C virus RNA. Lancet 336: 878-879.

Han JH, Shyamala V, Richman KH, Brauer MJ, Irvine B, Urdea MS, Tekamp-Olsen P, Kuo G, Choo Q-L, Hougthon M (1991): Characterization of the terminal regions of hepatitis C viral RNA: identification of conserved sequences in the 5' untranslated region and poly(A) tails at the 3' end. Proceedings of the National Academy of Science USA 88:1711-1715.

Houghton M, Weiner A, Han J, Kuo G, Choo Q-L (1991): Molecular biology or the Hepatitis C viruses: Implicationis for diagnosis, development and control of viral disease. Hepatology 14:381-387.

Hultman T, Bergh S, Moks T, Uhlén M (1991): Bidirectional solid-phase sequencing of in vitro-amplified plasmid DNA. BioTechniques 10:84-93.

Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugimura T, Shimotohno K (1990): Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proceedings of the National Academy of Science USA 87:9524-9528.

Kleter GEM, Brouwer JT, Heijtink RA, Schalm SW, Quint WGV (1993): Detection of hepatitis C virus RNA in patients with chronic hepatitis C virus infections during and after therapy with alpha-interferon. Antimicrobial Agents and Chemotherapy 37:595-597.

Kremsdorf D, Porchon C, Brechot C (1991): Hepatitis C virus (HCV)-RNA in non-A, non-B chronic hepatitis in France. Journal of Hepatology 13 (suppl.4):S24-S32.

Lee C-H, Cheng C, Wang J, Lumeng L (1992): Identification of hepatitis C viruses with a nonconserved sequence of the 5' untranslated region. Journal of Clinical Microbiology 30:1602-1604.

Mori S, Kato N, Yagyo A, Tanaka T, Ikeda Y, Petchclai B, Chiewsilp P, Kurimura T, Shimotohno K (1992): A new type of hepatitis C virus in patients in Thailand. Biochemical and Biophysical Research Communications 183:334-342.

Ogata N, Alter HJ, Miller RH, Purcell RH (1991): Nucleotide sequence and mutation rate of the H strain of hepatitis C virus. Proceedings of the National Academy of Science USA 88:3392-3396.

Okamoto H, Okada S, Sugiyama Y, Yotsumoto S, Tanaka T, Yoshizawa H, Tsuda F, Miyakawa Y, Mayumi M (1990): The 5' terminal sequence of the hepatitis C virus genome. Japanese

Journal of Experimental Medicine 60:167-177.

Okamoto H, Okada S, Sugiyami Y, Kurai K, Lizuka H, Machida A, Miyakawa Y, Mayumi M (1991): Nucleotide sequence of the genomic RNA of hepatitis C virus isolated from a human carrier: Comparison with reported isolates for conserved and divergent regions. Journal of General Virology 72:2697-2704.

Okamoto H, Kurai K, Okada S-I, Yamamoto K, Lizuka H, Tanaka T, Fukuda S, Tsuda F, Mishiro S (1992a): Full-length sequence of a hepatitis C virus genome having poor homology to reported isolates: Comparative study of four distinct genotypes. Virology 188:331-341.

Okamoto H, Kojima M, Okada S-I, Yoshizawa H, Lizuka H, Tanaka T, Muchmore EE, Peterson DA, Ito Y, Mishiro S (1992b): Genetic drift of hepatitis C virus during an 8.2-year infection in a chimpanzee: variability and stability. Virology 190:894-899.

Okamoto H, Sugiyama Y, Okada S, Kurai K, Akahane Y, Sugai Y, Tanaka T, Sato K, Tsuda F, Miyakawa Y, Mayumi M (1992c): Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. Journal of General Virology 73:673-679.

Pozatto G, Moretti M, Franzin F, Crocé LS, Tiribelli C, Masayu T, Kaneko S, Unoura M, Kobayashi K (1991): Severity of liver disease with different hepatitis C viral clones. The Lancet 338:509.

Takamizawa A, Mori C, Fuke I, Manabe S, Murakami S, Fujita J, Onishi E, Andoh T, Yoshida I, Okayama H (1991): Structure and organization of the hepatitis C virus genome isolated from human carriers. Journal of Virology 65:1105-1113.

Tsukiyama-Kohara K, Lizuka N, Kohara M, Nomoto A (1992): Internal ribosomal entry site within hepatitis C virus RNA. Journal of Virology 66:1476-1483.

Yohsioka K, Kakumu K, Wakita T, Ishikawa T, Itoh Y, Takayanagi M, Higashi Y, Shibata M, Morishima T (1992): Detection of hepatitis C virus by polymerase chain reaction and response to interferon-α therapy: relationship to genotypes of hepatitis c virus. Hepatology 16:293-299.

Yoo BJ, Spaete RR, Geballe AP, Selby M, Houghton M, Han JH (1992): 5' end-dependent translation initiation of hepatitis C virus RNA and the presence of putative positive and negative translational control elements within the 5' untranslated region. Virology 191:889-899.

CHAPTER 5

Analysis of Hepatitis C virus genotypes by a Line Probe Assay (LiPA) and correlation with antibody profiles

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ABSTRACT

The 5' untranslated regions (5' UTR) derived from 54 patients with a chronic hepatitis C virus infection were analyzed to determine the (sub)type of hepatitis C virus. Labelled polymerase chain reaction products from the 5' UTR were used as probes for reverse hybridization in a Line Probe Assay (Inno-LiPA) and results were validated by comparison with direct sequencing data. Five different genotypes could be distinguished based on 5' UTR sequence diversity. Results of typing by LiPA and direct sequencing were similar. Antibody responses against core, NS-3, NS-4 and NS-5 epitopes were detected by RIBA-4 and Inno-LIA HCV Ab II confirmatory assays. There was no consistent correlation between the genotype and the anti-HCV responses, although types 2 and 3 HCV isolates show poor reactivity with NS-4 epitopes.

INTRODUCTION

Hepatitis C virus (HCV), the main etiological agent of post-transfusion hepatitis, is a small enveloped virus, which contains a positive sense, single-stranded RNA genome of approximately 9400 nucleotides [Choo et al., 1989]. Based on genomic [Miller et al., 1990] and physico-chemical characteristics [Bradley et al., 1985] of the virus, HCV is classified as a distinct member of the Flaviviridae. From worldwide HCV isolates, several full length [Kato et al., 1990a; Takamizawa et al., 1991; Choo et al., 1991; Okamoto et al., 1991, 1992a; Chen et al., 1992] and numerous partial sequences [Chan et al., 1992; Han et al., 1991; Ogata et al., 1991; Takeuchi et al., 1990; Kato et al., 1990b; Mori et al., 1992; Enomoto et al., 1990; Weiner et al., 1991; Lee et al., 1992] have been obtained. Based on sequence diversity, several proposals were made to classify the different HCV isolates [Chan et al., 1992; Enomoto et al., 1990; Okamoto et al., 1992b; Houghton et al., 1991; Cha et al., 1992], but there is no consensus in HCV nomenclature so far. A useful HCV classification was proposed recently [Stuyver et al., 1993], based on phylogenetic trees determi-

ned by Chan and colleagues [Chan et al., 1992], differentiating between types (approx. 68% average sequence homology) as well as between subtypes (approx. 79% sequence homology). Homologies between isolates belonging to the same subtype usually exeed 90%. This system was further extended by a new type, provisionally designated as type 4 [Simmonds et al., 1993].

There are indications that infections caused by different HCV (sub)types may have different clinical implications [Okamoto et al., 1992b]. The effectiveness of antiviral treatment [Kanai et al., 1992; Pozatto et al., 1991; Yoshioka et al., 1992], efficiency of viral transmission, distribution among various patient populations and the development of hepatocellular carcinoma may also be subtype-related. Preliminary results urge HCV subtyping which complements the routine diagnostic antibody and reverse transcription polymerase chain reaction (RT-PCR) assays.

All available sequence data were used to develop type- and subtype-specific probes for the reverse hybridization Line Probe Assay (LiPA), which has recently been described in detail [Stuyver et al., 1993]. The assay is based on the observation that variation within the 5' untranslated region (5' UTR) is mainly restricted to two short (sub)type specific sequence motifs.

In this study HCV isolates were analyzed from 54 patients by the new LiPA which allows classification of isolates into types 1, 2, 3 [Chan et al., 1992; Stuyver et al., 1993], and 4 [Simmonds et al., 1993]. Results of reverse hybridization were compared with data from direct sequencing of the 5' UTR. Furthermore HCV antibody profiles, determined by recombinant immunoblot assay (RIBA)-4 and Inno-LIA HCV Ab II, were compared with the genotyping results.

MATERIALS AND METHODS

Patient sera

Blood samples from 54 patients were obtained by venipuncture. Ethylenediaminetetraacetic acid (EDTA)-plasma was prepared within 2 hours after collec-

tion, aliquotted, quickly frozen in liquid nitrogen and stored at -70°C. All patients had a chronic HCV infection with elevated alanine aminotransferase (ALT) levels, biopsy-proven liver abnormalities and were anti-HCV and HCV-RNA positive [Kleter et al, 1993].

RNA isolation, and RT-PCR

HCV-RNA was isolated from freshly frozen plasma samples by a modified version of the acid guanidinium-phenol-chloroform method as described [Kleter et al., 1993]. Briefly, cDNA was synthesized using antisense primer HCV19 (positions -1 to -20; 5'-GTGCACGGTCTACGAGACCT-3') and amplified by PCR using HCV19 and sense-primer HCV18 (positions -323 to -304; 5'-GGCGACAC-TCCACCATAGAT-3') or HCV35 (positions -318 to -296; TTGGCGGCCGCACT-CCACCATGAATCACTCCCC). PCR was performed for 40 cycles, consisting of 1 min. 94°C., 1 min. 55°C, and 1 min. 72°C. Amplification products were analyzed by agarose gelelectrophoresis and Southern blot hybridization using probe HCV17 (positions -88 to -69; 5'- GAGTAGTGTTGGGTCGCGAA-3').

Line Probe Assay (LiPA)

Based on all available 5' UTR sequences, several type-specific sequence motifs were recognized [see also Stuyver et al., 1993]. Motif 1 is located between positions -170 and -155, and motif 2 between -132 and -117. These motifs already allow discrimination between the different types, are partially complementary and can form a stable dsRNA stem structure [Brown et al., 1992]. A number of positions displaying more subtle, but consistent variations allow consolidation of typing by means of motif 1 and 2, or enable more detailed subtyping.

The LiPA (prototype version, Innogenetics, Ghent, Belgium) is based on the hybridization of labelled PCR amplification products to specific oligonucleotides directed against the variable regions of the 5' UTR. These probes were immobilized as parallel lines on membrane strips (reverse hybridization principle). During nested PCR, the product is biotinylated, which allows detection of hybrids by alkaline phosphatase labelled streptavidin. The HCV-LiPA (Fig. 1)

contains 15 probe lines, exposing 18 different 16-mer probes. Sixteen probes specifically recognize HCV (sub)types, and 2 (no. 21 and 22) are general HCV probes (location of the probes in the 5' UTR sequence is shown in Fig. 2). The development of the LiPA was recently described in detail [Stuyver et al., 1993].

From the first round PCR, 0.5 μl product was transferred into a new, 50 μl nested PCR reaction, containing primers HC3 (sense: -264 to -238: 5'-TCTAGCCATGGCGTTAGTRYGAGTGT-3'), HC4 (antisense: -29 to -54: 5'-CACTCGCAAGCACCCTATCAGGCAGT-3') and biotinylated ^{11}dUTP. The LiPA was performed according to the manufacturer's instructions. Briefly, biotinylated DNA was denatured by mixing 10-20 μl of the nested PCR product with NaOH and hybridized to the probes on the LiPA strip in the presence of tetramethylammoniumchloride. After stringent washing, hybridization was detected by alkaline phosphatase conjugated streptavidin and substrate. The results of the LiPA were determined by scoring the presence or absence of hybridization with each probe line.

Direct sequencing

PCR products were reamplified using sense primer NCR3 (positions -314 to -288; 5'-GGGGCGGCCGCCACCATARRATCACTCCCCTGTGAGG-3'; underlined sequence is non-HCV specific) and LD58 (positions -66 to -35; 5'-Bio-GGCCGG-GGCGGCCGCCAAGCACCCTATCAGGCAGTACCACAAGGC-3') carrying a 5' biotin moiety. Biotinylated nested PCR products were used as template for direct sequencing, using the protocol suggested by the manufacturer of the Dynabeads (Dynal, Norway). Briefly, nested PCR products were mixed with streptavidin-coated paramagnetic particles (Dynabeads M280, Dynal, Norway) in a binding and washing buffer (1x B&W buffer is 10 mM Tris-HCl, pH 7.5, 1 M NaCl, 1 mM EDTA), to allow the binding of the biotinylated DNA. Complementary strands were separated by addition of NaOH, and sequenced using the T7 DNA sequencing kit (Pharmacia, Uppsala, Sweden) and [α-^{32}P]-dATP (Amersham, Buckinghamshire, UK). DNA attached to the beads or in the supernatant was sequenced with either NCR3 as a sense primer on the captured minus-DNA strand or NCR4 (positions -66 to -47; 5'-CACTCTCGAGCACCCTATCAGGCAG-

TACC-3') as an antisense primer on the plus strand in the supernatant. Sequencing products were separated on an 8% polyacrylamide:bisacrylamide gel (19:1 w/w). DNA sequences were read manually from autoradiographs and analyzed with the PC/Gene computer program (Intelligenetics Inc., Mountain View, Calif., USA).

Anti-HCV assays

Antibodies to HCV were assayed by an Enzyme Immunosorbent assay (EIA; Abbott Chicago, Il, USA) and confirmed by RIBA-4 (Ortho diagnostics, Raritan, NJ, USA) and Inno-LIA HCV Ab II (Innogenetics, Gent, Belgium) according to the manufacturer's instructions.

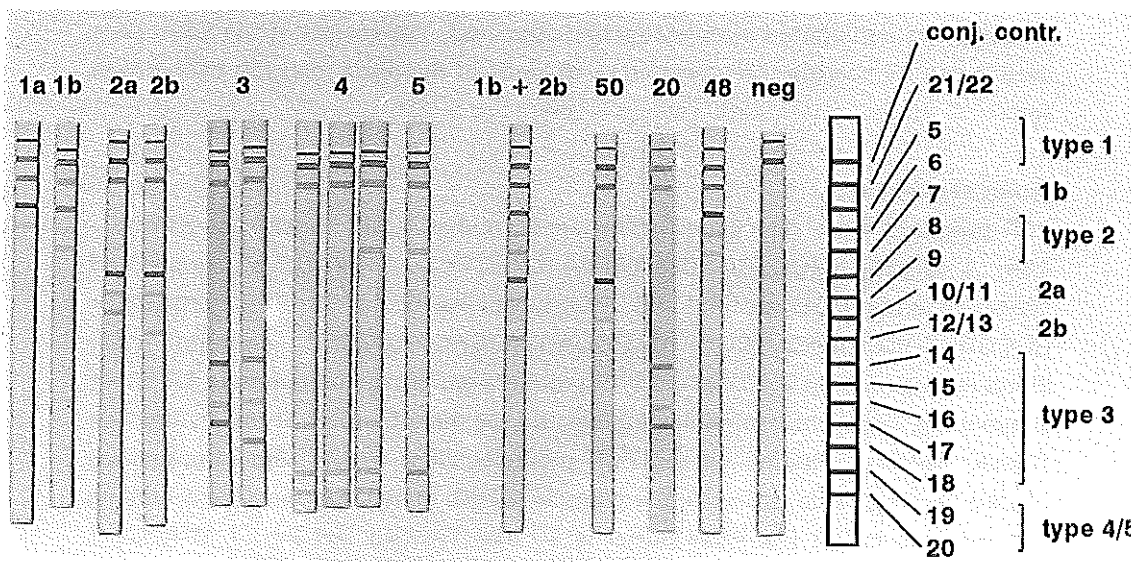


Figure 1. Positions of the probe lines on the LiPA strip. Probes 21 and 22 are general probes to identify general 5' UTR sequences. Probes 5-20 distinguish different types and subtypes of HCV as indicated. Representative results of isolates containing types 1a, 1b, 2a, 2b, and 3 are shown, together with all type 4 isolates. One isolate is provisionally classified as type 5. Furthermore, isolates 50 and 20, displaying sequence-specific variation and the single discrepant isolate 48, are shown.

RESULTS

HCV-RNA was isolated from 54 patients with a chronic HCV infection, and amplified by RT-PCR using 5' UTR specific primers. These PCR products were analyzed by direct sequencing as described elsewhere [Kleter et al., 1994]. Biotinylated amplification products from nested reactions were used as target to the probes in the reverse hybridization LiPA system. The location of the probes on the LiPA strip and typical LiPA results are shown in Fig. 1.

All labelled nested PCR products hybridized to the general probes 21/22 and with a subset of (sub)type-specific probes. Results of LiPA analysis of 54 patient isolates are summarized in Table 1. Four different types were detected and there were no isolates that could not be typed by LiPA. Observed hybridization patterns were consistent and classification into types and subtypes was obvious for all isolates, including one double infection with subtypes 1b and 2b. One isolate contained the SA1 sequence. This sequence can be classified provisionally as type 5 [Dr. P. Simmonds, personal communication].

Table 1. HCV types determined by the LiPA in 54 isolates

Type	No. of isolates	%
1a	7	13
1b	24	44
2a	7	13
2b	1	2
3	10	18
4	3	6
5	1	2
1b + 2b	1	2

Probes 17 and 18 were aimed to discriminate further between type 3 isolates, based on the presence of a G or A at -139 respectively. However, most type 1 isolates cross-reacted with probe 17, and to a lesser extent with probe 18. Overall background staining on the strips was very low or completely absent.

Reverse hybridization results from LiPA were compared with data obtained from direct sequencing. Sequences were determined on nested products derived from the same first round PCR products as used for LiPA. 5' UTR sequences and the positions of LiPA probes are shown in Fig. 2. Comparison of LiPA and direct sequencing results revealed a small discrepancy in only one sample. Amplified HCV-cDNA from this isolate (no. 48, containing the DK7 sequence figure 2.) hybridized with probe 7 (Fig. 1), indicating the presence of a G at position -99, but direct sequencing showed only an A. This isolate is therefore classified as 1b by LiPA but as 1a by direct sequencing. On the other hand, some aberrant hybridization patterns observed in LiPA were validated by direct sequencing results. Isolate 50 (containing sequence HC2-N2) failed to hybridize with probe 9. This is in perfect agreement with the presence of a G at position -127. Isolate 20 (containing sequence HC3-N2) hybridized only very weakly to type 3-specific probe 15, which can be explained by the presence of a C at position -118. The presence of these mutations in regions used for typing never complicated the classification because 2 or 3 type-specific probes are present in the LiPA. Probe 19 contains a C/T degeneracy at position -167, which allows hybridization to all type 4 sequences in this study, including SA1. Probe 20 does not hybridize to the SA1 sequence. In a number of isolates additional sequence variation outside the defined motifs 1 and 2 was detected by direct sequencing, but this did not affect the classification by the LiPA.

Antibodies against various HCV epitopes were assayed by RIBA-4 and LIA II. Results are shown in Table 2. One patient was negative by RIBA and LIA. One was indeterminate by LIA, as only a weak anti-core reaction was detected. From the single anti-HCV-negative sample, weak HCV-RNA signals were obtained. However, a sample from this patient, obtained one month earlier, was anti-HCV positive in RIBA. Only one patient specifically lacked core antibodies in

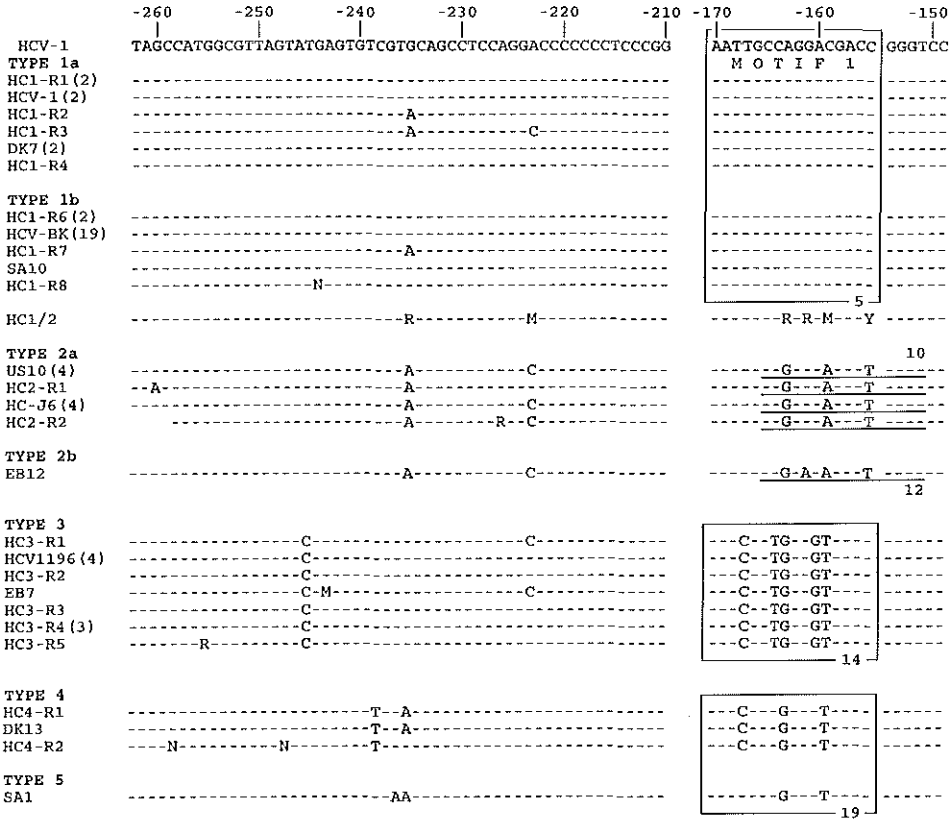
Table 2. Anti-HCV profiles of 54 patient isolates determined by RIBA-4 and Inno-HCV LIA HCV Ab II

		RIBA-4					Inno-LIA			
Type	# pat.	511 (NS4)	C-100 (NS4)	C33c (NS3)	C22-3 (core)	total	NS-4	NS-5	core	total
1a	8	8	8	8	8	8	8	5	8	8
1b	23	22	19	22	22	22	22	13	22	22
2a	7	1	2	7	7	7	2	5	6	6
2b	1	0	0	1	1	1	0	1	1	1
3	10	0	4	10	10	10	2	7	10	10
4	3	0	1	3	3	3	2	2	3	3
5	1	1	1	1	1	1	1	1	1	1
1+2	1	0	0	1	1	1	0	1	1	1

Anti-HCV detection by RIBA and LIA. 5-1-1 and C-100 are derived from the NS-4 region. C33c contains NS-3 and C22-3 covers core epitopes. Numbers in columns represent samples with a positive reaction against the specified epitope.

both confirmatory assays; all other anti-HCV positive isolates were anti-core positive in both assays. All patients contained anti-NS-3 antibodies as determined by RIBA.

Type 2- and type 3-infected patients showed a low response rate to type 1 NS-4 epitopes, both in RIBA and LIA. NS-5 antibodies were detected by LIA in the majority of patients. The only double infection isolate did not contain antibodies against NS-4 epitopes.


-260
-250
-240
-230
-220
-210
-170
-160
-150
HCV-1
TAGCCATGGCGTTAGTATGAGTGTCGTGCAGCCTCCAGGACCCCCCCTCCCGG
AATTGCCAGGACGACC
GGGTCC
M O T I F 1
TYPE 1a
HC1-R1(2)
HCV-1(2)
HC1-R2
HC1-R3
DK7(2)
HC1-R4
TYPE 1b
HC1-R6(2)
HCV-BK(19)
HC1-R7
SA10
HC1-R8
5
HC1/2
R-R-M-Y
TYPE 2a
10
US10(4)
HC2-R1
HC-J6(4)
HC2-R2
TYPE 2b
EB12
12
TYPE 3
HC3-R1
HCV1196(4)
HC3-R2
EB7
HC3-R3
HC3-R4(3)
HC3-R5
14
TYPE 4
HC4-R1
DK13
HC4-R2
TYPE 5
SA1
19

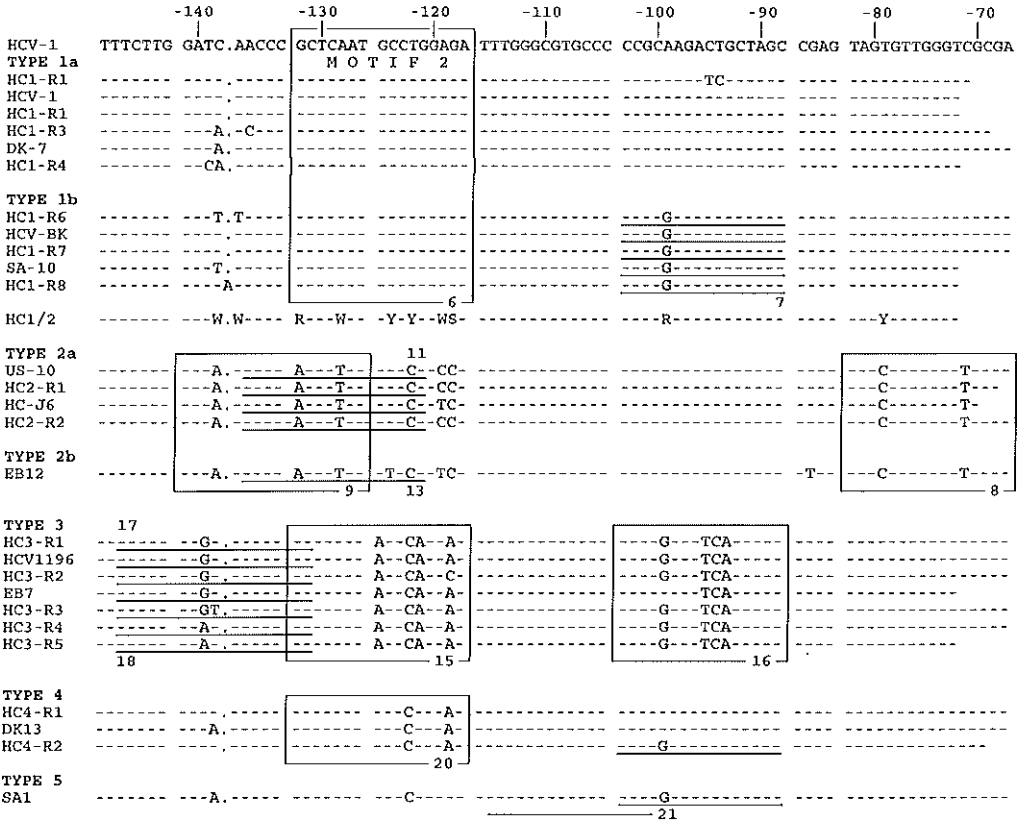


Figure 2. Alignment of 5' UTR sequences (positions -265 to -68) and positions of the Line Probe Assay (LiPA) probes. Sequences between positions -210 and -170 are completely conserved and omitted from the figure. Sequences are grouped into 5 different types. HCV-1 is the prototype sequence [Choo et al., 1990]. DK-7, SA10, US10, and DK13 [Bukh et al., 1992], HC-J6 [Okamoto et al., 1992], EB-7, EB-12 [Chan et al., 1992] were previously reported and identified by their original name. The positions of the LiPA probes 5-20 are identified by boxes type-specific) or are underlined (subtype-specific). General probe 21 is underlined and probe 22 (positions -178 to -194) is not shown in this figure. The SA1 sequence is tentatively classified as type 5.

DISCUSSION

This study describes the use of a new reverse hybridization Line Probe Assay to determine the (sub)type of HCV in 54 well characterized patient plasma isolates. The system was evaluated by direct sequening and results are markedly similar.

There are several advantages in using the 5' UTR of the HCV RNA genome for genotyping. First, the 5' UTR is generally used in diagnostic PCR assays with universal primers to detect viremia. Therefore it is convenient to perform subsequent typing analysis on the resulting DNA product. Secondly, the sequence variations within the 5' UTR are limited to specific regions, such as motifs 1 and 2, located between highly conserved flanking sequences. This allows the use of general PCR primers as well as (sub)type-specific probes. This relatively high degree of conservation in the 5' UTR omits the necessity of type-specific primers to classify HCV using more variable coding regions of the genome [Okamoto et al., 1992b]. A large number of 5' UTR sequences has been published so far [Chan et al., 1992; Stuyver et al., 1993; Bukh et al., 1992]. Discrimination between types has been described using amplified cDNA derived from the 5' UTR for restriction fragment length polymorphism (RFLP) [Simmonds et al., 1993; Nakao et al., 1991; McOmish et al., 1993]. Furthermore, the overall mutation rate of HCV has been estimated at approximately 1.5 x 10^{-3} base substitutions per site per year [Okamoto et al., 1992c; Ogata et al., 1991], but mutations occur unevenly along the genome. A hypervariable region located at the N-terminus of E2/NS1 has a high mutation rate, whereas the 5' UTR has a very low rate. Studies on genetic drift of HCV for more than 8 years in a chronically infected chimpanzee [Okamoto et al., 1992c] and over 13 years in a chronic patient [Ogata et al., 1991] showed complete conservation of the 5' UTR. Finally, the putative secondary structure of the 5' UTR [Simmonds et al., 1993; Brown et al., 1992; Tsukiyama-Kohara et al., 1992] implies functional conservation with respect to initiation of translation of the single open reading frame and genome replication [Yoo et al., 1992]. Therefore, there is probably high selective pressure on the function of this region. This is indicated by the existence of paired mutations (co-variants) in the complementary strands in the

stem of the putative RNA-hairpin. This covariance, observed among different types, conserves secondary RNA structure of motifs 1 and 2 [Simmonds et al., 1993]. Extensive sequence comparisons have shown [Chan et al., 1992] that sequence heterogeneity in the 5' UTR displays similar phylogenetic relationships between HCV types as other regions such as core, NS3 and NS5. Therefore, sequence analysis of the 5' UTR should allow consistent discrimination of HCV types.

HCV-RNA isolates from 54 chronic HCV patients were genotyped by 5' UTR analysis. Amplified cDNA from the 5'UTR was biotin-labelled during nested PCR and used as a probe for reverse hybridization in the LiPA. Reverse hybridization offers a fast method of screening for the presence of specific sequences in a PCR product. The probes used in the LiPA described here cover a considerable part of the entire 5' UTR sequence. The LiPA contains both general and (sub)type specific probes and therefore allows detection of known as well as unknown HCV types. Each of the 54 isolates described here hybridized to the general probes and could be further (sub)typed by LiPA. New HCV types will fail to hybridize with the current (sub)typing probes on the strip, but will hybridize to the general probes. Aberrant hybridization patterns can also be observed on the LiPA strip, as shown in isolates 20 and 50 (Fig. 2). Therefore the LiPA provides an instrument for rapid identification of new HCV types or subtypes.

To evaluate the efficiency of the LiPA system, the presence or absence of hybridization to each probe was compared with the corresponding results from direct sequencing. Single nucleotide differences were efficiently detected, e.g. to discriminate between type 2a and 2b by probes 10, 11, 12, and 13. In addition to published sequences, new variations were also detected by LiPA and confirmed by direct sequencing, as observed in isolates containing sequences HC3-N2 and HC2-N2. There was only one discrepancy between LiPA and direct sequencing with isolate 48, although it was typed correctly as type 1 by LiPA. The question remains whether this isolate contains 1a or 1b sequences. A possible explanation is a co-infection with types 1a and 1b. A large excess of 1a sequences over 1b sequences could explain the failure of direct sequencing to detect both A and G at position -99 and the weak hybridization of the PCR

product with probe 7. The directly obtained sequence represents the major sequence present in an isolate. Furthermore the LiPA identifies subtype 1a based on absence of hybridization with probe 7. Positive identification of each subtype could further improve the reliability of the LiPA. Probes 17 and 18 cross-reacted with most of the type 1 isolates. The reason for this is unclear. The value of probes 17 and 18 in discriminating between the presence of a G or A at position -139, is doubtful. The target sequence for these probes is located in the region forming a single stranded RNA loop in the putative secundary structure of the genomic RNA [Brown et al., 1992]. This might also explain the relatively high frequency of mutations detected in this region, including the insert in HC1-N8. Four isolates could not be classified as type 1, 2 or 3. Three of those are classified as type 4 and one (containing sequence SA1) is tentatively designated as type 5. More data from these isolates are necessary to justify this provisional classification.

One double infection with subtypes 1b and 2b was detected (Fig. 1 and 2; isolate HC1/2) both by LiPA and direct sequencing. It is much easier, both to detect and (sub)type the double infection by LiPA than by direct sequencing. Interpretation of double signals at one nucleotide position by direct sequencing can be difficult and may require advanced experience in reading sequence autoradiographs.

Although the version of the LiPA described here did not yet contain type 5 specific probes, it was possible to discriminate between type 4 and 5 sequences because probe 19 contains a degeneracy. Addition of type 5 specific probes onto the LiPA strip will further facilitate recognition of this type.

Profiles of antibodies against specific HCV epitopes were determined. Two confirmatory immunoblot assays, capable to detect antibodies against epitopes from core, NS-3, NS-4 and NS-5, could not discriminate between different types. RIBA-4 uses expression products of recombinant cDNA clones, derived from various parts of the HCV genome, whereas the Inno-LIA exposes a number of synthetic peptides. At present, as many more sequences of types 1, 2 and 3, and 4 became available, significant sequence heterogeneity in various parts of the genome [Chan et al., 1992; Cha et al., 1992] may be observed.

Consequently, infection with other HCV types may evoke antibodies against type-specific epitopes, which are not optimally recognized by type 1 epitopes in the RIBA and LIA. This is illustrated by the absence of antibodies against NS-4 epitopes in most type 2 and 3 isolates (Table 2) [Chan et al., 1991]. Antibodies against 5-1-1 are even completely absent in type 3 isolates. The single indeterminate LIA result on an isolate classified as type 2a may be partly explained by type-specific immune reactions.

Antibodies against core and NS-5 epitopes show a higher degree of cross-reactivity. It was possible to distinguish a number of type 1 and 2 isolates by assaying anti-core antibodies, directed against 2 different type-specific core peptides [Machida et al., 1992], although specificity was limited. Type-specific serological assays would considerably facilitate discrimination between different HCV types. Sequence information of antigenic regions is still limited. Therefore, many more sequence data from all different types must be obtained, to develop (sub)type-specific antigens for antibody assays. However, there are considerable differences in immune reactivity among individual patients. In some patients, antibody responses are poor; e.g. in isolate 23, antibodies against core were undetectable. Also in immunocompromised patients serological testing is difficult, due to lack of sufficient antibody titers. These conditions prevent serological HCV typing.

In summary, sequence analysis of the 5' UTR of HCV allows consistent genotyping of all presently known HCV isolates. Furthermore, reverse hybridization systems in a strip test format like the described LiPA, provide fast and reliable typing of HCV isolates and identification of new HCV types. This assay allows the use of the amplification products from 5' UTR after RT-PCR assays and therefore conveniently complements the routine HCV diagnosis. Screening of large patient populations is feasible, and could lead to rapid identification of new HCV types.

REFERENCES

Bradley DW, McCaustland KA, Cook EH, Schable CA, Ebert JW, Maynard JE (1985): Posttransfusion non-A, non-B hepatitis in chimpanzees. Physicochemical evidence that the tubule forming agent is a small, enveloped virus. Gastroenterology 88: 773-779.

Brown EA, Zhang H, Ping L-H, Lemon SM (1992): Secundary structure of the 5' nontranslated regions of hepatitis C virus and pestivirus genomic RNAs. Nucleic Acids Research 20, 19: 5041-5045.

Bukh J, Purcell RH, Miller RH (1992): Sequence analysis of the 5' noncoding region of hepatitis C virus. Proceedings of the National Academy of Sciences USA 89: 4942-4946.

Cha T-A, Beall E, Irvine B, Kolberg J, Chien D, Kuo G, Urdea MS (1992): At least five related, but distinct, hepatitis C viral genotypes exist. Proceedings of the National Academy of Sciences USA 89: 7144-7148.

Chan SW, Simmonds P, McOmish F, Yap PL, Mitchell R, Dow B, Follett E (1991): Serological responses to infection with three different types of hepatitis C virus. The Lancet 338: 1391.

Chan S-W, McOmish F, Holmes EC, Dow B, Peutherer JF, Follett E, Yap PL, Simmonds P (1992): Analysis of a new hepatitis C virus type and its phylogenetic relationship to existing variants. Journal of General Virology 73: 1131-1141.

Chen P-J, Lin M-H, Tai K-F, Liu P-C, Lin C-J, Chen D-S (1992): The Taiwanese hepatitis C virus genome: Sequence determination and mapping the 5' termini of viral genomic and antigenomic RNA. Virology 188: 102-113.

Choo Q-L, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M (1989): Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 244: 359-362.

Choo Q-L, Richman KH, Han JH, Berger K, Lee C, Dong C, Gallegos C, Coit D, Medina-Selby A, Barr PJ, Weiner AJ, Bradley DW, Kuo G, Houghton M (1991): Genetic Organization of the hepatitis C virus. Proceedings of the National Academy of Sciences USA 88: 2451-2455.

Enomoto N, Takada A, Nakao T, Date T (1990): There are two major types of hepatitis C virus in Japan. Biochemical and biophysical research communications 170, 3: 1021-1025.

Han JH, Shyamala V, Richman KH, Brauer MJ, Irvine B, Urdea MS, Tekamp-Olsen P, Kuo G, Choo Q-L, Houghton M (1991): Characterization of the terminal regions of hepatitis C viral RNA: Identification of conserved sequences in the 5' untranslated region and poly(A) tails at the 3' end. Proceedings of the National Academy of Sciences USA 88: 1711-1715.

Houghton M, Weiner A, Han J, Kuo G, Choo Q-L (1991): Molecular biology or the Hepatitis C viruses: Implications for diagnosis, development and control of viral disease. Hepatology 14, 2: 381-387.

Kanai K, Kako M, Okamoto H (1992): HCV genotypes in chronic hepatitis C and response to interferon. The Lancet 339: 1543.

Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugimura T, Shimothono K (1990a): Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proceedings of the National Academy of Sciences USA 87: 9524-9528.

Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Shimotohno K (1990b): Sequence diversity of hepatitis C viral genomes. Molecular Biological Medicine 7: 495-501.

Kleter GEM, Brouwer JT, Heijtink RH, Schalm SW, Quint WGV (1993): Detection of hepatitis C virus RNA in patients with chronic hepatitis C virus infections during and after therapy with alpha-interferon. Antimicrobial Agents and chemotherapy 37, 3: 595-597.

Kleter, GEM, van Doorn LJ, Brouwer JT, Schalm SW, Heijtink RH, and Quint, WGV (1994): At least 4 types of hepatitis C virus in the Netherlands: sequence analysis of the 5' untranslated region. Journal of Clinical Microbiology, in press.

Lee C-H, Cheng C, Wang J, Lumeng L (1992): Identification of hepatitis C viruses with a nonconserved sequence of the 5' untranslated region. Journal of Clinical Microbiology 30: 1602-1604.

Machida A, Ohnuma H, Tsuda F, Munekata E, Tanaka T, Akahane Y, Okamoto H, Mishiro S (1992): Two disctinct subtypes of hepatitis C virus defined by antibodies directed to the putative core protein. Hepatology 16: 886-891.

McOmish F, Chan SW, Dow BC, Gillon J, Frame WD, Crawford RJ, Yap PL, Follet EAC, Simmonds P (1993): Detection of three types of hepatitis C virus in blooddonors: investigation of type-specific differences in serological reactivity and rate of alanine aminotransferase abnormalities. Transfusion, in press.

Miller RH, Purcell RH (1990): Hepatitis C virus shares amino acid sequence similarity with Pestiviruses and Flaviviruses as well as members of two plant virus supergroups. Proceedings of the National Academy of Science USA 87: 2057-2061.

Mori S, Kato N, Yagyo A, Tanaka T, Ikeda Y, Petchclai B, Chiewsilp P, Kurimura T, Shimotohno K (1992): A new type of hepatitis C virus in patients in Thailand. Biochemical and biophysical research communications 183, 1: 334-342.

Nakao T, Enomoto N, Takada N, Date T (1991): Typing of hepatitis C virus genomes by restriction length polymorphism. Journal of General Virology 72: 2105-2112.

Okamoto H, Okada S, Sugiyami Y, Kurai K, Lizuka H, Machida A, Miyakawa Y, Mayumi M (1991): Nucleotide sequence of the genomic RNA of hepatitis C virus isolated from a human carrier: Comparison with reported isolates for conserved and divergent regions. Journal of General Virology 72: 2697-2704.

Okamoto H, Kurai K, Okada S-I, Yamamoto K, Lizuka H, Tanaka T, Fukuda S, Tsuda F, Mishiro S (1992a): Full-length sequence of a hepatitis C virus genome having poor homology to reported isolates: Comparative study of four distinct genotypes. Virology 188: 331-341.

Okamoto H, Sugiyama Y, Okada S, Kurai K, Akahane Y, Sugai Y, Tanaka T, Sato K, Tsuda F,

Miyakawa Y, Mayumi M (1992b): Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. Journal of General Virology 73: 673-679.

Okamoto H, Kojima M, Okada S-I, Yoshizawa H, Iizuka T, Tanaka E, Muchmore E, Peterson DA, Ito Y, Mishiro S (1992c): Genetic drift of hepatitis C virus during an 8.2-year infection in a chimpanzee: variability and stability. Virology 190: 894-899.

Ogata N, Alter HJ, Miller RH, Purcell RH (1991): Nucleotide sequence and mutation rate of the H strain of hepatitis C virus. Proceedings of the National Academy of Sciences USA 88: 3392-3396.

Pozatto G, Moretti M, Franzin F, Crocé LS, Tiribelli C, Masayu T, Kaneko S, Unoura M, Kobayashi K (1991): Severity of liver disease with different hepatitis C viral clones. The Lancet 338: 509.

Stuyver L, Rossau R, Wyseur A, Duhamel M, Vanderbroght B, Van Heuverswyn H, Maertens G (1993): Typing of HCV isolates and characterization of new (sub)types using a line probe assay. Journal of General Virology 74, 1093-1102.

Simmonds P, McOmish F, Yap PL, Chan SW, Lin CK, Dusheiko G, Saeed AA, Holmes EC (1993): Sequence variability in the 5' non-coding region of hepatitis C virus: identification of a new virus type and restrictions on sequence diversity. Journal of General Virology 74:661-668.

Takamizawa A, Mori C, Fuke I, Manabe S, Murakami S, Fujita J, Onishi E, Andoh T, Yoshida I, Okayama H (1991): Structure and organization of the hepatitis C virus genome isolated from human carriers. Journal of Virology 65: 1105-1113.

Takeuchi K, Kubo Y, Boonmar S, Watanabe Y, Katayama T, Choo, Q-L, Kuo G, Houghton M, Saito I, Miyamury T (1990): Nucleotide sequence of core and envelope genes of the Hepatitis C virus genome derived directly from human healthy carriers. Nucleic acids Research 18, 15: 4626.

Tsukiyama-Kohara K, Iizuka N, Kohara M, Nomoto A (1992): Internal ribosome entry site within Hepatitis C virus RNA. Journal of Virology 66: 1476-1483.

Weiner AJ, Christopherson C, Hall JE, Bonino F, Saracco G, Brunetto MR, Crawford K, Marion CD, Crawford KA, Venkatakrishna S, Miyamura T, McHutchinson J, Cuypers T, Houghton M (1991): Sequence variation in hepatitis C viral isolates. Journal of Hepatology 13 (suppl. 4): S6-S14.

Yohsioka K, Kakumu K, Wakita T, Ishikawa T, Itoh Y, Takayanagi M, Higashi Y, Shibata M, Morishima T (1992): Detection of hepatitis C virus by polymerase chain reaction and response to interferon-α therapy: relationship to genotypes of hepatitis C virus. Hepatology 16, 2: 293-299.

Yoo BJ, Spaete RR, Geballe AP, Selby M, Houghton M, Han JH (1992): 5' end-dependent translation initiation of hepatitis C viral RNA and the presence of putative positive and negative translational control elements within the 5' untranslated region. Virology 191: 889-899.

CHAPTER 6

Sequence analysis of hepatitis C virus genotypes 1 to 5 reveals multiple novel subtypes in the Benelux countries

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ABSTRACT

Hepatitis C virus (HCV) isolates from a cohort of 315 patients from the Benelux countries (Belgium, The Netherlands, Luxembourg) with a chronic infection were genotyped by means of reverse hybridization Inno-LiPA (Line Probe Assay). HCV (sub)types 1a, 1b, 2a, 2b, 3a, 4a, and 5a were detected. From the cohort, isolates representing all types and those showing an aberrant LiPA pattern were further analyzed by sequencing parts of the 5' UTR, the core (nt 1 to 326: aa residues 1 to 108) and core/E1 (nt 477 to 924: aa residues 159 to 308) regions. Typing by LiPA was completely confirmed by 5' UTR sequencing. Molecular evolutionary analysis of the core and the core/E1 regions allowed discrimination between known and additional subtypes, especially within types 2 and 4. The core region is not suitable for classification of new subtypes because of the relatively high level of conservation. The core/E1 region displays a higher level of sequence variation and allows much more distinct discrimination between subtypes. Types 2 and 4 are particularly heterogeneous, with at least 7 and 10 subtypes, respectively. In contrast to previous reports from Europe, HCV isolates from this study cohort constituted a highly heterogeneous population of virus variants, especially within types 2 and 4.

INTRODUCTION

Hepatitis C virus (HCV), the major etiologic agent of parenterally transmitted non-A, non-B hepatitis [Choo et al., 1989; Kuo et al., 1989], is classified as a separate genus within the Flaviviridae [Miller & Purcell, 1990; Houghton et al., 1991]. The virus contains a positive-sense, single stranded RNA genome of approximately 9400 nt. Sequence comparisons revealed the existence of

multiple HCV strains or types. Since there are no methods available for biological characterization of HCV, due to the lack of an in vitro culture system, classification relies almost entirely upon nucleotide sequence analysis. Recently, a classification system has been proposed [Chan et al., 1992; Simmonds et al., 1994a; Stuyver et al., 1993], differentiating between types, subtypes and isolates. HCV genotyping may have clinical relevance, such as the efficacy of interferon therapy [Takada et al., 1992; Yoshioka et al., 1992; Tsubota et al., 1994].

Analysis of different HCV strains can be performed by either serotyping or genotyping. Current serotyping methods allow identification of the major viral types [Simmonds et al., 1993b; Tsukiyama-Kohara et al., 1993] but do not discriminate between subtypes. Genotyping would be most reliable if complete genomes were analyzed. However, since this is not feasible on a routine basis, genotyping can also be accomplished by partial analysis of the genome, such as the 5'UTR [Stuyver et al., 1993; Simmonds et al., 1993a] and the core region [Okamoto et al., 1992b]. Phylogenetic studies of the core [Bukh et al., 1994], the E1 [Bukh et al., 1993] and the NS5 regions [Chayama et al., 1993; Simmonds et al., 1994b] suggested that any region of the genome could be used for classification of HCV isolates [Chan et al., 1992], although the level of sequence heterogeneity differs considerably between different parts of the genome. More detailed studies indicated that the NS5B is probably most suited for classification of novel isolates [Stuyver et al., 1994].

A reverse hybridization Line Probe Assay (LiPA) has been developed for genotyping of HCV isolates by 5' UTR analysis [Stuyver et al., 1993; van Doorn et al., 1994a]. The 5'UTR is highly conserved but shows significant sequence variation, mainly in two small 'motifs' (nt -132 to -117 and nt -170 to -155) [Kleter et al., 1994; van Doorn et al., 1994a]. 5'UTR sequences of types 1 to 6 as well as of several subtypes are distinct, which allows the use of this region

for HCV genotyping [Stuyver et al., 1994].

In this study, 315 HCV isolates, obtained from patients living in the Benelux area of Western Europe were all genotyped by LiPA. Randomly selected isolates as well as isolates showing an aberrant LiPA pattern were subjected to sequence analysis of the 5'UTR, N-terminal core and part of the core/E1 regions, in order to validate the 5'UTR classification in coding regions of the genome and to determine the genomic variability of HCV isolates from the Benelux region.

MATERIALS AND METHODS

Patients

HCV isolates described in this study were obtained from participants in the trial for treatment with interferon-*α*, coordinated by the Benelux Study Group on treatment of chronic hepatitis C. All patients resided in either Belgium, the Netherlands or Luxembourg (Benelux), although their ethnic origin was diverse. All samples used in this study were obtained before the onset of therapy.

HCV-RNA detection and genotyping

HCV RNA was isolated by a modification of the guanidinium thiocyanate method [Chomczynski & Sacchi, 1987; Kleter et al., 1993]. A small number of heparinized plasma samples were analyzed by an HCV RNA capture method as described earlier [van Doorn et al., 1994b] since these samples could not be analyzed by conventional methods [Willems et al., 1993]. Detection of HCV RNA was performed by RT-PCR with primers HCV35 (sense, positions -318 to -296; 5'-TTGGCGGCCGCACTCCACCATGAATCACTCCCC-3'; underlined sequence is non-HCV specific) and HCV19 (antisense, positions -1 to -20; 5'-GTGCACGGTCTACGAGACCT-3'). Genotyping was performed by a prototype

Inno-LiPA HCV genotyping assay [Stuyver et al., 1993].

Sequence analysis of the 5'UTR, core and core/E1 regions

PCR products from the 5'UTR were reamplified in a nested PCR with primers NCR3 (sense, positions -314 to -288; 5'-GGGGCGGCCGCCAACCA TARRATCACTCCCCTGTGAGG-3'; R= A or G) and LD58b (antisense, positions -35 to -64; 5'Bio-GGCCGGGGCGGCCGCCAAGCACCCTATCAGGCAGTACCAC-AAGGC-3').

RT-PCR was also performed with primers HCV983 (antisense, positions 963 to 983; 5'-GGIGACCAGTTCATCATCAT-3'; I=inosine) and biotinylated LD58c (sense, positions -57 to -34; 5'-Bio-GGTACTGCCTGATAGGGTGCTTGC 3'). cDNA synthesis was primed with primer HCV983. First round PCR products were reamplified with LD58c and 186c (antisense, positions 410 to 391; 5'-ATITACCCCATGAGITCGGC-3') in a semi-nested PCR for analysis of the core region. To analyze core/E1 sequences, first round PCR products were reamplified by HCV983 and HCV720b (5'Bio-GCCGACCTCATGGGGTACAT-3').

All biotinylated nested PCR fragments were subjected to direct sequence analysis as described earlier [Kleter et al., 1994; van Doorn et al., 1994a]. Sequences were analyzed by the PCGene software (Intelligenetics) and phylogenetic trees were constructed using the Phylogeny Inference Package (PHYLIP; version 3.5c; Felsenstein, 1993).

Nucleotide sequence accession number

The sequence data reported here have been deposited in the Genome Sequence Data Base and assigned the following accession numbers: 5'UTR, X78856-X78867 and X58937-X58953; core, Z29444-Z29474; core/E1, L39280-L39318.

RESULTS

A cohort of 315 HCV isolates was genotyped in the 5'UTR by means of LiPA. HCV (sub)types 1a, 1b, 2a, 2b, 3a, 4a, and 5a were detected and the prevalence of each (sub)type is shown in Table 1. Only four mixed infections were detected. One isolate (NE92) could be typed, but subtyping was not possible (designated type 2*). One isolate (NL96) resulted in an aberrant LiPA pattern and could not be typed. Within type 1, the prototype LiPA allowed positive identification of subtype 1b only, and typing the remaining isolates as type 1.

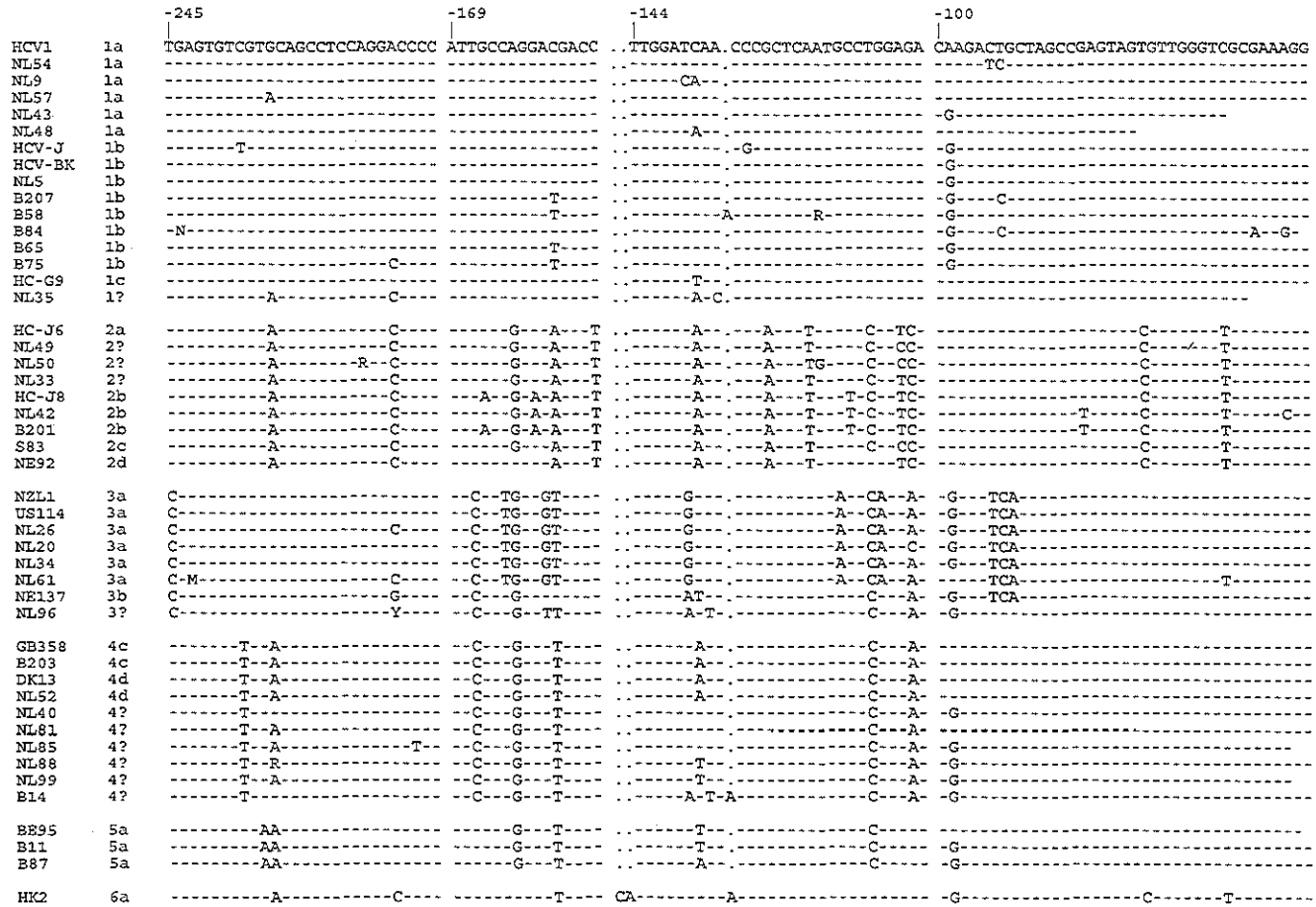
First, a total of 37 isolates (nine of type 1, four of 1b, three of 2a, two of 2b, one of 2*, four of 3a, eight of 4a, five of 5a, and one untypeable, according to LiPA) were further analyzed by sequencing of the 5' UTR (Fig. 1). LiPA patterns were in complete agreement with 5' UTR sequence results for each isolate.

Secondly, part of the core region sequence (nucleotides 1-326) was amplified from 36 isolates (12 of type 1, four of 1b, three of 2a, two of 2b, one of 2*, four of 3a, four of 4a, five of 5a, and one untypeable, according to LiPA) and subjected to direct sequence analysis. Phylogenetic distances among these and several reference sequences were calculated. The phylogenetic distances did not segregate into three non-overlapping distance ranges (results not shown) for types, subtypes and isolates. Further analysis revealed that isolates originally typed as HCV subtypes 2a or 4a were much more heterogeneous than other subtypes, suggesting the existence of multiple subtypes within these groups. Therefore, 36 isolates (seven of type 1, four of 1b, five of 2a, one of 2b, one of 2*, four of 3a, eight of 4a, five of 5a, and one untypeable, according to LiPA) were subjected to sequencing of the more variable C-terminal core/N-terminal E1 region (nucleotides 477 to 924). Phylogenetic distances were calculated and

Table 1. Prevalence of HCV (sub)types in a cohort of 315 patients from the Benelux countries as determined by LiPA.

Type	Number of patients	Prevalence (%)
1	32	10.1
1b	187	59.4
2a	20	6.3
2b	3	0.9
2*	1	0.3
3a	45	14.3
4a	15	4.8
5a	7	2.2
multiple	4	1.3
untypeable	1	0.3

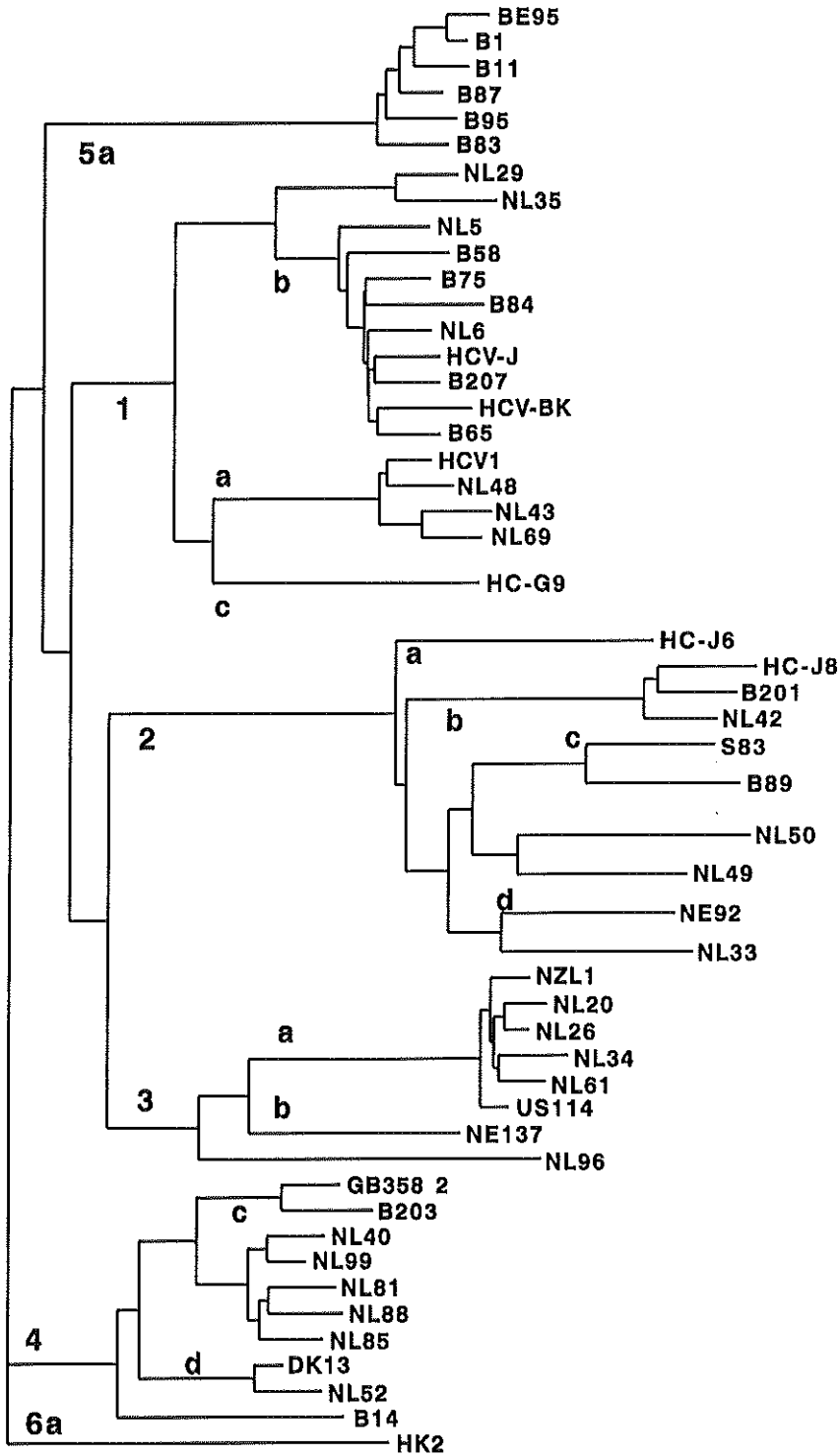
the resulting phylogenetic tree is represented in Fig. 2. The frequency distribution of pair-wise phylogenetic distances in the core/E1 region showed some overlap (Fig. 3), but the segregation into types, subtypes and isolates was much better than for the N-terminal core region. Remarkably, the average pairwise phylogenetic distance of type 2 isolates compared to the other types was 0.68 ± 0.05, whereas for all remaining intertype distances this was only 0.57 ± 0.08. The frequency peak of 0.24 is mainly related to pairwise distances within type 4 (0.23 ± 0.04) compared to 0.36 ± 0.05 between subtypes of all other types. Although overlap regions were small, it was impossible to calculate exact border values for types, subtypes and isolates.


-245
-169
-144
-100
HCV1 1a TGAGTGTCGTGCAGCCTCCAGGACCCC ATTGCCAGGACGACC ..TTGGATCAA.CCCGCTCAATGCCTGGAGA CAAGACTGCTAGCCGAGTAGTGTTGGGTCGCGAAAGG
NL54 1a ....TC.....
NL9 1a ....CA-- .....
NL57 1a ....A..... ....
NL43 1a .... -G.....
NL48 1a ....A-- .....
HCV-J 1b .....T..... ....G..... -G.....
HCV-BK 1b .... -G.....
NL5 1b .... -G.....
B207 1b ....T.... ..... -G....C.....
B58 1b ....T.... .....A.....R..... -G.....
B84 1b -N..... .... -G....C.....A--G-
B65 1b ....T.... ..... -G.....
B75 1b .....C.... .....T.... ..... -G.....
HC-G9 1c ....T-- .....
NL35 1? ....A.....C.... ....A-C. .....
HC-J6 2a ....A.....C.... .....G---A---T ..-----A--.---A---T-----C--TC- .....C-------T--------
NL49 2? ....A.....C.... .....G---A---T ..-----A--.---A---T-----C--CC- .....C-------T--------
NL50 2? ....A.....R--C.... .....G---A---T ..-----A--.---A---TG----C--CC- .....C-------T--------
NL33 2? ....A.....C.... .....G---A---T ..-----A--.---A---T-----C--TC- .....C-------T--------
HC-J8 2b ....A.....C.... ---A--G-A-A---T ..-----A--.---A---T---T-C--TC- .....C-------T--------
NL42 2b ....A.....C.... .....G-A-A---T ..-----A--.---A---T---T-C--TC- .....T-----C-------T-----C--
B201 2b ....A.....C.... ---A--G-A-A---T ..-----A--.---A---T---T-C--TC- .....T-----C-------T--------
S83 2c ....A.....C.... .....G---A---T ..-----A--.---A---T-----C--CC- .....C-------T--------
NE92 2d ....A.....C.... .....A---T ..-----A--.---A---T--------TC- .....C-------T--------
NZL1 3a C..... --C--TG--GT---- ..-----G---.----------A--CA--A- -G---TCA.....
US114 3a C..... --C--TG--GT---- ..-----G---.----------A--CA--A- -G---TCA.....
NL26 3a C.....C.... --C--TG--GT---- ..-----G---.----------A--CA--A- -G---TCA.....
NL20 3a C..... --C--TG--GT---- ..-----G---.----------A--CA--C- -G---TCA.....
NL34 3a C..... --C--TG--GT---- ..-----G---.----------A--CA--A- -G---TCA.....
NL61 3a C-M..... C.... --C--TG--GT---- ..-----G---.----------A--CA--A- -----TCA.....T--------
NE137 3b C.....G.... --C---G------- ..-----AT--.----------C---A- -G---TCA.....
NL96 3? C.....Y.... --C---G--TT---- ..-----A-T-.----------C---A- -G.....
GB358 4c .....T--A..... --C---G---T---- ..------A--.----------C---A- .....
B203 4c .....T--A..... --C---G---T---- ..------A--.----------C---A- .....
DK13 4d .....T--A..... --C---G---T---- ..------A--.----------C---A- .....
NL52 4d .....T--A..... --C---G---T---- ..------A--.----------C---A- .....
NL40 4? .....T..... --C---G---T---- ..----------.----------C---A- -G.....
NL81 4? .....T--A..... --C---G---T---- ..----------.----------C---A- .....
NL85 4? .....T--A.....T-- --C---G---T---- ..----------.----------C---A- -G.....
NL88 4? .....T--R..... --C---G---T---- ..------T--.----------C---A- -G.....
NL99 4? .....T--A..... --C---G---T---- ..------T--.----------C---A- -G.....
B14 4? .....T..... --C---G---T---- ..------A-T-A----------C---A- -G.....
BE95 5a .....AA..... -----G---T---- ..------T--.----------C----- .....
B11 5a .....AA..... -----G---T---- ..------T--.----------C----- -G.....
B87 5a .....AA..... -----G---T---- ..------A--.----------C----- -G.....
HK2 6a ....A.....C.... ----------T---- CA---------A..... -G.....C-------T--------

Therefore, phylogenetic distances were also compared with the ranges obtained by analysis of a larger core/E1 region of which NS5B sequences were also known [Stuyver et al., 1994]. Final assignments were based on nucleotide distances, border values from the larger core/E1 region [Stuyver et al., 1994] and the position in the phylogenetic tree.

Two isolates, NL43 and NL69, were identified as 1b by LiPA but appeared to belong to subtype 1a after core and core/E1 analysis. The isolates NL29 and NL35 were obtained from patients who probably contracted their HCV infection in Morocco. These two isolates could be classified into a separate subtype of type 1, and are distinct from the proposed subtype 1c isolates [HCG9, Okamoto et al., 1994; Td-6, Td-34/92, Hotta et al., 1994a and 1994b] from Indonesia. Two isolates (B84 and B207) were identified as type 1, but were not typed by LiPA as subtype 1b, due to a mutation at position -94 in the 5' UTR (Fig. 1). Four isolates (B58, B65, B75 and B207) contained a C/T mutation at position -158, which resulted in an aberrant LiPA pattern, which did not influence typing.

Figure 1. Nucleotide sequence alignment of the HCV 5' UTR sequences together with representative isolates for each (sub)type. Parts of the 5'UTR that were completely conserved among all isolates are not shown. Reference sequences are: type 1a, HCV1 [Choo et al., 1991]; type 1b, HCV-J [Kato et al., 1990] and HCV-BK [Takamizawa et al., 1991]; type 1c, HC-G9 [Okamoto et al., 1994]; type 2a, HC-J6 [Okamoto et al., 1991]; type 2b, HC-J8 [Okamoto et al., 1992a]; type 2d, NE-92 [Stuyver et al., 1994]; type 2c, S83 [Bukh et al., 1993]; type 3a, NZL1 [Sakamoto et al., 1994]; type 3a, US114 [Okamoto et al., 1993]; type 3b, NE137 [Tokita et al., 1994]; type 4c, GB358 [Stuyver et al., 1994]; type 4d, DK13 [Bukh et al., 1993]; type 5a, BE95 [Stuyver et al., 1994]; type 6a, HK2 [Bukh et al., 1993].


BE95
B1
B11
B87
B95
B83
5a
NL29
NL35
NL5
B58
b
B75
B84
NL6
HCV-J
B207
1
HCV-BK
B65
HCV1
a
NL48
NL43
NL69
HC-G9
c
HC-J6
a
HC-J8
B201
NL42
b
2
c
S83
B89
NL50
NL49
d
NE92
NL33
NZL1
NL20
a
NL26
NL34
NL61
3
b
US114
NE137
NL96
GB358 2
c
B203
NL40
NL99
NL81
NL88
4
NL85
d
DK13
NL52
B14
6a
HK2

Based on core/E1 sequences, three type 2 isolates (NL33 and NL49 from Surinam, and NL50 from the Netherlands) could each be classified into a novel subtype, apart from subtype 2a (HCJ-6), 2b (HCJ-8), 2c (S83) and 2d (NE92). Isolate NL50 contained a mutation at position -127, whereas the other two subtypes were indistinguishable from subtype 2a in the 5'UTR. Subtypes 2b and 2d could both be identified by unique covariant mutations in the 5'UTR sequence motifs, resulting in specific LiPA patterns. The single type 2 isolate that could not be subtyped by LiPA was also analyzed in the NS5B region; it has been described earlier as NE92, classified as subtype 2d [Stuyver et al., 1994]. Isolate B89 (from Belgium) appeared to contain an insertion of 2 amino acids between residues 197-198 in the E1 region. No insertions have been observed yet in any of the previously described E1 sequences.

The four selected type 3 isolates all belonged to HCV subtype 3a. However, the single untypeable isolate, NL96, could be classified as a seventh subtype of type 3, different from subtypes 3a to 3f [Tokita et al., 1994]. This isolate has also been analyzed in the E1/E2 region and the NS5 region (data not shown), confirming classification as a separate subtype within type 3. The characteristic type 3 'TCA motif' at position -95 to -92 is not present in this

Figure 2. Phylogenetic tree drawn from the phylogenetic analysis of nucleotide sequences by the neighbor-joining method [Saitou & Nei, 1987]. reference sequences are: type 1a, HCV1 [Choo et al., 1991]; type 1b, HCV-J [Kato et al., 1990] and HCV-BK [Takamizawa et al., 1991]; type 1c, HC-G9 [Okamoto et al., 1994]; type 2a, HC-J6 [Okamoto et al., 1991]; type 2b, HC-J8 [Okamoto et al., 1992a]; type 2d, NE-92 [Stuyver et al., 1994]; type 2c, S83 [Bukh et al., 1993]; type 3a, NZL1 [Sakamoto et al., 1994]; type 3a, US114 [Okamoto et al., 1993]; type 3b, NE137 [Tokita et al., 1994]; type 4c, GB358 [Stuyver et al., 1994]; type 4d, DK13 [Bukh et al., 1993]; type 5a, BE95 [Stuyver et al., 1994]; type 6a, HK2 [Bukh et al., 1993].

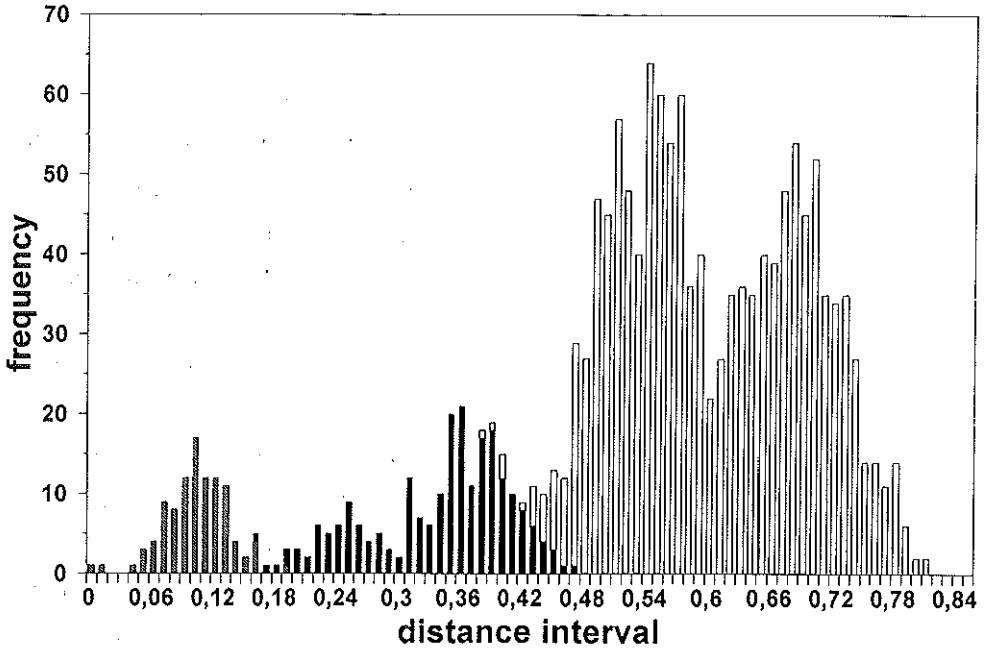


Figure 3. Frequency distribution of pair-wise molecular evolutionary distances in the core/E1 region (nt 477 to 924) among 37 selected HCV isolates and several reference sequences.

isolate. A new sequence motif is present between position -167 and -159.

The two sequence motifs in the 5'UTR of type 4 isolates were completely conserved in our group of patients. Additional point mutations, located outside the motifs, were present. Since the frequency distribution (Fig. 3) showed some overlap between isolates and subtypes, several of our type 4 isolates could not be formally classified as different subtypes after core/E1 analysis. However, among eight type 4 isolates, at least 4 subtypes could be distinguished. B203

clustered with subtype 4c isolates, and NL52 with the subtype 4d isolate DK13. Comparison with published type 4 sequence data [Stuyver et al., 1994; Bukh et al., 1993 and 1994) indicated that the remaining six isolates (from Egypt, Zaire and the Netherlands) clustered into two novel subtypes. Five of these isolates belong to the same newly identified subtype, which seems to be the predominant type 4 subtype in the Benelux countries.

The 5 isolates of HCV type 5 are highly homogeneous, and were classified into a single subtype (5a). The isolates from South Africa (e.g. SA1; [Bukh et al., 1992]) also belong to the same subtype (data not shown).

DISCUSSION

Hepatitis C virus isolates from 315 chronically infected HCV patients living in the Benelux area of Western Europe were genotyped by analysis of the 5'UTR with LiPA. The genotypic distribution (Table 1) differs from earlier reports [Dusheiko et al., 1994; McOmish et al., 1994] and illustrates that the major HCV types are all present in Western Europe including types 4 and 5 which were regarded as 'African' types. The version of LiPA used in this study did not permit the recognition of type 6, which seems to be exclusively present in the Far-East.

In order to investigate sequence variation in coding regions of HCV isolates from the Benelux area, we analyzed parts of the core and core/E1 regions from a number of randomly selected isolates as well as from isolates with an aberrant LiPA pattern. Phylogenetic analyses indicated a remarkable heterogeneity of HCV in the Benelux isolates.

Within type 1, three subtypes were recognized. Two isolates from patients, probably infected in Morocco, could be classified as a novel subtype.

Initial phylogenetic analyses have suggested the importance of the G at position -99 in the 5'UTR for identification of subtype 1b isolates. Recently, some 1b isolates containing an A at -99, have been reported [Bukh et al., 1992 and 1993], suggesting that this position cannot be consistently used for recognition of subtype 1b. In our limited number of isolates we observed two discrepancies (isolates NL43 and NL69). These two isolates were identified as 1b by LiPA, but as 1a by sequence analyses of the core and core/E1 regions. Low discrepancy levels have also been reported by others [Mahaney et al., 1994; Gianinni et al., 1994].

Type 2 isolates show remarkable heterogeneity in the core and core/E1 regions. Most of these are indistinguishable in the 5'UTR and were initially identified as subtype 2a by the LiPA, together with HC-J6. From phylogenetic analysis of the core/E1 region six subtypes can be detected in this group of patients. All type 2 isolates from our randomly selected group were different from the Japanese subtype 2a. This has also been confirmed for most of the remaining type 2 isolates of the entire cohort of 315 patients, by subtype-specific PCR in the core region, as described by Okamoto (data not shown). Taken together, type 2 appears to comprise at least 7 different subtypes. Similar to observations by Bukh et al. [1993] our study also revealed that type 2 is the most distant group among the HCV types 1 to 5 and confirmed the high degree of heterogeneity of subtypes within this type.

Type 3 isolates from Benelux patients appear to be highly homogeneous, except for isolate NL96 which can be classified as a separate subtype. This isolate was obtained from a patient who probably contracted the HCV infection in Indonesia, which may explain the rarity of such isolates in Western Europe. Recently, this isolate was found to be highly homologous to the Indonesian isolate Td-3/93 [Hotta et al., 1994b] in the NS5B region (data not shown).

Like type 2, type 4 isolates are a highly heterogeneous group. Patients in

this group originate from Egypt, Zaire, Belgium and the Netherlands. The average phylogenetic distance between putative subtypes in this group is remarkably lower than within other types (0.23 ± 0.04 vs. 0.36 ± 0.05). Therefore, in some cases, the core/E1 region could not accurately predict the precise division of type 4 isolates into separate subtypes. The existence of at least eight subtypes within type 4 has been reported earlier [Stuyver et al., 1994], and comparison with these data led to the identification of at least two new subtypes. Therefore the total number of subtypes within type 4 increases to at least 10. Some of these subtypes can be identified by specific 5'UTR sequences. However, formal classification of all type 4 subtypes requires additional sequence analyses, preferably in the NS5B region [Stuyver et al., 1994].

Classification of isolates into the major types based on either 5'UTR, core or E1 was completely consistent. It is remarkable that analyses of 5'UTR and the core/E1 regions result in similar classifications. The 5'UTR is a non-coding region containing a highly conserved, presumably functional, element such as an internal ribosomal entry site with stringent constrains on the secondary structure of the RNA [Yoo et al., 1992; Wang et al., 1993]. In contrast, the core and E1 regions encode the putative nucleocapsid and envelop proteins, and would therefore be subjected to completely different selective pressures than the 5'UTR.

A HCV typing system based on subtype-specific primers in the core region has been described by Okamoto [Okamoto et al., 1992b and 1993]. Comparison of core sequences described in this study and subtype-specific primers as described by Okamoto [Okamoto et al., 1992b] revealed that specific primer target sequences contained several mismatches, especially in type 2 isolates. Experiments have shown that this PCR typing system does not allow efficient typing of type 2 isolates [Kleter et al. unpublished observations]. Type-

specific PCR primers for types 4 and 5 have not yet been described and could be designed now. However, it seems likely that subtype-specific amplification with multiple different subtype-specific primers will not be an efficient genotyping method in heterogeneous HCV populations.

HCV types 1 to 5 can be distinguished by differences in the 5'UTR. Very similar discrimination between viral variants is possible by analysis of the relatively well conserved core region as shown in this study. The more variable E1 region revealed identical, although more precise, classification of isolates. This indicates that genotypic differences are maintained throughout the HCV genome in all major types of HCV, although the degree of sequence variation differs considerably between the different regions of the HCV genome. It appears that the majority of types known today can be differentiated in the 5'UTR. However, sequence variation in this region is not sufficient to recognize every single subtype. Whereas the 5'UTR and core region are very conserved, the E1 and NS5 regions reveals additional variation, and the hypervariable region in the E2 region would allow identification of almost every single HCV isolate [Weiner et al., 1991]. However, discrimination between single isolates might be only useful for epidemiological studies. At present, it is unclear whether detailed subtyping of HCV isolates has any clinical significance. This is subject of further study.

The classification system of HCV into types, subtypes and isolates and the nomenclature of the major types appears now to be generally accepted. However, classification and nomenclature of novel isolates is still a problem that requires consensus among the scientific community. Distinct criteria should be defined, in order for a new type or subtype to be classified. Based on this and other studies [Bukh et al., 1993; Stuyver et al., 1994] the E1 and NS5B regions might be the most appropriate regions to use for detailed phylogenetic analysis and classification.

In conclusion, genotyping of HCV isolates from a large patient cohort revealed the presence of a highly heterogeneous population of HCV types and subtypes in the Benelux countries of Western Europe. However, it should be noted that the Benelux region has a multiracial population, which might explain detection of HCV variants that had only been reported in distant regions of the world. It can be speculated that most HCV types have a worldwide distribution, although the relative regional prevalence of each type may vary considerably. Therefore, detection of minor subtypes may require analysis of larger patient cohorts.

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REFERENCES

Bukh J, Purcell R.H, Miller RH (1992): Sequence analysis of the 5' noncoding region of hepatitis C virus. Proceedings of the National Academy of Sciences U.S.A. 89:4942-4946.

Bukh J, Purcell RH, Miller RH (1993): At least 12 genotypes of hepatitis C virus predicted by sequence analysis of the putative E1 gene of isolates collected worldwide. Proceedings of the National Academy of Sciences U.S.A. 90: 8234-8238.

Bukh J, Purcell RH, Miller RH (1994): Sequence analysis of the core gene of 14 hepatitis C virus genotypes. Proceedings of the National Academy of Sciences U.S.A. 91:8239-8243.

Chan S-W, McOmish F, Holmes EC, Dow B, Peutherer JF, Follett E, Yap PL, Simmonds P (1992): Analysis of a new hepatitis C virus type and its phylogenetic relationship to existing variants. Journal of General Virology 73:1131-1141.

Chayama K, Tsubota A, Arase Y, Saitoh S, Koida I, Ikeda K, Matsumoto T, Kobayashi M, Iwasaki S, Koyama S, Morinaga T, Kumada H (1993): Genotypic subtyping of hepatitis C virus. Journal of Gastroenterology and Hepatology 8:150-156.

Chomczynski P, Sacchi N (1987): Single step method of RNA isolation by guanidiniumthiocyanate-phenol-chloroform extraction. Analytical Biochemistry 162:152-159.

Choo Q-L, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M (1989): Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 244:359-362.

Choo Q-L, Richman KH, Han JH, Berger K, Lee C, Dong C, Gallegos C, Coit D, Medina-Selby A, Barr PJ, Weiner AJ, Bradley DW, Kuo G, Houghton M (1991): Genetic organization and diversity of the hepatitis C virus. Proceedings of the National Academy of Sciences USA 88:2451-2455.

Dusheiko G, Schmilovitz-Weiss H, Brown D, McOmish F, Yap PL, Sherlock S, McIntyre N, Simmonds P (1994): Hepatitis C virus genotypes: an investigation of type-specific differences in geographic origin and disease. Hepatology 19:13-18.

Felsentstein J (1993): PHILIP, Phylogeny Inference Package, version 3.5c. distributed by the author. Department of Genetics, University of Washington, Seattle, USA.

Gianinni G, Thiers V, Nousbaum JB, Stuyver L, Maertens G, Brèchot C (1994): Comparative evaluation of type-specific primers and type-specific probes based assays for hepatitis c virus (HCV) genotyping. Hepatology 20:243A.

Hotta H, Doi H, Hayashi T, Purwanta M, Soemarto W, Mizokami M, Ohba K, Homma M (1994a): Analysis of the core and E1 envelope region sequences of a novel variant of hepatitis C virus obtained in Indonesia. Archives of Virology 136:53-62.

Hotta H, Handajani R, Lusida MI, Soemarto W, Doi H, Miyajami H, Homma M (1994b): Subtype analysis of hepatitis C virus in Indonesia on the basis of NS5b region sequences. Journal of Clinical Microbiology 32:3049-3051.

Houghton M, Weiner AJ, Han J, Kuo G, Choo Q-L (1991): Molecular biology of the hepatitis C viruses: implications for diagnosis, development and control of viral disease. Hepatology 14:382-388.

Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugimura T, Shimotohno K (1990): Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proceedings of the National Academy of Sciences U.S.A. 87:9524-9528.

Kleter GEM, Brouwer JT, Heijtink RA, Schalm SW, Quint WGV (1993): Detection of hepatitis C

virus RNA in patients with chronic hepatitis C virus infections during and after therapy with alpha-interferon. Antimicrobial Agents and Chemotherapy 37:595-597.

Kleter GEM, van Doorn LJ, Brouwer JT, Schalm SW, Heijtink RA, Quint WGV (1994): Sequence analysis of the 5' untranslated region in isolates of at least four genotypes of hepatitis C virus in the Netherlands. Journal of Clinical Microbiology 32:306-310.

Kuo G, Choo Q-L, Alter HJ, Gitnick GL, Redeker AG, Purcell RH, Miyamura T, Dienstag JL, Alter MJ, Stevens CE, Tegtmeier GE, Bonino F, Colombo M, Lee W-S, Kuo C, Berger K, Shuster JR, Overby LR, Bradley DW, Houghton M (1989): An assay for circulating antibodies to a major etiologic agent of human non-A, non-B hepatitis. Science 244:362-364.

Mahaney K, Tadeschi V, Maertens G, Di Bisceglie AM, Vergalla J, Hoofnagle JH, Sallie R (1994): Genotypic analysis of hepatitis C virus in American patients. Hepatology 20:1405-1411.

McOmish F, Yap PL, Dow BC, Follett EAC, Seed C, Keller AJ, Cobain TJ, Krusius R, Kolho E, Naukkarinen R, Lin C, Lai C, Leong S, Medgyesi GA, Hejjas M, Kiyokawa H, Fukada K, Cuypers T, Saeed AA, Al-Rasheed AM, Lin M, Simmonds P (1994): Geographical distribution of hepatitis C virus genotypes in blood donors: an international collaborative survey. Journal of Clinical Microbiology 32:884-892.

Miller RH, Purcell RH (1990): Hepatitis C virus shares amino acid sequence similarity with pestiviruses and flaviviruses as well as members of two plant virus supergroups. Proceedings of the National Academy of Sciences U.S.A. 87:2057-2061.

Okamoto H, Okada S, Sugiyama Y, Kurai K, Iizuka H, Machida A, Miyakawa Y, Mayumi M (1991): Nucleotide sequence of the genomic RNA of hepatitis C virus isolated from a human carrier: comparison with reported isolates for conserved and divergent regions. Journal of General Virology 72:2697-2704.

Okamoto H, Kurai K, Okada S-I, Yamamoto K, Iizuka H, Tanaka T, Fukuda S, Tsuda F, Mishiro S (1992a): Full-length sequence of a hepatitis C virus genome having poor homology to reported isolates: comparative study of four distinct genotypes. Virology 188:331-341.

Okamoto H, Sugiyama Y, Okada S, Kurai K, Akahane Y, Sugai Y, Tanaka T, Sato K, Tsuda F, Miyakawa Y, Mayumi M (1992b): Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. Journal of General Virology, 73:673-679.

Okamoto H, Tokita H, Sakamoto M, Horikita M, Kojima M, Iizuka H, Mishiro S (1993): Characterization of the genomic sequence of type V (or 3a) hepatitis C virus isolates and PCR primers for specific detection. Journal of General Virology 74:2385-2390.

Okamoto H, Kojima M, Sakamoto M, Iizuka H, Hadiwandowo S, Suwignyo S, Miyakawa Y, Mayumi M (1994): The entire nucleotide sequence and classification of a hepatitis C virus isolate of a novel genotype from an Indonesian patient with chronic liver disease. Journal of General Virology 75:629-635.

Saitou N, Nei M (1987): The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406-425.

Sakamoto M, Akahane Y, Tsuda F, Tanaka T, Woodfield DG, Okamoto H (1994): Entire nucleotide sequence and characterization of a hepatitis C virus of genotype V/3a. Journal of General Virology 75:1761-1768.

Simmonds P, McOmish F, Yap PL, Chan SW, Lin CK, Dusheiko G, Saeed AA, Holmes EC (1993a): Sequence variability in the 5' non-coding region of hepatitis C virus: identification of a new virus type and restrictions on sequence diversity. Journal of General Virology 74:661-668.

Simmonds P, Rose KA, Graham S, Chan SW, McOmish F, Dow BC, Follett EAC, Yap PL, Marsden H (1993b): Mapping of serotype-specific, immunodominant epitopes in the NS-4 region of hepatitis C virus (HCV): Use of type-specific peptides to serologically differentiate infections with HCV types 1, 2, and 3. Journal of Clinical Microbiology: 31:1493-1503.

Simmonds P, Alberti A, Alter HJ, et al. (1994a): A proposed system for nomenclature of hepatitis C viral genotypes. Hepatology, 19:1321-1324.

Simmonds P, Smith DB, McOmish F, Yap PL, Kolberg J, Urdea MS, Holmes EC (1994b): Identification of genotypes of hepatitis C virus by sequence comparisons in the core, E1 and NS-5 regions. Journal of General Virology 75:1053-1061.

Stuyver L, Rossau R, Wyseur A, Duhamel M, Vanderborght B, Van Heuverswyn H, Maertens G (1993): Typing of hepatitis C virus isolates and characterization of new subtypes using a line probe assay. Journal of General Virology 74:1093-1102.

Stuyver L, van Arnhem W, Wyseur A, Hernandez F, Delaporte E, Maertens G (1994): Classification of hepatitis C viruses based on phylogenetic analysis of the envelope 1 and nonstructural 5B regions and identification of five additional subtypes. Proceedings of the National Academy of Sciences U.S.A. 91:10134-10138

Takada N, Takase S, Enomoto N, Takada A, Date T (1992): Clinical background of the patients having different types of hepatitis C virus genomes. Journal of Hepatology 14:35-40.

Takamizawa A, Mori C, Fuke I, Manabe S, Murakami S, Fujita J, Onishi E, Andoh T, Yoshida I, Okayama H (1991): Structure and organization of the hepatitis C virus genome isolated from

human carriers. Journal of Virology 65:1105-1113.

Tokita H, Shresta SM, Okamoto H, Sakamoto M, Horikita M, Iizuka H, Shresta S, Miyakawa Y, Mayumi M (1994): Hepatitis C virus variants from Nepal with novel genotypes and their classification into the third major group. Journal of General Virology 75:931-936.

Tsubota A, Chayama K, Ikeda K, Yasuji A, Koida I, Saitoh S, Hashimoto M, Iwasaki S, Kobayashi M, Hiromitsu K (1994): Factors predictive of response to interferon-α therapy in hepatitis C virus infection. Hepatology 19:1088-1094.

Tsukiyama-Kohara K, Yamaguchi K, Maki N, Ohta Y, Miki K, Mizokami M, Ohba KI, Tanaka S, Hattori N, Nomoto A, Kohara M (1993): Antigenicities of groups I and II hepatitis C virus polypeptides. Molecular basis of diagnosis. Virology 192:430-437.

van Doorn LJ, Kleter GEM, Stuyver L, Maertens G, Brouwer JT, Schalm SW, Heijtink RA, Quint WGV (1994a): Analysis of hepatitis C virus genotypes by a Line Probe Assay (LiPA) and correlation with antibody profiles. Journal of Hepatology 21:122-129.

van Doorn LJ, Kleter B, Voermans J, Maertens G, Brouwer H, Heijtink R, Quint W (1994b). Rapid detection of hepatitis C virus RNA by direct capture from blood samples. Journal of Medical Virology, 42:22-28.

Wang C, Sarnow P, Siddiqui A (1993): Translation of human hepatitis C virus RNA in cultured cells is mediated by an internal ribosome-binding mechanism. Journal of General Virology 67:3338-3344.

Weiner AJ, Brauer MJ, Rosenblatt J, Richman KH, Tung J, Crawford K, Bonino F, Saracco G, Choo QL, Houghton M, Han JH (1991): Variable and hypervariable domains are found in the regions of HCV corresponding to the Flavivirus envelope and NS1 proteins and the Pestivirus envelope glycoproteins. Virology 180:842-848.

Willems M, Moshage H, Nevens F, Fevery L, Yap SH (1993): Plasma collected from heparinized blood is not suitable for HCV-RNA detection by conventional RT-PCR. Journal of Virological Methods 43:127-130.

Yoo BJ, Spaete RR, Geballe AP, Selby M, Houghton M, Han JH (1992): 5'end-dependent translation initiation of hepatitis C viral RNA and the presence of putative positive and negative translational control elements within the 5' untranslated region. Virology 191:889-899.

Yoshioka K, Kakumu S, Wakita T, Ishikawa T, Itoh Y, Takayanagi M, Higashi Y, Shibata M, Morishima T (1992): Detection of hepatitis C virus by polymerase chain reaction and response to interferon-alpha therapy: relationship to genotypes of Hepatitis C virus. Hepatology 16:293-299.

CHAPTER 7

Rapid genotyping of hepatitis C virus RNA-isolates obtained from patients residing in Western Europe.

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ABSTRACT

Two rapid genotyping methods for hepatitis C virus (HCV), the Line probe assay (Inno-LiPA) and the subtype-specific core amplification system [Okamoto et al., (1992b) Journal of General Virology 73:673-679], were applied on 58 HCV isolates which were typed as type 1 (n = 37) and type 2 (n = 21) by sequence analysis of the 5' untranslated region (5'UTR). The Line probe assay, targets the 5'UTR and recognized 12 subtype 1a, 25 subtype 1b, 18 subtype 2a, 2 subtype 2b and 1 subtype 2d in accordance to sequence analysis of this region. Subtype-specific core amplification revealed 7 discrepancies among the 37 type 1 isolates when compared to LiPA. A different subtype was observed in 3 isolates (1a versus 1b), 2 isolates remained untyped and 2 isolates showed a coinfection of subtypes 1a and 1b. The first 5 discrepancies were confirmed by sequence analysis of the core region whereas the coinfection could not be confirmed. Of the 21 type 2 isolates only one could be typed by subtype-specific core amplification. HCV RNA was detected in all 21 cases after the general first round of polymerase chain reaction (PCR). Direct sequencing in the core region indicated sequence variation as a source of failure. It is concluded that LiPA results are conclusive for typing of HCV. However, LiPA is hampered occasionally for subtyping by lack of subtype-specific sequence variation in 5'UTR. Subtyping results by subtype-specific core amplification were accurate. However, it seems that this assay is not suitable for identification of type 2 isolates that circulate in patients living in Western Europe.

INTRODUCTION

Hepatitis C virus (HCV) is the major etiologic agent of post-transfusion non-A,

non-B hepatitis [Choo et al., 1989] and is classified as a distinct genus of the Flaviviridae [Miller and Purcell, 1990]. Several full-length [Choo et al., 1991; Kato et al., 1990; Okamoto et al., 1991, 1992a; Takamizawa et al., 1991] and numerous partial [Bukh et al., 1992, 1993; Chan et al., 1992; Enomoto et al., 1990; Kleter et al., 1994; Mori et al., 1992; Simmonds et al., 1993a; Stuyver et al., 1993b] HCV sequences have been reported and comparison of those sequences revealed considerable heterogeneity between isolates. Recently, a useful classification system, based on phylogenetic analyses, was proposed and has been accepted by the scientific community [Simmonds et al., 1994]. This system discriminates between types and subtypes. Genotypic variation seems to be maintained throughout the entire viral genome, although the level of heterogeneity differs considerably between the various regions of the genome. Nucleotide sequence variation ranges from approximately 10% in the 5′ UTR [Chan et al., 1992] to nearly 50% in the E1 [Bukh et al., 1993] and NS5 [Simmonds et al., 1993b] regions. Currently, there is evidence for the existence of at least 9 major types [Tokita et al., 1994b].

Genotyping of HCV isolates is of interest for epidemiological studies [Dusheiko et al., 1994; McOmish et al., 1993]. The success of antiviral treatment of chronic HCV infections appears to be related to the viral genotype [Hino et al., 1994; Kobayashi et al., 1993; Tsubota et al., 1994; Yoshioka et al., 1992] and the level of viremia [Hagiwara et al., 1993; Kobayashi et al., 1993; Lau et al., 1993; Yoshioka et al., 1992].

Genotyping can be undertaken by sequence analysis but this is tedious and time-consuming. Recently, several rapid methods for genotyping of HCV have been reported, including subtype-specific polymerase chain reaction (PCR) in either the core region [Okamoto et al., 1992b, 1993] or the NS5B region [Chayama et al., 1993], restriction fragment length polymorphism (RFLP) [McOmish et al., 1993; Murphy et al., 1994; Nakao et al., 1991], hybridization

of PCR products with type-specific probes [Cha et al., 1992; Enomoto et al., 1990] and reverse hybridization [Stuyver et al., 1993a; van Doorn et al., 1994].

In the present study, two of these rapid methods were applied to 58 type 1 and 2 isolates sequenced in 5'UTR origin. HCV Genotyping was carried out by reverse hybridization with the Line Probe Assay (LiPA), which is aimed at the 5'UTR, and by the subtype-specific core amplification system [Okamoto et al., 1992b]. Discrepant results were analyzed.

MATERIALS AND METHODS

Patients

Plasma samples were obtained by venepuncture and stored at -70°C. All 58 patients had elevated alanine aminotransferase (ALT) levels, histological changes compatible with HCV infection, antibodies to hepatitis C virus, no recent history of infection with hepatitis B virus, hepatitis A virus, cytomegalovirus or Epstein-Barr virus, and were between 26 and 74 years of age. The analyzed patients are living in the Netherlands or Belgium.

HCV RNA PCR

HCV RNA and cDNA were prepared as described previously [Kleter et al., 1993]. For 5' UTR analysis, PCR (40 cycles, 1 min at 94°C, 2 min at 48°C, 3 min at 72°C) was undertaken with antisense primer HCV19 (GTGCACGGTC-TACGAGACCT, positions -1 to -20) and sense primer HCV18 (GGCGACACT-CCACCATAGAT, positions -304 to -324) or sense primer HCV35 (TTGGCGG-CCGCACTCCACCATGAATCACTCCCC, positions -296 to -318; underlined sequences are not complementary to HCV).

Genotyping by LiPA

Rapid genotyping of HCV isolates by analysis of 5'UTR was carried out with the Line Probe Assay (Inno-LiPA HCV, Innogenetics, Gent, Belgium) [Stuyver et al., 1993a]. Briefly, first round 5'UTR PCR products were subjected to nested PCR with primers HC3 (TCTAGCCATGGCGTTAGTRYGAGTGT; positions -264 to -238; R=A or G; Y=T or C) and HC4 (CACTCGCAAGCACCCTATCAGGCAGT; positions -29 to -54) in the presence of bio-11-dUTP. After nested PCR the biotinylated DNA products were denatured by alkaline treatment. Reverse hybridization of the DNA products to general and type-specific HCV probes which are applied onto a cellulose membrane strip was carried out in the presence of tetramethyl ammoniumchloride. After stringent washing, alkaline phosphatase and streptavidin conjungate were added. Finally, the HCV isolates were identified and classified by scoring the presence or absence of a purple precipitate at the probe lines.

Genotyping by subtype-specific core amplification

This rapid typing system was developed by Okamoto et al. [1992b] and is based on a universal first round PCR, followed by subtype-specific nested PCR. Four antisense primers in the second PCR yield subtype-specific amplification products of distinct lengths, allowing identification of subtypes 1a, 1b, 2a and 2b by agarose gelelectrophoresis. In this study the originally described general sense primers 256 and 104 [Okamoto et al., 1992b] were replaced by, respectively, LD58c (5'-bio-GGTACTGCCTGATAGGGTGCTTGC; positions -57 to -34) and LD58s (GCCTGATAGGGTGCTTGC; positions -51 to -34). These new general sense primers are located in a completely conserved part of the 5'UTR whereas the original sense primers 256 and 104 were not. The first round of PCR (40 cycles, 1 min at 94°C, 2 min at 48°C, 3 min at 72°C) was performed with LD58c and antisense primer 186c (ATITACCCCATGAGITCGGC; positions

410 to 391). One microliter of the first round PCR product was subjected to subtype-specific nested PCR (40 cycles, 1 min at 94°C, 1 min at 60°C, 1 min at 72°C) with universal sense primer LD58s and the four antisense primers 132 to 135 as described [Okamoto et al., 1992b], exept for primer 133 which was slightly modified by a degeneracy (T and C) at position 273. The antisense primers 132, 133, 134 and 135 are specific for HCV subtypes 1a, 1b, 2a and 2b respectively. In addition, antisense primer 296 (GGATAGGCTGACGTCTA-CCT; positions 196 to 177), which is subtype 1a-specific, was also used for subtype-specific core amplification [Kinoshita et al., 1993] and, together with subtype 1b-specific primer 235 (CGTGGAAGGCGACAAC; positions 175 to 190) used as a ^{32}P radioactive labelled probe in Southern blot analysis. All PCR reactions were undertaken in a Biomed 60 PCR processor (Bittfurth, Germany).

Direct sequencing of PCR products

First round PCR products from the 5' UTR were subjected to nested PCR with primers NCR3 (GGGGCGGCCGCCACCATRRATCACTCCCCTGTGAGG, positions -288 to -314) and antisense primer LD58 (5'-bio-GGCCGGGGCGGCCGCCAAG-CACCCTATCAGGCAGTACCACAAGGC, positions -37 to -64). The first round PCR products from the core region had a biotin moiety at the 5' end of primer LD58c. Biotinylated PCR products were captured onto streptavidin-coated paramagnetic particles (Dynabeads M-280, Dynal, Oslo, Norway) and processed as described previously [Hultman et al., 1991; Kleter et al., 1994].

Phylogenetic analysis

Molecular evolutionary distances between individual isolates were determined by the DNADIST program of the PHYLIP program version 3.5c [Felsenstein, 1993].

Nucleotide sequence accession number

The nucleotide sequences have been deposited in the EMBL data library (accession numbers X58937-X58953, X78858-X78860 and X78862 for 5'UTR sequences; Z29444-Z29474 and for core sequences).

RESULTS

HCV isolates were obtained from a large population of patients participating in a study on treatment of chronic hepatitis C, organized by the Benelux Study Group [Brouwer et al., 1993]. All patients lived in the Netherlands or Belgium and were born in different countries (Table 1). After sequence analysis on 5'UTR, 37 isolates were assigned as type 1 and 21 as type 2.

Table 1. Country of birth of patients infected with HCV types 1 and 2

	HCV type	
Country of birth	1	2
Belgium	5	3
Germany	1	
Indonesia	2	
Italy	1	3
Morocco	2	
Nigeria		1
Spain	2	
Surinam		9
the Netherlands	21	5
Turkey	3	

5'UTR genotyping

HCV genotyping as performed by sequence analysis of the 5' UTR [Kleter et al., 1994] and LiPA [van Doorn et al., 1994] resulted in identical subtyping for type 1 as well as type 2 isolates (Table 2). One type 2 isolate (NE92) has been provisionally classified as "2d" (accession number X78862) because novel covariant mutations were observed at positions -163 and -122 [Kleter et al., 1994]. Sequence analysis of the E1 and NS5B coding regions of this particular isolate confirmed classification as a separate subtype within type 2 [Stuyver et al., 1994].

Table 2. Genotyping of 58 HCV type 1 and 2 isolates by LiPA (5'UTR) and subtype-specific core amplification

LiPA		Core		
			132[a]	296[b]
Type	N	Type	N	N
1a	12	1a	8	7
		1b	1	1
		1a + 1b	2	2
		untyped[c]	1	2
1b	25	1a	2	2
		1b	23	23
2a	18	untyped	18	nd [d]
2b	2	2b	1	nd
		untyped	1	nd
2d	1	untyped	1	nd

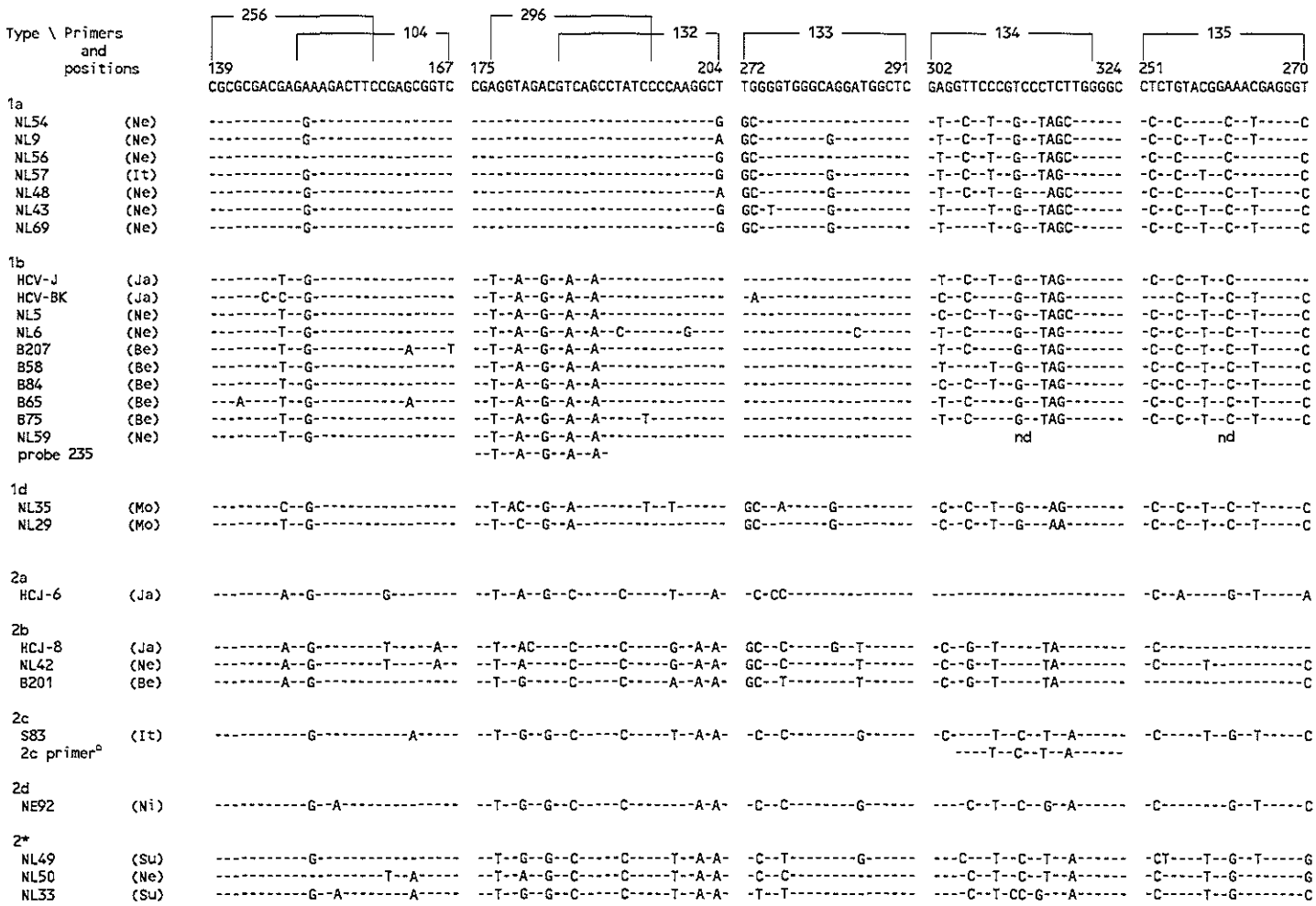
[a]Subtype-specific core amplification with antisense primers 132/133/134/135
[b]Subtype-specific core amplification with antisense primers 296/133/134/135
[c]No reaction in the subtype-specific nested PCR
[d]not determined

Subtype-specific core amplification

The 58 type 1 and 2 isolates were analyzed subsequently also by a modified version of the subtype-specific core amplification method [Okamoto et al., 1992b]. In this study the originally described general sense primers 256 and 104 were replaced by primers LD58c and LD58s respectively. This modification was introduced to circumvent possible failure of amplification by the general primers due to primer target mismatches (Fig. 1, B207), a phenomenon which was also noticed by Okamoto et al. [1993]. First round PCR products were obtained and subsequent subtype-specific amplification was completely dependent on the antisense primers employed in the nested PCR. The core typing results are summarized and compared with LiPA in Table 2.

Type 1 isolates. In 2 of the 37 type 1 HCV isolates, coinfections of subtypes 1a and 1b were detected by subtype-specific core amplification, whereas these isolates were typed as subtype 1a by LiPA (Table 2). In order to confirm this finding, nested PCR products were analyzed by Southern blot hybridization with subtype 1a- and 1b-specific probes (Fig. 2). Probe 235, which is 1b-specific, did not hybridize to the 1b fragment of 342 bp. Probe 296, which is 1a-specific, hybridized to the 255 bp (1a) as well as the 342 bp (1b) PCR fragment. This result indicated the presence of 1a sequences also in the 342 bp (1b) PCR fragment (Fig. 2, lane 12 and 13) and therefore, the subtype 1b primer 133 had falsely amplified the subtype 1a sequence. Additionally, direct sequencing of the N-terminal core region indicated that these two isolates contain only subtype 1a sequences. Direct sequencing of 17 of the 37 type 1 isolates confirmed the subtype-specific core amplification results (Fig. 1).

Two other isolates, NL29 and NL35, remained untyped by subtype-specific core amplification (Table 2). Isolate NL35, typed as subtype 1a by 5'UTR analysis, could not be typed by subtype-specific core amplification (Fig 2, lane 25). Moreover, Southern blot hybridizations of the first round PCR


Type \ Primers and positions
256
104
296
132
133
134
135
139
167
175
204
272
291
302
324
251
270
CGCGCGACGAGAAAGACTTCCGAGCGGTC
CGAGGTAGACGTCAGCCTATCCCCAAGGCT
TGGGGTGGGCAGGATGGCTC
GAGGTTCCCGTCCCTCTTGGGGC
CTCTGTACGGAAACGAGGGT
1a
NL54 (Ne)
NL9 (Ne)
NL56 (Ne)
NL57 (It)
NL48 (Ne)
NL43 (Ne)
NL69 (Ne)
1b
HCV-J (Ja)
HCV-BK (Ja)
NL5 (Ne)
NL6 (Ne)
B207 (Be)
B58 (Be)
B84 (Be)
B65 (Be)
B75 (Be)
NL59 (Ne)
nd
nd
probe 235
1d
NL35 (Mo)
NL29 (Mo)
2a
HCJ-6 (Ja)
2b
HCJ-8 (Ja)
NL42 (Ne)
B201 (Be)
2c
S83 (It)
2c primer°
2d
NE92 (Ni)
2*
NL49 (Su)
NL50 (Ne)
NL33 (Su)

product with either subtype 1a- or 1b-specific probes (296 and 235 respectively) were also negative (Fig. 2, lane 24). Isolate NL29, typed as subtype 1a by 5'UTR analysis, was also identified as 1a by subtype-specific core amplification with primer 132 (Fig. 2, lane 23). However, nested PCR with primer 296 (1a) instead of primer 132 did not yield a PCR fragment (Table 2). Southern blot hybridizations of first and second round PCR products with subtype 1a- and 1b-specific probes were also negative (Fig. 2, lane 22 and 23). The isolates NL29 and NL35 were obtained from patients born in Morocco (Table 2). Phylogenetic analysis of 310 nucleotides of the N-terminal core region revealed that these two isolates possibly belong to an additional type 1 subtype (Table 3). Further analysis of the E1 and NS5B regions confirmed classification into subtype 1d (unpublished observation).

In 3 of the 37 type 1 isolates different subtypes were detected when comparing 5'UTR and Core typing. Two isolates (NL69 and NL43) were typed as subtype 1b by LiPA but as subtype 1a by subtype-specific core amplification (Fig. 2, lane 16 and 18). In one isolate (NL59) the opposite was observed (Fig. 2, lane 20). Sequence analysis of the N-terminal core region (nt 1-310) confirmed the results of subtype-specific core amplification (Fig. 1).

Figure 1. Comparison of type 1 and type 2 nucleotide sequences in the target region of the 2 universal sense (256 and 104) and the 4 subtype-specific antisense (132 [1a], 133 [1b], 134 [2a] and 135[2b]) core primers as described by Okamoto et al. [1992b]. Probes 296 and 235 are subtype 1a- and 1b-specific, respectively. Dashes indicate identical nucleotides. The origin of the HCV isolates is given in parentheses: Be, Belgium; It, Italy; Ja, Japan; Mo, Morocco; Ne, the Netherlands; Ni, Nigeria and Su, Surinam. HCV-J [Kato et al., 1990]; HCV-BK [Takamizawa et al., 1991]; HC-J6 and HC-J8 [Okamoto et al., 1991, 1992a]); S83 [Bukh et al., 1994]; ^ subtype "2a" primer for European isolates as described by Silini et al. [1993].

Type 2 isolates. All 21 samples of type 2 isolates as deduced from sequence analysis of 5'UTR were HCV RNA positive after the first round of PCR with general primers LD58c and 186c. After the subtype-specific nested PCR, only one isolate was typed as subtype 2b (Fig. 2, lane 4) whereas all other type 2 isolates remained untyped (Table 2). Five of the 20 untyped samples were sequenced and 3 to 6 mismatches were found to the type 2 subtype-specific primers 134 (2a) or 135 (2b; Fig. 1). The obtained sequences showed also mismatches to the HCV subtype "2a" primer for European isolates as described by Silini et al.[1993]. This primer appeared to be specific for HCV subtype 2c (Fig. 1, S83). The subtype 2b isolate that was identified by PCR contained only a single mismatch at the very 5' end of subtype-specific 2b primer 135 (Fig 1, B201).

Type 2 core sequences were subjected to phylogenetic analysis and molecular evolutionary distances were determined between our and published HCV subtype 2a (n=5), 2b (n=8), 2c (S83) and 2d (NE92) sequences (Table 3). From this analysis it appeared that type 2 sequences are highly heterogeneous. The 3 isolates [typed as subtype 2a by LiPA (Fig 1, NL33, NL49 and NL50)] had greater molecular evolutionary distances to subtype 2a than the distances observed between subtypes 2c and 2d (Table 3). The distances among the 3 isolates were also greater (range 0.0647-0.0765) than the maximum distance between confirmed subtype 2a sequences (Table 3, 0.0605). These results suggest that these 3 isolates belong to 3 additional HCV type 2 subtypes. This observation has been confirmed by sequence analysis of the E1 and NS5B regions (unpublished observations). Interestingly, 2 of the 3 new subtypes were detected in patients born in Surinam. In contrast to this heterogeneity, the core sequence of the two isolates typed as 2b by LiPA, were highly homologous to published 2b core sequences (Table 3).

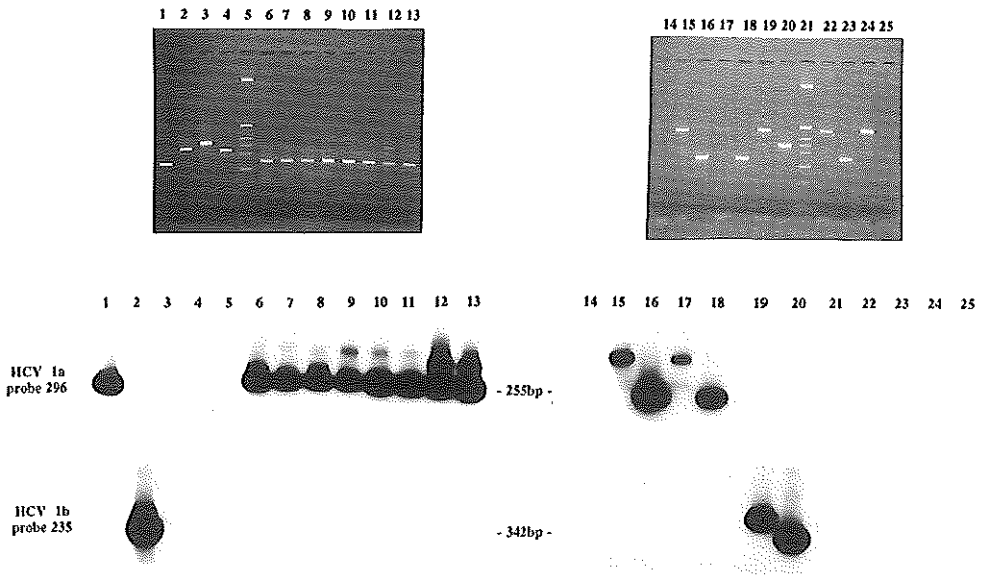


Figure 2. Identification of subtype-specific nested PCR products obtained with sense primer LD58c and antisense primers 132, 133, 134 and 135. **Top:** ethidiumbromide staining of agarose gels. **Middle:** Southern blot hybridization with subtype 1a-specific probe 296. **Bottom:** Southern blot hybridization with subtype 1b-specific probe 235. Typical results of subtype 1a (255 bp), 1b (342 bp), 2a (372 bp) and 2b (321 bp) are shown in lanes 1 to 4, respectively. Lanes 5 and 21 contain marker DNA, i.e., pBR322 digested by HinfI. Lanes 6 to 11 show subtype 1a sequences. Lanes 12 and 13 show a coinfection of HCV subtypes 1a and 1b. Lane 14, negative PCR control. Lanes 15, 17, 19, 22 and 24 show the first round PCR product of primer set LD58c/186c and lanes 16, 18, 20, 23 and 25 show nested PCR products of isolate NL69 (1a), NL43 (1a), NL59 (1b), NL29 (1d) and NL35 (1d), respectively.

DISCUSSION

The most reliable approach for genotypic classification of HCV isolates would be nucleotide sequencing of the entire RNA genome. Genotypic variation seems to be maintained throughout the viral genome [Chan et al., 1992] and therefore, only part of the genome needs to be analyzed. However, for analysis of a large number of samples this is laborious and rapid HCV genotyping assays are therefore required. In this study subtyping of type 1 and type 2 HCV isolates was compared by two rapid assays, i.e., the LiPA and subtype-specific core amplification.

5' UTR genotyping by LiPA

For detection of HCV viremia RT-PCR is generally aimed at the 5'UTR. Therefore, LiPA offers a convenient method for genotyping of HCV isolates supplementary to HCV RNA detection in a routine diagnostic setting. The 58 isolates in this study showed that all LiPA results were in accordance with 5'UTR sequence analysis. However, in 7 (19%) of the 37 type 1 isolates subtyping results by LiPA were not similar to that obtained by subtype-specific core amplification. In the case of 5 HCV isolates this observation may be explained by the minimal sequence difference between subtypes 1a and 1b at position -99. At this particular position subtype 1a contains an Adenosine whereas subtype 1b contains a Guanosine. This minor difference should be considered as lack of sufficient sequence variation in the highly conserved 5' UTR between subtypes 1a and 1b, rather than by recombination between these subtypes. On the other hand, nucleotide variations in the 5'UTR are analyzed thoroughly by LiPA. Each HCV type is recognized by 2 or more probes. By assessing hybridization to multiple probes 26-41% of the 5'UTR sequence is determined [Stuyver et al., 1993a]. This contrasted the use of one subtype-specific primer for HCV genotyping in the subtype-specific core amplification system.

Table 3. Molecular evolutionary distances (range and mean ± SD) between HCV subtypes and isolates in the core region (nt 1-310)

HCV type 1 isolates				
subtype/ isolate	1a	1b	1c	NL29
1a	0.0065-0.0436			
	0.0259 ± 0.0092			
1b	0.0682-0.1290	0.0197-0.0641		
	0.0895 ± 0.0128	0.0369 ± 0.0130		
1c	0.0572-0.0868	0.0716-0.1242	0.0000-0.0230	
	0.0738 ± 0.0080	0.0941 ± 0.0112	0.0177 ± 0.0066	
NL29	0.0596-0.0712	0.0489-0.0859	0.0593-0.0743	
	0.0633 ± 0.0037	0.0659 ± 0.0109	0.0653 ± 0.0057	
NL35	0.0900-0.1097	0.0757-0.1009	0.0900-0.1053	
	0.0947 ± 0.0035	0.0853 ± 0.0106	0.0990 ± 0.0057	.0524
HCV type 2 isolates				
subtype/ isolate	2a	2b	2c	2d
2a	0.0262-0.0605			
	0.0384 ± 0.0096			
2b	0.0719-0.1236	0.0065-0.0431		
	0.1018 ± 0.0135	0.0258 ± 0.0094		
2c	0.0711-0.0928	0.0858-0.1079		
	0.0805 ± 0.0100	0.0937 ± 0.0065		
2d	0.0600-0.0817	0.0860-0.1079		
	0.0672 ± 0.0107	0.0955 ± 0.0056	0.0467	
NL33	0.0817-0.1037	0.1044-0.1271		
	0.0891 ± 0.0093	0.1100 ± 0.0124	0.0572	0.0533
NL49	0.0683-0.0975	0.0947-0.1093		
	0.0821 ± 0.0127	0.1020 ± 0.0056	0.0616	0.0649
NL50	0.0716-0.1008	0.0978-0.1166		
	0.0861 ± 0.0112	0.1068 ± 0.0079	0.0717	0.0824

Subtype-specific core amplification

Two problems were encountered with subtype-specific core amplification in this study population of residents in Western Europe. First, although identification of type 1 isolates was clearly possible, 2 (5.5%) of the 37 HCV type 1 isolates were diagnosed as a 1a and 1b coinfection. Theoretically, this phenomenon could be explained by the presence of a small amount of subtype 1b sequences that could have been detected by the highly sensitive nested PCR. However, direct sequencing and Southern blot analyses with subtype 1a- and 1b-specific probes revealed that these isolates only contained subtype 1a sequences. A more likely explanation therefore seems to be imperfect annealing of primer 133 to the subtype 1a genome, resulting in aspecific amplification [Andonov and Chaudhary, 1994]. Non-specific amplification occurred only in HCV subtype 1a isolates (Fig. 1 and 2). The opposite phenomenon, i.e., nonspecific amplification of '1a' sequences in subtype 1b isolates was not observed.

Secondly, identification of type 2 isolates by subtype-specific core amplification was a major problem. Of the 21 HCV type 2 isolates, only 1 isolate could be subtyped as subtype 2b although a positive result was obtained in all cases after the first round of PCR. The remaining 20 untyped isolates did not yield any nested PCR fragment, even after reducing the annealing temperature in the nested PCR to 55°C (data not shown). The antisense core primers are subtype-specific. Therefore, failure of the nested PCR may be explained by mismatches between the subtype-specific primers and their target sequences as is illustrated in Figure 1 (i.e. NL42: 3 mismatches to primer 135).

Recently, identical 5'UTR sequences were described for subtypes 2a and 2c [Bukh et al., 1993]. Therefore, it is possible that the 18 isolates, typed as 2a by LiPA, belong to HCV subtype 2c. However, phylogenetic analysis carried out on 3 of these 18 samples suggested that each of these isolates could be classified into yet another subtype. This observation suggests the existence of

multiple subtypes among type 2 isolates in Western Europe and requires confirmation by sequence analysis of the E1 and NS5B regions. Theoretically, the subtype-specific "2a" primer as described by Silini et al. [1993] would not allow typing of the 3 type 2 isolates due to mismatches at essential positions (Fig. 1). These results indicate that HCV type 2 isolates in our study population are considerably different from the published type 2 isolates from Japan [Okamoto et al., 1991, 1992b]. Technically, subtype-specific core amplification is a feasible assay for subtyping of HCV isolates. However, improvement of the subtype-specific core amplification assay for typing of HCV isolates will be difficult due to the extended variation among HCV subtypes. At present more than 29 HCV subtypes have been reported [Bukh et al., 1994; Stuyver et al., 1994; Tokita et al., 1994a, 1994b].

In summary, it appeared that several HCV RNA isolates remained untyped by subtype-specific core amplification, indicating that this typing method is restricted to the patient population to be analyzed. Furthermore, rapid genotyping results obtained by either LiPA or subtype-specific core amplification were generally in accordance with sequence analysis at respectively the 5'UTR and core regions. However, the specificity of both rapid genotyping assays is occasionally hampered due to a lack or excess of sequence variation in their respective target regions as is sequence analysis for HCV typing if restricted to one of these regions.

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REFERENCES

Andonov A, Chaudhary RK (1994): Genotyping of Canadian hepatitis C virus isolates by PCR. Journal of Clinical Microbiology 32:2031-2034.

Brouwer JT, Benelux Multicentre Trial Study Group (1993): Benelux multicentre trial of alpha interferon treatment for chronic hepatitis C: Standard versus high-dose therapy monitored by biochemical and virological markers (interim analysis). Gut, supplement S119-120.

Bukh J, Purcell RH, Miller RH (1992): Sequence analysis of the 5′ noncoding region of hepatitis C virus. Proceedings of the National Academy of Sciences of the USA 89:4942-4946.

Bukh J, Purcell RH, Miller RH (1993): At least 12 genotypes of hepatitis C virus predicted by sequence analysis of the putative E1 gene of isolates collected worldwide. Proceedings of the National Academy of Sciences of the USA 90:8234-8238.

Bukh J, Purcell RH, Miller RH (1994): Sequence analysis of the core gene of 14 hepatitis C virus genotypes. Proceedings of the National Academy of Sciences of the USA 91:8239-8243.

Cha T-A, Beall E, Irvine B, Kolberg J, Chien D, Kuo G, Urdea MS (1992): At least five related, but distinct, hepatitis C viral genotypes exist. Proceedings of the National Academy of Sciences of the USA 89:7144-7148.

Chan S-W, McOmish F, Holmes EC, DowB, Peutherer JF, Follett E, Yap PL, Simmonds P (1992): Analysis of a new hepatitis C virus type and its phylogenetic relationship to existing variants. Journal of General Virology 73:1131-1141.

Chayama K, Tsubota A, Arase Y, Saitoh S, Koida I, Matsumoto T, Kobayashi M, Iwasaki S, Koyama S, Morinaga T, Kumada H (1993): Genotypic subtyping of hepatitis C virus. Journal of Gastroenterology and Hepatology 8:150-156.

Choo Q-L, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M, (1989): Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 244:359-362.

Choo Q-L, Richman KH, Han JH, Berger K, Lee C, Dong C, Gallegos C, Coit D, Medina-Selby A, Barr PJ, Weiner AJ, Bradley DW, Kuo G, Houghton M (1991): Genetic Organization of the hepatitis C virus. Proceedings of the National Academy of Sciences of the USA 88:2451-2455.

Dusheiko G, Schmilovitz-Weiss H, Brown D, McOmish F, Yap P-L, Sherlock S, McIntyre N, Simmonds P (1994): Hepatitis C virus genotypes: An investigation of type-specific differences in

geographic origin and disease. Hepatology 19:13-18.

Enomoto N, Takada A, Nakao T, Date T (1990): There are two major types of hepatitis C virus in Japan. Biochemical and Biophysical Research Communications 170:1021-1025.

Felsenstein J (1993): PHYLIP (Phylogenetic inference Package) version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle, WA, USA.

Hagiwara H, Hayashi N, Mita E, Takehara T, Kasahara A, Fusamoto H, Kamada T (1993): Quantitative analysis of hepatitis C virus RNA in serum during interferon alfa therapy. Gastroenterology 104:877-883.

Hino K, Sainokami S, Shimoda K, Iino S, Wang Y, Okamoto H, Miyakawa Y, Mayumi M (1994): Genotypes and titers of hepatitis C virus for predicting the response to interferon in patients with chronic hepatitis C. Journal of Medical Virology 42:299-305.

Hultman T, Bergh S, Moks T, Uhlén M (1991): Bidirectional solid-phase sequencing of in vitro-amplified plasmid DNA. BioTechniques 10:84-93.

Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugimura T, Shimothono K (1990): Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proceedings of the National Academy of Sciences of the USA 87:9524-9528.

Kinoshita T, Miyake K, Okamoto H, Mishiro S (1993): Imported hepatitis C virus genotypes in Japanese haemophiliacs. Journal of Infectious Diseases 168:249-250.

Kleter GEM, Brouwer JT, Heijtink RA, Schalm SW, Quint WGV (1993): Detection of hepatitis C virus RNA in patients with chronic hepatitis C virus infections during and after therapy with alpha-interferon. Antimicrobial Agents and Chemotherapy 37:595-597.

Kleter GEM, van Doorn L-J, Brouwer JT, Schalm SW, Heijtink RA, Quint WGV (1994): Sequence analysis of the 5'untranslated region in isolates of at least four genotypes of hepatitis C virus in The Netherlands. Journal of Clinical Microbiology 32:306-310.

Kobayashi Y, Watanabe S, Konishi M, Yokoi M, Kakehashi R, Kaito M, Kondo M, Hayashi Y, Jomori T, Suzuki S (1993): Quantitation and typing of serum hepatitis C virus RNA in patients with chronic hepatitis C treated with interferon-ß. Hepatology 18:1319-1325.

Lau JYN, Davis GL, Kniffen J, Qian K-P, Urdea MS, Chan CS, Mizokami M, Neuwald PD, Wilber JC (1993): Significance of serum hepatitis C virus RNA levels in chronic hepatitis C. Lancet 341:1501-1504.

McOmish F, Chan S-W, Dow BC, Gillon J, Frame WD, Crawford RJ, Yap PL, Follett EAC, Simmonds P (1993): Detection of three types of hepatitis C virus in blood donors: investigation of type-specific differences in serologic reactivity and rate of alanine aminotransferase abnormalities. Transfusion 33:7-13.

Miller RH, Purcell RH (1990). Hepatitis C virus shares amino acid sequence similarity with Pestiviruses and Flaviviruses as well as members of two plant virus supergroups. Proceedings of the National Academy of Sciences of the USA 87:2057-2061

Mori S, Kato N, Yagyo A, Tanaka T, Ikeda Y, Petchclai B, Chiewsilp P, Kurimura T, Shimotohno K (1992): A new type of hepatitis C virus in patients in Thailand. Biochemical and Biophysical Research Communications 183:334-342.

Murphy D, Willems B, Delage G (1994): Use of 5' noncoding region for genotyping hepatitis C virus. Journal of Infectious Diseases 169:473-475.

Nakao T, Enomoto N, Takada N, Takada A, Date T (1991): Typing of hepatitis C virus genomes by restriction fragment length polymorphism. Journal of General Virology 72:2105-2112.

Okamoto H, Okada S, Sugiyami Y, Kurai K, Lizuka H, Machida A, Miyakawa Y, Mayumi M (1991): Nucleotide sequence of the genomic RNA of hepatitis C virus isolated from a human carrier: Comparison with reported isolates for conserved and divergent regions. Journal of general Virology 72:2697-2704.

Okamoto H, Kurai K, Okada S-I, Yamamoto K, Lizuka H, Tanaka T, Fukuda S, Tsuda F, Mishiro S (1992a): Full-length sequence of a hepatitis C virus genome having poor homology to reported isolates: Comparative study of four distinct genotypes. Virology 188:331-341.

Okamoto H, Sugiyama Y, Okada S, Kurai K, Akahane Y, Sugai Y, Tanaka T, Sato K, Tsuda F, Miyakawa Y, Mayumi M (1992b): Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. Journal of General Virology 73:673-679.

Okamoto H, Tokita H, Sakamoto M, Horikita M, Kojima M, Iizuka H, Mishiro S (1993): Characterization of the genomic sequence of type V (or 3a) hepatitis C virus isolates and PCR primers for specific detection. Journal of General Virology 74:2385-2390.

Silini E, Bono F, Cerino A, Piazza V, Solicia E, Mondelli MU (1993): Virological features of hepatitis C virus infection in hemodialysis patients. Journal of Clinical Microbiology 31:2913-2917.

Simmonds P, McOmish F, Yap PL, Chan S-W, Lin CK, Dusheiko G, Saeed AA, Holmes EC

(1993a): Sequence variability in the 5' non-coding region of hepatitis C virus: identification of a new virus type and restrictions on sequence diversity. Journal of General Virology 74:661-668.

Simmonds P, Holmes EC, Cha T-C, Chan S-W, McOmish F, Irvine B, Beall E, Yap PL, Kolberg J, Urdea MS (1993b). 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 74:2391-2399.

Simmonds P, Alberti A, Alter HJ, et al. (1994). A proposed system for nomenclature of hepatitis C viral genotypes. Hepatology 19:1321-1324.

Stuyver L, Rossau R, Wyseur A, Duhamel M, Vanderborght B, Van Heuverswyn H, Maertens G (1993a). Typing of hepatitis C virus isolates and characterization of new subtypes using a line probe assay. Journal of General Virology 74:1093-1102.

Stuyver L, Van Arnhem W, Wyseur A, DeLeys R, Maertens G (1993b): Analysis of the putative E1 envelope and NS4a epitope regions of HCV type 3. Biochemical and Biophysical Research Communications 192:635-641.

Stuyver L, Van Arnhem W, Wyseur A, Hernandez F, Delaporte E, Maertens G (1994): Classification of hepatitis C viruses based on phylogenetic analysis of the E1 and NS5b regions and identification of 5 new subtypes. Proceedings of the National Academy of Sciences of the USA 91:10134-10138.

Takamizawa A, Mori C, Fuke I, Manabe S, Murakami S, Fujita J, Onishi E, Andoh T, Yoshida I, Okayama H (1991): Structure and organization of the hepatitis C virus genome isolated from human carriers. Journal of Virology 65:1105-1113.

Tokita H, Man Shrestha SM, Okamoto H, Sakamoto M, Horikita M, Iizuka H, Shrestha S, Miyakawa Y, Mayumi M (1994a): Hepatits C virus variants from Nepal with novel genotypes and their classification into the third major group. Journal of General Virology 75:931-936.

Tokita H, Okamoto H, Tsuda, F, Song P, Nakata S, Chosa T, Iizuka H, Mishiro S, Miyakawa Y, Mayumi M (1994b): Hepatitis C virus variants from Vietnam are classifiable into the seventh, eighth and ninth major genetic groups. Proceedings of the National Academy of Sciences of the USA 91:11022-11026.

Tsubota A, Chayama K, Ikeda K, Yasuji A, Koida I, Saitoh S, Hashimoto M, Iwasaki S, Kobayashi M, Hiromitsu K (1994): Factors predictive of response to interferon-α therapy in hepatitis C infection. Hepatology 19:1088-1094.

van Doorn L-J, Kleter B, Stuyver L, Maertens G, Brouwer H, Schalm S, Heijtink R, Quint W

(1994): Analysis of hepatitis C virus genotypes by a Line Probe Assay (LiPA) and the correlation with antibody profiles. Journal of Hepatology 21:122-129.

Yohsioka K, Kakumu K, Wakita T, Ishikawa T, Itoh Y, Takayanagi M, Higashi Y, Shibata M, Morishima T (1992). Detection of hepatitis C virus by polymerase chain reaction and response to interferon-α therapy: relationship to genotypes of hepatitis c virus. Hepatology 16:293-299.

CHAPTER 8

Hepatitis C virus types 1 to 5 and the relationship with geographical regions, routes of transmission, clinical characteristics and liver disease

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ABSTRACT

HCV RNA isolates from 292 chronically infected patients living in the Benelux countries were genotyped by the Line Probe Assay (LiPA). Subsequently, the relationship with geographical origin of the patient, route of transmission, clinical characteristics and severity of liver disease was analyzed. Patients of European origin were predominantly infected with HCV subtype 1b (164/257, 64%, 95%CI 58-70). Also, the 13 patients of Asian origin were most frequently infected with HCV subtype 1b (54%). The ten patients originating from Surinam (South America) had predominantly type 2 (90%) while this was only 5% in Europeans. Africans were mainly infected with type 4 (7/12, 59%); this was only 4% in Europeans. Blood transfusion was established as the most suspected route of transmission in 145 (49%) patients; in these HCV subtype 1b was predominant (68%, 95%CI 61-76). In the group of 40 (14%) patients that might have contracted their infection by intravenous drug abuse (IVDA) HCV subtype 1b was only 17% ($P<0.001$). Patients infected by IVDA had mainly subtype 1a and type 3, respectively 10 (25%) and 18 (45%) individuals. Occupational needle accident or tattoo was a possible mode of transmission in 11 (4%) patients. In 96 (33%) patients risk factors for exposure to the virus could not be identified. Cirrhosis was observed in 70 (24%) patients. No significant relationship was found between a specific HCV (sub)type and the presence of cirrhosis, although 10 (43%) of the 23 HCV type 2 infected patients had cirrhosis and this was absent in the seven patients with HCV type 5 ($P=0.09$). In summary, the HCV types 1 to 5 as detected in this population were related to the country of origin and the route of transmission. No evidence was found that HCV (sub)types have an influence on the clinical characteristics or on the severity of liver disease.

INTRODUCTION

Sequence analysis of the hepatitis C virus (HCV) RNA genome revealed

considerable heterogeneity between patient isolates. Currently, there is evidence for the existence of at least 9 major HCV types [Tokita et al., 1994a]. These types are classified according to an useful system based on nucleotide variations [Simmonds et al., 1993]. By sequence analysis of 222 nucleotides in the NS5 region, HCV isolates can be provisionally classified as a new HCV type when sequence similarities are less than 72% with any known sequence whereas sequence similarities of 75% to 86% would provide evidence for a new HCV subtype. Classification could be confirmed by both sequence analysis of another coding region and phylogenetic analysis, which would indicate the relationship with already characterized HCV (sub)types. This method for classification of HCV isolates has been accepted by the international community [Simmonds et al., 1994].

For identification of HCV isolates several rapid typing assays have been designed. They include techniques such as (sub)type-specific reverse hybridization at the 5' untranslated region (5'UTR) by a Line Probe Assay [LiPA; Stuyver et al., 1993; van Doorn et al., 1994], restriction fragment length polymorphism (RFLP) at 5'UTR [McOmish et al., 1993] and subtype-specific amplification of either the core region [Okamoto et al., 1992] or the NS5B region [Chayama et al., 1993].

Fast typing of HCV isolates revealed that the distribution of HCV (sub)types was different between various countries or geographical areas [Takada et al., 1993]. In Japan HCV subtype 1b is most prevalent (70-80%) [Takada et al., 1993; Chayama et al., 1993; Yamada et al., 1994] and in the USA HCV subtypes 1a and 1b were detected in 74% of the patients [Mahaney et al., 1994]. HCV type 2 co-exists with subtype 1b at an incidence of about 20% in Japan as well as in the USA [Takada et al., 1993; Chayama et al., 1993; Yamada et al., 1994; Mahaney et al., 1994]. In European countries the prevalence of HCV type 1 as determined in blood donors was 47% in Scotland and as high as 98% in Hungary [McOmish et al., 1994]. Approximately 40% of the Scottish blood donors were infected with HCV type 3 [McOmisch et al., 1994]. This type was also frequently found in small populations from Nepal and Thailand [Mori et al., 1992; Tokita et al 1994b; Apichartpiyakul et al., 1994].

HCV type 4 was observed in the majority of patients from the African countries [Stuyver et al., 1993; Dusheiko et al., 1994; Xu et al., 1994], and HCV types 5, 6 and 7-9 were locally detected in, respectively South-Africa, Hong-kong and Vietnam [Bukh et al., 1992; Simmonds et al., 1994; Tokita et al., 1994a]. Since different HCV (sub)types exist, it is of interest to know whether differences in prevalence of HCV types are related to the mode of transmission. High rates of HCV antibodies have been found among patients infected by blood transfusion [Alter et al., 1989; Esteban et al., 1989; Kuo et al., 1989; van der Poel et al., 1989], haemophiliacs [Esteban et al., 1989; Roggendorf et al., 1989; van der Poel et al., 1991], intravenous drug addicts [Esteban et al., 1989; Roggendorf et al., 1989] and in non-A, non-B hepatitis patients without identified risk factors, so called sporadic hepatitis C [Kuo et al., 1989; Roggendorf et al., 1989]. In contrast, rather low prevalence figures have been recorded among sexual partners and in children from HCV positive mothers [Brettler et al., 1992; Reinus et al., 1992; Wejstal et al., 1992; Bresters et al., 1993; Lam et al., 1993].

HCV causes a chronic infection in the majority of cases [Alter et al., 1989; Gerber 1993] and may progress towards liver cirrhosis and hepatocellular carcinoma [Bruix et al., 1989; Colombo et al., 1989; Kew et al., 1990; Saito et al., 1990; Bukh et al., 1993]. This process of liver damage may take decades [Yano et al., 1993], although some patients developed cirrhosis within 5-10 years after detection of onset of the disease [Realdi et al., 1982; Hay et al., 1985]. The clinical implications of different HCV (sub)types were only partially analyzed in either small patient cohorts or in a selected number of (sub)types [Dusheiko et al., 1994; Qu et al., 1994; McOmish et al., 1993; Takada et al., 1992; Yamada et al., 1994]. It has been suggested that HCV subtype 1b causes a more serious liver disease than other (sub)types in either uncompromised patients [Pozatto et al., 1991; Dusheiko et al., 1994] or in patients after liver transplantation [Feray et al., 1995].

In the present study we used LiPA (Innogenetics, Gent, Belgium) for typing of 292 HCV RNA isolates from chronically infected patients and correlated the typing data with geographical origin of the patients, route of transmission, clinical characteristics and severity of liver disease.

MATERIALS AND METHODS

Patients

The 292 patients investigated in the present study were derived from a population of 355 patients with a chronic HCV infection who enrolled in a multi-centre randomized trial organized by the Benelux Study Group on treatment of hepatitis C [Brouwer et al., 1993]. Results of interferon therapy will be described elsewhere. The patients lived in the Benelux (Belgium, the Netherlands or Luxembourg) area of Western Europe and were between 26 and 74 years of age, had elevated alanine aminotransferase (ALT) levels of at least twice the upper limit of normal (i.e. 30IU/L) for more than 6 months, confirmed antibodies to HCV except for one, detectable HCV RNA and liver histology compatible with chronic non-A, non-B hepatitis. Patients with concomittant other causes of liver disease were excluded.

HCV genotyping was performed in 320 (90%) of the 355 cases. Information on the background of the HCV infection was received in 312 (88%) of the 355 patients. In 14 of these 312 cases the HCV type was not determined due to reconsideration of diagnosis in 5, and no blood being available in 9. Additionally, in 6 cases, liver histology could not be determined because liver biopsy was not done. The clinical characteristics of the 63 patients excluded did not differ from the 292 patients discussed in this study.

Questionnaire

The patients were specifically orally questioned by their local physician for the backgrounds of the HCV infection with the use of a questionnaire. Questions were related to: (i) the ethnic origin (race, nationality, country of birth and living abroad for 6 months or more), (ii) the mode and date of acquisition: i.e. type of surgery in the past, use of blood-products and occurrence of jaundice; intravenous drug abuse; receipt of tattoos; promiscuity. Subjects who had no risk factors, identified were scored as sporadic hepatitis C.

Liver biopsy

Liver tissue was obtained by needle biopsy and examined by the local pathologist for inflammatory activity and stage of fibrosis. From the 292 liver biopsies 246 (84%) were re-evaluated by one central pathologist. Discordant results for cirrhosis were found in 22 (8.9%) cases. The kappa value was 0.74 meaning a good correlation. Therefore, biopsy results from the local pathologist were used in the analysis.

Antibodies

Antibodies to HCV, as locally determined by screening assays, were confirmed by RIBA-2 (Ortho Diagnostics, Raritan, NJ, USA) and/or Inno-LIA HCV Ab II and/or III (Innogenetics, Gent, Belgium) according to the instructions of the manufacturer. Hepatitis B core antibodies were detected by EIA (Abbott, North Chicago, IL, USA).

Blood samples

For HCV RNA detection and HCV genotyping, plasma samples obtained prior to interferon treatment were analyzed. EDTA-blood was collected by venipuncture and plasma was prepared within 2 hours after sampling. Samples were stored at -70 °C and shipped on frozen carbondioxide.

HCV genotyping

The viral RNA was isolated as described previously [Kleter et al., 1993] and RT-PCR (40 cycles, 1 min at 94°C, 2 min at 48°C, 3 min at 72°C) was performed at the 5'UTR with sense primer HCV35 (TTGGCGGCCGCACTCCACCATGAAT-CACTCCCC, positions -296 to -318; the underlined sequence is not complementary to HCV) and anti-sense primer HCV19 (GTGCACGGTCTACGAGACCT, positions -1 to -20). The amplification products were used in the Line Probe Assay (Inno-LiPA, Innogenetics, Gent, Belgium) for HCV typing [Stuyver et al., 1993].

Statistics

Percentages were compared using the Chi-square test, or Fisher's exact test if appropriate. Continous data were compared using the t-test. Logistic regression was used to investigate the relation between the percentage of cases with cirrhosis and various characteristics simultaneously. $P=0.05$ (two-sided) was considered as the limit of significance.

RESULTS

HCV (sub)types

By means of LiPA, 286 (98%) of the 292 HCV isolates were identified as type 1 to 5 (Table 1). Four HCV isolates were provisionally classified as type "1*". Two of these isolates did not react with the type 1 subtype probes and two isolates from patients originating from Morocco could be classified as subtype 1d as indicated by core, core/E1 and NS5B sequence analysis (unpublished observations). Mixed HCV infections were observed in two patients. One was infected with HCV subtypes 1a and 1b whereas the other had HCV types 1, 3 and 4.

Geographical regions

In the 292 patients analyzed, a significant association between HCV type and origin was noted. HCV (sub)types 1a, 1b, 2, 3, 4 and 5 were found in 257 European patients (Table 1). In this group HCV subtype 1b was predominant and identified in 164 cases (64%, 95%CI 58-70%). The other HCV (sub)types were detected at frequencies ranging from 3-15% (Table 1). Similarly, HCV subtype 1b was documented in 7 of the 13 Asian patients. The distribution of HCV types in the 13 Asian patients was not significantly different from that observed in the 257 Europeans. In contrast, HCV type 2 was predominantly present in the ten patients originating from Surinam, a former Dutch colony in South America (90%; 95%CI 67-100). This type was observed in only 5% of the Europeans. Similarly, HCV type 4 was most prevalent in the 12 Africans

Table 1. Distribution of HCV types per geographic origin[a] of the patient

HCV (sub)type	Total 292	Europe 257	Asia 13	Africa 12	South America 10
1a	24 (8%)	22 (8%)	2 (15%)	0	0
1b	172 (59%)	164 (64%)	7 (54%)	1 (8%)	0
1*	4 (1%)	2 (0.7%)	0	2 (17%)	0
2	23 (8%)	13 (5%)	0	1 (8%)	9 (90%)
3	44 (15%)	39 (15%)	4 (31%)	0	1 (10%)
4	16 (5%)	9 (4%)	0	7 (59%)	0
5	7 (2%)	7 (3%)	0	0	0
multiple	2 (1%)	1 (0.3%)	0	1 (8%)	0

[a]origin was determined by country of birth

(59%; 95%CI 26-91) while this was only found in 4% of the patients of European origin. The seven African patients infected with HCV type 4 were born in Egypt (n = 4), Zaire (n = 2) and Morocco (n = 1).

Mode of acquisition

HCV transmission by blood transfusion was established in 145 (49%) of the 292 cases (Table 2). In these patients HCV types 1 to 5 were present but subtype 1b was predominant (68%, 95%CI 61-76). Contraction of the HCV infection by intravenous drug abuse (IVDA) was documented in 40 cases (Table 2). In contrast to blood transfusion subtype 1b was found in only 17% of the IVDA group ($P<0.001$). Patients infected by IVDA had mainly subtype 1a (25%) and type 3 (45%). IVDA was the major route of transmission for subtype 1a (42% of the cases) and type 3 (41% of the cases), whereas it was a minor cause of infection for all other types (mean 6%).

Table 2. Prevalence of HCV (sub)types per route of transmission

HCV (sub)type	Transfusion 145 (49%)	IVDA[a] 40 (14%)	Needle[b] 11 (4%)	Sporadic[c] 96 (33%)	Total 292
1a	9 (6%)	10 (25%)	0	5 (5%)	24 (8%)
1b	99 (68%)	7 (17%)	3 (27%)	63 (66%)	172 (59%)
1*	1 (1%)	0	0	3 (3%)	4 (1%)
2	10 (7%)	3 (7%)	1 (9%)	9 (9%)	23 (8%)
3	15 (11%)	18 (45%)	4 (37%)	7 (7%)	44 (15%)
4	3 (2%)	2 (5%)	3 (27%)	8 (8%)	16 (5%)
5	6 (4%)	0	0	1 (1%)	7 (2%)
multiple	2 (1%)	0	0	0	2 (1%)

[a]intravenous drug abuse
[b]occupational needle accident or tattoo
[c]including promiscuity

The route of HCV transmission remained unknown in 96 (33%) cases, therefore these infections were classified as sporadic hepatitis C (Table 2). In 6 sporadic cases, patients had marked promiscuity as a possible source of their HCV infection. In these so-called sporadic cases, HCV (sub)types 1a, 1b, 2, 3, 4, and 5 were found at a similar incidence rate as in patients who had undergone blood transfusion. Similarly, clinical characteristics (age, gender, anti-HBc) and liver disease (ALT and histology) were comparable in both groups. The median age of the patients infected by blood transfusion was 48 (range 24-73) years and it was 47 (range 24-72) years for those with sporadic hepatitis C. The prevalence of a previously resolved HBV infection was 14% (95%CI 9-20) in transmission by blood transfusion and 21% (95%CI 13-29) in sporadic hepatitis C. The median serum levels of ALT were 131 (range 45-926) IU/L and 114 (range 37-505) IU/L for respectively, blood transfusion and sporadic

Table 3. Comparison of clinical characteristics with different HCV (sub)types

HCV (sub)type	Male 177 (61%)	Jaundice 34 (12%)	Anti-HBc 57 (20%)	Age[a] 45 (22-73)	Duration[a,b] 13 (1-45)
1a	18 (75%)	1 (4%)	6 (25%)	35 (22-67)	10 (1-24)
1b	93 (54%)	18 (10%)	24 (14%)	49 (24-72)	13 (1-45)
1*	4 (100%)	0	2 (50%)	45 (24-68)	25 (17-33)
2	15 (65%)	3 (13%)	12 (52%)	47 (26-68)	17 (4-38)
3	30 (68%)	5 (11%)	8 (18%)	37 (22-73)	10 (1-31)
4	13 (81%)	3 (19%)	4 (25%)	37 (24-68)	15 (5-27)
5	3 (43%)	3 (43%)	1 (14%)	49 (30-65)	16 (1-28)
multiple	1 (50%)	1 (50%)	0	34 (34-35)	2 (1-2)

[a]Data expressed as median (range) in years

[b]Duration of infection could be determined in 193 patients

hepatitis C. Cirrhosis was found in 26% (95%CI 19-34) of the patients infected by blood transfusion and in 25% (95%CI 17-35) of those infected by sporadic hepatitis C.

Clinical characteristics

Gender and jaundice were not significantly related to a specific HCV (sub)type (Table 3). A previously resolved HBV infection, anti-HBc positivity, was found in 57 (20%) of the 292 patients (Table 3). A high incidence of hepatitis B core antibodies was observed in HCV type 2 infected patients (52% for type 2 versus 16% in all other types, $P<0.001$). An association between age and HCV (sub)type was also observed (Table 3). Older patients appeared to be infected with HCV (sub)types 1b, 2 and 5, whereas the younger ones were infected with 1a, 3 and 4 ($P<0.001$).

Table 4. Comparison of the activity of liver inflammation and liver histology with different HCV (sub)types

HCV (sub)type	ALT[a] 147 (37-927)	CPH 68 (23%)	CAH 154 (53%)	Cirrhosis 70 (24%)
1a	141 (42-291)	6 (25%)	14 (58%)	4 (17%)
1b	144 (38-927)	39 (23%)	90 (52%)	43 (25%)
1*	144 (98-194)	1 (25%)	1 (25%)	2 (50%)
2	145 (37-439)	3 (14%)	10 (43%)	10 (43%)
3	165 (54-505)	9 (20%)	26 (60%)	9 (20%)
4	161 (53-322)	5 (31%)	9 (56%)	2 (13%)
5	132 (50-272)	3 (43%)	4 (57%)	0
multiple	65 (57-72)	2 (100%)	0	0

[a]ALT, alanine aminotransferase; data expressed as median (range) in IU/l
CPH, chronic persistent hepatitis; CAH, chronic active hepatitis

Liver disease

Neither the activity of liver cell necrosis (as measured by ALT concentrations in serum) nor the presence or absence of cirrhosis ($P=0.09$) were related to any of the HCV (sub)types (Table 4). In the study population, 70 (24%) patients had cirrhosis and the duration of infection could be established in 193 (66%) patients (Table 4). Multivariant regression analysis of the probability of the presence of cirrhosis was significantly independently associated with both older age ($P<0.001$) and longer duration of infection ($P=0.009$). The median age in cirrhotic patients was 55 (range 36-73) years whereas it was 42 (range 22-71) years in those without cirrhosis. The median duration of infection in patients with cirrhosis was 16 (range 2-42) years whereas it was 7 (range 1-45) years when cirrhosis was absent ($P<0.001$).

DISCUSSION

In this report HCV isolates from 292 chronically infected patients were investigated for the HCV (sub)type. Subsequently, the relationship with the geographical region of origin, routes of transmission, clinical characteristics and liver disease was studied.

HCV typing

Typing of HCV RNA isolates was performed by means of LiPA. We previously reported on the similarity between the results of LiPA and direct sequencing of the 5'UTR [van Doorn et al., 1994]. Furthermore, sequence data of the coding regions core, E1 and NS5B obtained from several of our HCV isolates, indicated that they were correctly divided into the major HCV types [Stuyver et al,. 1994; van Doorn et al., 1995]. However, subtype-specific core amplification revealed that 3 (8.1%) of 37 analyzed HCV type 1 isolates were not correctly subdivided by LiPA into the subtypes 1a and 1b [Kleter et al., 1995]. In addition, the LiPA version used did not discriminate between types 1 and 6. This limitation of LiPA was not of influence on our results because none of the studied patients originated from Hong-Kong or other areas with a high prevalence of HCV type 6.

Distribution and Transmission

A significant difference was found in the distribution of HCV (sub)types according to the geographical region of origin as defined by birth. HCV subtype 1b was predominant in Europeans and Asians, whereas HCV type 2 was more common in Surinam patients (South-America) and HCV type 4 in Africans. The observed predominance of subtype 1b in Europe is in accordance with previous reports on smaller European populations [Qu et al., 1994; Nousbaum et al., 1993; McOmish et al., 1994; Pistello et al., 1994; Yun et al., 1994]. In these studies the majority of HCV isolates from European origin belonged to HCV type 1. HCV subtype 1b was most prevalent when HCV typing was performed by subtype-specific core amplification, except for Sweden [Yun et al., 1994]. The similarity with other European studies indicates that the somewhat reduced

reliability of dividing HCV type 1 isolates into 1a and 1b by LiPA did not influence final results.

Seven (54%) of the 13 patients born in Asia were infected with HCV subtype 1b. In Japan, approximately 70% of the HCV infected patients have subtype 1b and 18% have subtype 2a [Chayama et al., 1993; Yamada et al., 1994]. HCV typing results indicated that type 1 is the most abundant type in the western world [McOmish et al., 1994; Chayama et al., 1993; Yamada et al., 1994; Mahaney et al., 1994], although it cannot be excluded that the prevalence of other HCV types is underestimated. Indeed, the first generation anti-HCV screening assays appeared to be less sensitive for detection of HCV type 2 and 3 [Chan et al., 1991; van Doorn et al., 1994] since the applied recombinant antigens, i.e., clones 5-1-1 and C100-3, were derived from HCV type 1 sequences.

The ten patients from Surinam origin were all except one infected with HCV type 2. This type appeared to be rather rare in the neighbor country Brasil, since most patients studied from Brasil were infected with HCV type 1 [Stuyver et al., 1993]. In the 12 patients of African origin, HCV type 4 was predominant. This had also been observed by others in small African populations, e.g in patients originating from Burundi [Stuyver et al., 1993], Egypt [Dusheiko et al., 1994] or Gabon [Xu et al., 1994]. These data suggests that HCV type 4 is the most common type in Northern and Central African countries.

Analysis of HCV transmission routes revealed that HCV transmission by IVDA was clearly correlated with the HCV subtype 1a and and type 3. Within our population of 40 drug addicts the prevalence of subtype 1a and type 3 was respectively 25% and 45%. This observation is in accordance with others who reported also a high frequency of either HCV subtype 1a [Andonov et al., 1994; Apichartpiyakul et al., 1994; Qu et al., 1994] or HCV type 3 [McOmish et al., 1993; Apichartpiyakul et al., 1994] transmission by IVDA. In these studies the groups of drug addicts ranged from 9 to 34 patients. The similarity found for HCV subtype 1a, might be an indication for the reliability of HCV subtyping into 1a and 1b by LiPA. This is because HCV typing was performed by either subtype-specific amplification or subtype-specific hybridization of the core or

NS5 regions [Andonov et al., 1994; Apichartpiyakul et al., 1994; Qu et al., 1994]. In contrast, patients who acquired their infection by blood transfusion and those without identified risk factors, i.e. sporadic hepatitis C, were predominantly infected with HCV subtype 1b (Table 2). No significant differences were found between so-called posttransfusion and sporadic cases in the prevalence of HCV (sub)types nor in the clinical characteristics or the severity of liver disease. Although non-parenteral HCV transmission has been reported [Thaler et al., 1991], the risk for vertical as well as horizontal transmission is probably less than 5% [Brettler et al., 1992; Reinus et al., 1992; Wejstal et al., 1992; Bresters et al., 1993; Lam et al., 1993]. Therefore, it could be speculated that hidden parenteral factors such as non-single use of needles in e.g. vaccination are involved in patients with sporadic hepatitis C.

Clinical characteristics and liver disease

Neither gender nor jaundice were statistically associated with a specific HCV (sub)type. The jaundice incidence of 12% (Table 3) was similar to that reported by others who found this in 6-17% of the cases [Miyamura et al., 1990; van der Poel et al., 1991; Crawford et al., 1994].

Young individuals were more frequently infected with HCV (sub)type(s) 1a, 3 and 4 than older ones. This observation could be caused by the fact that younger patients had acquired the HCV infection more often by IVDA. The median age for HCV infection by IVDA was 32 (range 22-43) years whereas it was 47 (range 24-73) years for other modes of transmission ($P<0.001$).

The activity of liver cell damage as estimated by ALT was not related to any of the HCV types 1 to 5. Similar results are reported for HCV types 1, 2 and 3 [McOmish et al., 1993; Yamada et al., 1994]. From a small study it has been suggested that HCV subtype 1b causes a more serious liver disease than other (sub)types [Pozatto et al., 1991] and therefore the presence of this subtype could have clinical implications. This finding was supported by others [Nousbaum et al., 1993; Dusheiko et al., 1994, Pistello et al., 1994]. In our study no significant relation was observed between the HCV (sub)types and the presence of cirrhosis. Recently, Mahaney et al. [1994] reported a more severe

liver disease in patients with HCV type 2 ($P=0.0027$). In our study, 10 (43%) out of the 23 patients with HCV type 2 had cirrhosis, but this might also be caused by the high percentage of hepatitis B infection in this group (52%). The presence of cirrhosis appeared to be related to older age and longer duration of infection as indicated by multivariant analysis. The fact that elderly are more often infected with HCV subtype 1b or HCV type 2 might explain why small studies using univariant analysis appear to find a relation between the HCV type and cirrhosis [Pozatto et al., 1991; Nousbaum et al., 1993; Dusheiko et al., 1994, Pistello et al., 1994; Mahaney et al., 1994]. In two Japanese studies with respectively 251 and 148 patients similar results as ours were obtained concerning the severity of liver disease [Yamada et al., 1994; Mita et al., 1994]. The HCV isolates analyzed by these investigators contained mainly the HCV subtypes 1b and 2a. These subtypes were not related with ALT and cirrhosis. These and our findings suggests that the HCV (sub)type is not an important factor in the progression towards cirrhosis. Since these studies are based on retrospective analysis, a final statement on this point can only be made when large cohorts of hepatitis C patients have been prospectively followed for over 20 years.

In conclusion, this retrospective analysis revealed no evidence for a marked influence of HCV (sub)type on clinical characteristics nor on the presence of cirrhosis. However, HCV genotyping seems to be a valuable tool to study the epidemiology of hepatitis C.

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REFERENCES

Alter HJ, Purcell RH, Shih JW, Melpolder JC, Houghton M, Choo Q-L, Kuo G (1989): Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A non-B hepatitis. New England Journal of Medicine 321:1494-1500.

Andonov A, Chaudhary RK (1994): Genotyping of Canadian hepatitis C virus isolates by PCR. Journal of Clinical Microbiology 32:2031-2034.

Apichartpiyakul C, Chittivudikarn C, Miyajima H, Homma M, Hotta H (1994): Analysis of hepatitis C virus isolates among healthy blood donors and drug addicts in Chiang Mai, Thailand. Journal of Clinical Microbiology 32:2276-2279.

Bresters D, Mauser-Bunschoten EP, Reesink HW, Roosendaal G, Van der Poel CL, Chamuleau RAFM, Jansen PLM, Weegink CJ, Cuypers HTM, Lelei PN, van den Berg HM (1993): Sexual

transmission of hepatitis C virus. Lancet 342: 210-211.

Brettler DB, Mannucci PM, Gringeri A, Rasko JE, Forsberg AD, Rumi MG, Garcia RJ, Rickard KA, Colombo M (1992): The low risk of hepatitis C virus transmission among sexual partners of hepatitis C-infected hemophilic males: an international, multicenter study. Blood 80:540-543.

Brouwer JT, Benelux Multicentre Trial Study Group (1993): Benelux multicentre trial of alpha interferon treatment for chronic hepatitis C: Standard versus high-dose therapy monitored by biochemical and virological markers (interim analysis). Gut, supplement S119-S120.

Bruix J, Barrera JM, Calvet X, Ercilla G, Costa J, Sanchez-Tapias JM, Ventura M, Vall M, Brugeuera M, Bru C, Castillo R, Rodes J (1989): Prevalence of antibodies to hepatitis C virus in Spanish patients with hepatocellular carcinoma and hepatic cirrhosis. Lancet ii:1004-1006.

Bukh J, Purcell RH, Miller RH (1992): Sequence analysis of the 5' noncoding region of hepatitis C virus. Proceedings of the National Academy of Sciences of the USA 89:4942-4946.

Bukh J, Miller RH, Kew MC, Purcell RH (1993): Hepatitis C virus RNA in southern African Blacks with hepatocellular carcinoma. Proceedings of the National Academy of Sciences of the USA 90:1848-1851.

Chan SW, Simmonds P, Mcomish F, Yap PL, Mitchell R, Dow B, Follett E (1991): Serological responses to infection with three different types of hepatitis C virus. Lancet 338:1391.

Chayama K, Tsubota A, Arase Y, Saitoh S, Koida I, Matsumoto T, Kobayashi M, Iwasaki S, Koyama S, Morinaga T, Kumada H (1993): Genotypic subtyping of hepatitis C virus. Journal of Gastroenterology and Hepatology 8:150-156.

Colombo M, Kuo G, Choo Q-L, Donato MF, Del Nino E, Tommasini MA, Dioguardi N, Houghton M (1989): Prevalence of antibodies to hepatitis C virus in Italian patients with hepatocellular carcinoma. Lancet ii:1006-1008.

Crawford RJ, Gillon J, Yap PL, Brookes E, McOmish F, Simmonds P, Dow BC, Follett EAC (1994): Prevalence and epidemiological characteristics of hepatitis C in Scottish blood donors. Transfusion Medicine 4:121-124.

Dusheiko G, Schmilovitz-Weiss H, Brown D, McOmish F, Yap P-L, Sherlock S, McIntyre N, Simmonds P (1994): Hepatitis C virus genotypes: An investigation of type-specific differences in geographic origin and disease. Hepatology 19:13-18.

Esteban JI, Esteban R, Viladomiu L, Lopez-Talavera JC, Gonzalez A, Hernandez JM, Roget M, Vargas V, Genesca J, Buti M, Guardia J, Houghton M, Choo Q-L, Kuo G (1989): Hepatitis C virus antibodies among risk groups in Spain. Lancet ii:294-297.

Féray C, Gigou M, Samuel D, Paradis V, Mishiro S, Maertens G, Reynes M, Okamoto H, Bismuth H, Bréchot C (1995): Influence of the genotypes of hepatitis C virus on the severity of recurrent liver disease after liver tranplantation. Gastroenterology 180:1088-1096.

Gerber MA (1993): Relation of hepatitis C virus to hepatocellular carcinoma. Journal of

Hepatology 17:S108-S111.

Hay CRM, Presto FE, Triger DR, Underwood JCE (1985): Progressive liver disease in haemophilia: an underestimated problem? Lancet i:1495-1497.

Kew MC, Houghton M, Choo QL, Kuo (1990): Hepatitis C virus antibodies in Southern African blacks with hepatocellular carcinoma. Lancet 335:873-874.

Kleter GEM, Brouwer JT, Heijtink RA, Schalm SW, Quint WGV (1993): Detection of hepatitis C virus RNA in patients with chronic hepatitis C virus infections during and after therapy with alpha-interferon. Antimicrobial Agents and Chemotherapy 37:595-597.

Kleter GEM, van Doorn LJ, L Stuyver, G Maertens, Brouwer JT, Schalm SW, Heijtink RA, Quint WGV (1995): Experiences with rapid genotyping of hepatitis C virus RNA-isolates obtained from patients residing in Western Europe. Journal of Medical Virology 47:35-42.

Kuo G, Choo Q-L, Alter HJ, Gitnick GL, Redeker AG, Purcell RH, Miyamura T, Dienstag JL, Alter MJ, Stevens CE, Tegtmeier GE, Bonino F, Colombo M, Lee W-S, Kuo C, Berger K, Shuster JR, Overby LR, Bradley DW, Houghton M (1989): An assay for circulating antibodies to a major etiologic agent of human non-A, non-B hepatitis. Science 244:362-364.

Lam JPH, McOmish F, Burns SM, Yap PL, Mok JYQ, Simmonds P (1993): Infrequent vertical transmission of hepatitis C virus. Journal of Infectious Diseases 167:572-576.

Mahaney K, Tedeschi V, Maertens G, Di Bisceglie AM, Vergalla J, Hoofnagle JH, Sallie R (1994): Genotypic analysis of hepatitis C virus in American patients. Hepatology 20:1405-1411.

McOmish F, Chan S-W, Dow BC, Gillon J, Frame WD, Crawford RJ, Yap PL, Follett EAC, Simmonds P (1993): Detection of three types of hepatitis C virus in blood donors: investigation of type-specific differences in serologic reactivity and rate of alanine aminotransferase abnormalities. Transfusion 33:7-13.

McOmish F, Yap PL, Dow BC, Follett EAC, Keller AJ, Cobain TJ, Krusius T, Kolho E, Naukkarinen R, Lin C, Lai C, Leong S, Medgyesi GA, Hejjas M, Kiyokawa H, Fukada K, Cuypers T, Saeed AA, Al-Rasheed AM, Lin M, Simmonds P (1994): Geografical distribution of hepatitis C virus genotypes in blood donors: an international collaborative survey. Journal of Clinical Microbiology 32:884-892.

Mita E, Kanazawa Y, Hagiwara H, Ueda K, Kasahara A, Fusamoto H, Kamada T (1994): hepatitis C virus genotype and RNA titer in the progression of type C chronic liver disease. Journal of Hepatology 21:468-473.

Miyamura T, Saito I, Katayama T, Kikuchi S, Tateda A, Houghton M, Choo Q-L, Kuo G (1990): Detection of antibody against antigen expressed by molecularly cloned hepatitis C virus cDNA: application to diagnosis and blood screening for posttransfusion hepatitis. Proceedings of the National Academy of Sciences of the USA 87:983-987.

Mori S, Kato N, Yagyo A, Tanaka T, Ikeda Y, Petchclai B, Chiewsilp P, Kurimura T, Shimotohno K (1992): A new type of hepatitis C virus in patients in Thailand. Biochemical and Biophysical

Research Communications 183:334-342.

Nousbaum JB, Pol S, Gigou M, Okamoto H, Feray C, Poussin K, Paterlini P, Berthelot P, Mishiro S, Brechot C (1993): HCV genotype II is associated to liver cirrhosis, cancer and low response to interferon alpha in European patients. Hepatology 18:88A.

Okamoto H, Sugiyama Y, Okada S, Kurai K, Akahane Y, Sugai Y, Tanaka T, Sato K, Tsuda F, Miyakawa Y, Mayumi M (1992): Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. Journal of General Virology 73:673-679.

Pistello M, Maggi F, Vatteroni L, Cecconi N, Panicucci F, Bresci GP, Gambardella L, Taddei M, Bionda A, Tuoni M, Bendinelli M (1994): Prevalence of hepatitis C virus genotypes in Italy. Journal of Clinical Microbiology 32:232-234.

Pozatto G, Moretti M, Franzin F, Crocé LS, Tiribelli C, Masayu T, Kaneko S, Unoura M, Kobayashi K (1991): Severity of liver disease with different hepatitis C viral clones. Lancet 338:509.

Qu D, Li J-S, Vitvitski L, Mechia S, Berby F, Tong S-P, Bailly F, Wang Q-S, Martin J-L, Trepo C (1994): Hepatitis C virus genotypes in France: comparison of clinical features of patients infected with HCV type I and type II. Journal of Hepatology 21:7075.

Realdi G, Alberti A, Rugge M, Rigoli AM, Tremolada F, Schivazappa L, Ruol A (1982): Long-term follow-up of acute and chronic non-A, non-B post-transfusion hepatitis: evidence of progression to liver cirrhosis. Gut 23:270-275.

Reinus JF, Leikin EL, Alter HJ, Cheung L, Shindo M, Jett B, Piazza S, Shih JW-K (1992): Failure to detect vertical transmission of hepatitis C. Ann. Int. Med. 117:881-886.

Roggendorf M, Deihardt F, Rasshofer R, Eberle J, Hopf U, Moller B, Zachoval R, Pape G, Scramm W, Rommel F (1989): Antibodies to hepatitis C virus. Lancet ii:324-325.

Saito I, Miyamura T, Ohbayashi A, Harada H, Katayama T, Kikuchi S, Watanabe Y, Koi S, Onji M, Ohta Y, Choo QL, Houghton M, and Kuo G. (1990) Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. Proceedings of the National Academy of Sciences USA 87:6547-6549.

Simmonds P, Holmes EC, Cha T-C, Chan S-W, McOmish F, Irvine B, Beall E, Yap PL, Kolberg J, Urdea MS (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 74:2391-2399.

Simmonds P, Alberti A, Alter HJ, et al. (1994). A proposed system for nomenclature of hepatitis C viral genotypes. Hepatology 19:1321-1324.

Stuyver L, Rossau R, Wyseur A, Duhamel M, Vanderborght B, Van Heuverswyn H, Maertens G (1993). Typing of hepatitis C virus isolates and characterization of new subtypes using a line probe assay. Journal of General Virology 74:1093-1102.

Stuyver L, van Arnhem W, Wyseur A, Hernandez F, Delaporte E, Maertens G (1994): Classification of hepatitis C viruses based on phylogenetic analysis of the envelope 1 and nonstructural 5B regions and identifation of additional subtypes. Proceedings of the National Academy of Sciences of the USA 91:10134-10138.

Takada N, Takase S, Enomoto N, Takada A, and Date T (1992): Clinical backgrounds of the patients having different types of hepatitis C virus genomes. Journal of Hepatology 14:35-40.

Takada N, Takase S, Takada A, Date T (1993): Differences in the hepatitis C virus genotypes in different countries. Journal of Hepatology 17:277-283.

Thaler MM, Park C-K, Landers DV, Wara DW, Houghton M, Veereman-Wauters G, Sweet RL, Han JH (1991): Vertical transmission of hepatitis C virus. Lancet 338:17-18.

Tokita H, Okamoto H, Tsuda, F, Song P, Nakata S, Chosa T, Iizuka H, Mishiro S, Miyakawa Y, Mayumi M (1994a): Hepatitis C virus variants from Vietnam are classifiable into the seventh, eighth and ninth major genetic groups. Proceedings of the National Academy of Sciences of the USA 91:11022-11026.

Tokita H, Man Shrestha SM, Okamoto H, Sakamoto M, Horikita M, Iizuka H, Shrestha S, Miyakawa Y, Mayumi M (1994b): Hepatits C virus variants from Nepal with novel genotypes and their classification into the third major group. Journal of General Virology 75:931-936.

van Doorn L-J, Kleter B, Stuyver L, Maertens G, Brouwer H, Schalm S, Heijtink R, Quint W (1994): Analysis of hepatitis C virus genotypes by a Line Probe Assay (LiPA) and the correlation with antibody profiles. Journal of Hepatology 21:122-129.

van Doorn L-J, Kleter B, Stuyver L, Maertens G, Brouwer H, Schalm S, Heijtink R, Quint W (1995): Sequence analysis of hepatitis C virus genotypes 1 to 5 reveals multiple novel subtypes in the Benelux countries. Journal of General Virology 76:1871-1876.

van der Poel CL, Reesink HW, Lelie PN Leenvaart-Kuypers A, Choo Q-L, Kuo G, Houghton M (1989): Anti-hHepatitis C antibodies and non-A, non-B post-transfusion hepatitis in the Netherlands. Lancet ii:297-298.

van der Poel CL, Reesink HW, Mauser-Bunschoten EP, Kaufmann RH, Leentvaart-Kuypers A, Chamuleau RAFM, Schaasberg W, Bakker E, Exel-Oehlers PJ, Theobalds I, van Boven JJP, Cameron A, Lelie PN (1991): Prevalence of anti-HCV antibodies confirmed by recombinant immunoblot in different population subsets in the Netherlands. Vox Sanguinis 61:30-36.

Wejstal R, Widell A, Mansson A-S, Hermodsson S, Norkrans G (1992) Mother-to-infant transmission of hepatitis C virus. Ann. Int. Med. 177:887-890.

Xu L-Z, Larzul D, Delaporte E, Brechot C, Kremsdorf D (1994): Hepatits C virus genotype 4 is highly prevalent in central Africa (Gabon). Journal of general Virology 75:2393-2398.

Yamada M, Kakumu S, Yoshioka K, Higashi Y, Tanaka K, Ishikawa T, Takayanagi M (1994): Hepatitis C virus genotypes are not responsible for development of serious liver disease. Digestive Diseases and Sciences 39:234-239.

Yano M, Yatsuhashi H, Inoue O, Inokuchi, Koga M (1993): Epidemiology and long term prognosis of hepatitis C virus infection in Japan. Gut supplement:S13-S16.

Yun ZB, Reichard O, Chen M, Lundeberg J, Norkrans G, Fryden A, Sonnerborg A, O Weiland (1994): Serum hepatitis C virus RNA levels in chronic hepatitis C: Importance for outcome of interferon alfa-2b treatment. Scandinavian Journal of Infectious diseases 26:263-270.

CHAPTER 9

Summary and general discussion

(samenvatting en discussie)

Hepatitis C virus detection

After the discovery of hepatitis C virus (HCV) as the causative agent for parenterally transmitted non-A non-B hepatitis (NANBH), several detection assays were developed for diagnosis of HCV infection. Firstly, HCV screening and confirmation assays (such as RIBA-2 and LIA-II) allow detection of HCV antibodies to peptides or cloned HCV antigens. HCV antibody assays were used because the level of circulating HCV antigens in serum is too low to be detected by conventional enzyme immunoassays. The disadvantage of antibody detection for HCV diagnosis is the problem of discrimination between an ongoing and a resolved infection. Furthermore, HCV antibodies can only be detected after a window period of seronegativity (i.e. absence of HCV antibodies) and are hardly present in immuno-compromised patients. Currently, HCV RNA detection is the only feasible method to determine HCV viremia. Confirmation of HCV viremia by other methods such as cell culture is yet not available, therefore reliable HCV RNA test results are required for adequate diagnosis.

For direct detection of HCV viremia an HCV RNA assay based on the reverse transcription polymerase chain reaction (RT-PCR) technique, with primers directed to the highly conserved 5'untranslated region (UTR), was developed. 5'UTR primer sets appear to be superior in universal detection of hepatitis C when compared with primer sets derived from coding regions such as core, NS4 or NS5 [Garson et al., 1990 and 1991; Cristiano et al., 1991; Inchauspe et al., 1991; Bukh et al., 1992; Cuypers et al., 1992]. Since qualitative data are produced by PCR, 'negative' test results (i.e. HCV RNA undetectable) will have an additional value if the detection limit of the PCR assay is known. Analysis of HCV dilution series by the branched DNA assay (HCV-Quantiplex, Chiron, Emeryvile, CA) revealed a detection level for our HCV RNA PCR assay of 500-1000 HCV genome equivalents per ml. Besides sensitivity, false positivity is a well-known problem of highly sensitive amplification methods. Therefore, our "in-house" developed HCV RNA PCR assay was evaluated in two quality control panels (Chapter 2). Both proficiency panels, "1992" and "1993", each consisted of 10 undiluted samples and two dilution series. In these quality control

studies, our laboratory had produced results of high sensitivity and specificity [Zaaijer et al., 1993; Cuypers et al., 1994] and no false-positive results were obtained. Furthermore, the strategy followed for HCV RNA detection was useful since highly reliable HCV RNA results were produced. HCV samples were analyzed in two independent experiments and confirmed results were accepted as valid and formed the final test result. In case of a discordant result, sample analysis was repeated in two independent experiments. Test results that remained discordant were classified as indeterminate.

The advantage of powerful amplification techniques for detection of small quantities of DNA or RNA is also their disadvantage. Therefore, in assays for HCV RNA detection by RT-PCR or other amplification methods like nucleic acid system based amplification (NASBA) and ligase chain reaction (LCR), positive as well as negative controls are crucial to determine the quality and sensitivity in each experiment. RT-PCR test results are dependent on enzyme activity and pipetting accuracy of laboratory workers [Mahony et al., 1994]. A low level positive control is necessary to ensure the reproducebility of each experiment and sufficient negative controls are required to exclude false-positive results which are mostly caused by cross-contamination. Interestingly, even the application of a semi-automatic PCR assay (HCV-Amplicor, Roche) did not guarantee exclusion of false-positive results [Cuypers et al., 1994]. The quality of the Roche Amplicor HCV-test results seems to be related with a good performance of the in-house developed PCR assay. These results indicated that besides a good strategy for analysis, only well trained laboratory workers are able to produce reliable PCR results.

For treatment of HCV, alpha interferon (IFN) has been used as the standard drug for several years. The effect of IFN therapy was initially determined by monitoring alanine aminotransferase (ALT) levels in serum. From these studies it appeared that 50% of the patients with normalizing ALT during treatment (i.e. biochemical response) relapsed after cessation of therapy. Monitoring HCV RNA revealed that alpha IFN suppresses viral replication in most of the patients who had a biochemical response during treatment [Chayama et

al., 1991; Shindo et al., 1991; Chapter 3]. Additionally, relapse of the disease, defined as ALT elevation after treatment, is observed in some patients with undetectable HCV RNA and in almost all patients with detectable HCV RNA levels at the end of therapy. Therefore, the conflict in these results indicated that monitoring ALT levels during therapy is insufficient to define the outcome of treatment. A better definition for a sustained response would be both normalization of ALT (i.e. biological) and an undetectable level of HCV RNA (i.e. virological) in blood. The absence of detectable HCV RNA for a prolonged period after treatment suggests the possible eradication of the virus. Interestingly, HCV RNA is still undetectable after 2-3 years of IFN treatment in all four patients with both a biochemical and virological response at 6-9 months of follow-up (Chapter 3).

In order to predict the effect of IFN on viral replication in the early phase of treatment, HCV RNA was measured at week 4 as well as other time points (Chapter 3). From this study it appeared that HCV RNA assessment at 4 weeks of treatment has clinically relevant and prognostic value; patients still HCV RNA positive at week 4 have a very low chance of sustained remission.

Characterization of hepatitis C virus RNA by genotyping

Initial full-length sequence comparisons from HCV isolates of patients living in the United States and Japan revealed the existence of different viral strains or types. Comparison of large numbers of virus isolates revealed sequence heterogeneity at different levels throughout the viral genome. The 5'UTR and core region are conserved, E1 and NS5 regions are variable, whereas part of the N-terminal E2 region is hypervariable. Sequencing of complete HCV genomes is the most reliable approach for classification of different HCV (sub)types. However, this is not feasible for analysis of large groups of patients since sequence analysis is tedious and time consuming. For characterization of HCV isolates a useful classification system, based on different levels of sequence homology between HCV isolates, has recently been proposed by Simmonds [Simmonds et al., 1993a]. From sequence data of a 222 bp fragment of the NS5B region, it appeared that HCV isolates could be provisionally classified as a new HCV type

when sequence similarities were less than 72% with known sequences, whereas sequence similarities of 75% to 86% would provide evidence for a new HCV subtype. Final classification of new HCV (sub)types should be confirmed by both sequence analysis of another coding region (e.g. E1) and phylogenetic analysis. The branching order will indicate the relationship between previously classified isolates [Simmonds et al., 1994]. Comparison of 8 full-length sequences gave rise to the proposed classification system, although different degrees of sequence variation along the genome have been observed. Sequence similarities of 67.1% to 68.6% were found between isolates belonging to different types whereas this was 77.0% to 78.9% between those of different subtypes.

Chapters 4 to 7 describe the analyses and comparisons of small regions (200 to 500 nt) of the HCV genome. HCV sequences from chronically infected patients were investigated in order to identify prognostic markers for the outcome of IFN therapy and to determine the clinical relevance of distinct HCV (sub)types.

In chapter 4, 5'UTR sequences were determined to discriminate between HCV types. Sequence data indicated that the 5'UTR is highly conserved and sequence variation was mainly detected in two "motifs". These sequence motifs appear to be essential for the formation of a stable hairpin structure which was identified as an internal ribosomal entry site (IRES; possible starting point for viral protein translation) [Brown et al., 1992; Tsukiyama-Kohara et al., 1992]. Variations in these motifs were (sub)type-specific and occurred mainly by covariance (=pair-wise). Therefore, 5'UTR allows consistent discrimination between HCV types.

In chapter 5 evaluation of a reverse hybridization assay, the Line Probe Assay (LiPA), was described. Biotin labelled PCR products from the 5'UTR were used as probes for reverse hybridization with general as well as type- and subtype-specific probes which are applied onto a strip. By LiPA, sequence variation is detected at the level of a single nucleotide difference. Typing results of both sequence analysis and LiPA were identical for all 54 HCV isolates, except in 1. The discordant isolate was typed as subtype 1a by direct sequencing and as subtype 1b by LiPA. After repeating the LiPA, subtype 1a was

found.

After typing 315 HCV isolates by LiPA, 5'UTR typing results were validated by sequence analysis of part of the core and core/E1 regions (Chapter 6). For this study 40 samples belonging to types 1 to 5 were selected. Sequence data were analyzed for their phylogenetic relations by pair-wise comparisons. The phylogenetic tree from both regions showed 5 branches with clusters of sequences. This result indicated that HCV typing into types 1 to 5 by LiPA was in accordance with sequence variation in the core and E1 regions. However, phylogenetic analysis of the core and core/E1 regions allowed discrimination between known and new HCV subtypes. Although HCV subtypes can be recognized at the core region, confirmation for classification of new HCV (sub)types is not possible. The core region is relatively well conserved. The core/E1 region displayed a higher level of sequence variation and allowed a much better discrimination between HCV subtypes. Analysis of HCV isolates belonging to types 2 and 4 revealed the existence of several subtypes. In the selected 7 HCV type 2 and 8 HCV type 4 samples respectively, 6 and 4 subtypes were identified whereas the selected type 3 and type 5 samples belonged to only one subtype. Some of our type 4 isolates could not be properly classified as different subtypes, since the frequency distribution of pair-wise molecular evolutionary distances in the core/E1 region showed a slight overlap. For final classification these isolates should be analyzed in another coding region, preferably NS5B [Simmonds et al., 1993a]. However, phylogenetic analysis of large numbers of NS5B sequences revealed also some overlap in the frequency distribution when analyzing the 222 bp fragment [Stuyver et al., 1994]. Recently, studies by Stuyver [1994] and Tokita [1994] revealed that a fragment of at least 340 bp in the NS5B region should be sequenced for reliable classification. The introduced classification system is useful and comparison of novel HCV sequences to reported data is still in progress. However, there is still a clear need for international agreement on classification of new HCV (sub)types to overcome the problems of nomenclature. For classification of new HCV (sub)types at least two regions of the HCV genome should be sequenced. Both the NS5B region and the E1 region seems to be good. The E1 region would have the

advantage of possible correlation with biological differences.

To identify HCV (sub)types, different fast genotyping assays have been described and are based on PCR, either using subtype-specific primers, or analysis of the PCR product in (reverse) hybridization assays with type and subtype specific probes or by digestion of the PCR product with several combinations of restriction enzymes (restriction fragment length polymorphism [RFLP]). In general, genotyping systems recognize a small sequence which is representative for an HCV type and/or HCV subtype. Recently, HCV isolates can also be typed by detection of type-specific antibodies [Machida et al., 1992; Simmonds et al., 1993b; Stuyver et al., 1993]. Serotyping assays aimed at either the core or the NS4 region are based on amino acid sequence variation. Therefore, type-specific HCV antibodies are a reflection of the nucleotide sequence variation. Serotyping has the advantage over genotyping in terms of speed, simplicity and special facilities are not required. However, besides problems with low or absence of antibody responses serotyping allows only discrimination between HCV samples at the level of types whereas genotyping assays can discriminate between subtypes. Serotyping results have not yet been evaluated by comparison to either genotyping assays or sequence data.

In order to assess the reliability of HCV subtyping by 5'UTR analysis, the rapid HCV typing assays LiPA and subtype-specific core amplification were compared (Chapter 7). From this study it appeared that 5 of the 37 (14%) type 1 isolates were mistyped at the subtype level by LiPA. Other studies on European cohorts infected with type 1 showed a frequency of approximately 5% in mistyping between HCV subtypes 1a and 1b [Stuyver et al., 1994; Gianinni et al., 1994]. This difference can be explained by the heterogeneity of our studied population. Two of the five wrongly subtyped isolates were obtained from patients born in Morocco. Sequence analysis of the E1 and NS5B regions revealed that these two isolates could be classified as a new HCV subtype, 1d. These two samples were recognized as 1a by LiPA whereas they remained correctly untyped by the subtype-specific core amplification assay. By subtype-specific core amplification two mixed infections of HCV subtypes 1a and 1b

were detected but could not be confirmed. This false amplification of 1a sequences by the 1b specific primer was also observed by others [Andonov et al., 1994].

Samples typed as 2a (n = 18) by LiPA appeared to be a rather heterogeneous group. None of these 18 samples could be typed as 2a by subtype-specific core amplification and therefore remained untyped. Core sequence analysis as performed in 3 of these 18 cases confirmed the core amplification result. Interestingly, additional sequence analysis of the E1 region revealed that the three samples could be classified as three new type 2 subtypes besides the already characterized subtypes 2a-2d.

Our typing studies revealed that both LiPA as well as subtype-specific core amplification have different strengths and weaknesses. Typing at the 5'UTR has the advantage over core in terms of HCV RNA detection by general PCR in diagnosis of infection. The limited nucleotide differences found at the 5'UTR allow discrimination between the different HCV types but differentiation between subtypes is sometimes hampered. In contrast, subtype-specific core amplification allowed accurate discrimination between subtypes, due to a higher degree in sequence variation when compared to 5'UTR. However, a large number of samples (n = 22) remained untyped. By subtype-specific core amplification, an HCV subtype is recognized by a subtype-specific primer of 20 nucleotides at the target site, whereas HCV isolates are typed by LiPA with multiple type- and subtype-specific probes.

Recently, several research groups reported modifications for typing by subtype-specific core amplification assay. The improvements include adaption of subtype-specific as well as general primers [Kinoshita et al., 1993; Okamoto et al., 1993; Silini et al., 1993]; addition of new subtype-specific primers for detection of novel HCV subtypes [Okamoto et al., 1993; Xu et al., 1994]; additional analysis for confirmation of 1a/1b mixed infections [Andonov et al., 1994]; equalization of all primer melting temperatures to increase the stringency in the subtype-specific nested PCR [Widell et al., 1994]. Technically, subtype-specific core amplification is an elegant and accurate assay for identification of HCV subtypes. However, it is very likely that subtype-specific core amplification

with multiple subtype-specific primers will not be an efficient method in heterogeneous HCV populations. Currently, 52 HCV subtypes have been described. For more precise and efficient typing of HCV isolates it would be useful to apply the strength of the LiPA test format on sequence variation in the 5'UTR as well as the core region [Stuyver et al., 1995].

HCV (sub)types and patient related parameters

In order to asses the clinical relevance of HCV genotyping we investigated the relationship of HCV (sub)types with geographical regions, routes of transmission, clinical characteristics and liver disease. Nowadays there is evidence for the existence of 9 HCV types. In chapter 8 a study is described on 292 HCV RNA isolates from chronically infected patients. This study and other reports revealed that the distribution of HCV (sub)types vary considerably per geographical region. HCV types 1, 2 and 3 are distributed worldwide, whereas the other types generally appear to be restricted to more specific geographical areas. Type 4 was found in several Northern- and Central-African countries, types 5 and 6 were detected in respectively South-Africa and Hong-Kong, and recently types 7-9 were identified in patients from Vietnam. In European patients HCV subtype 1b was predominant (64%) and the (sub)types 1a, 2, 3, 4, and 5 were prevalent at frequencies ranging from 3-15%.

Remarkably, HCV types 4 and 5 were detected at a frequency of 3-4% in patients originating from Belgium and the Netherlands. Neither type has ever been detected in Japanese patients and it seems that they are highly prevalent in Africans. Besides historical data of the patient, knowledge about routes of transmission could be useful in understanding the distribution of HCV (sub)types. The study described in chapter 8 revealed that HCV subtype 1b was predominantly transmitted by blood transfusion whereas HCV subtype 1a and type 3 were correlated with transmission by IV drug abuse. This study also revealed that the HCV types 1-5 were both parenterally as well as inapparent parenterally transmitted. Furthermore, when comparing both modes of transmission (i.e. blood transfusion versus no risk factors identified) similar clinical

characteristics, ALT levels and frequencies of cirrhosis were found. Since the incidence of non-parenteral transmission is very low, it could be speculated that hidden parenteral risk factors such as injection with contaminated needles are included in the population classified as sporadic hepatitis C. Interestingly, when the patients were recruited, approximately 40% reported to have no known parenteral cause of transmission. However, after more extensive questioning, over 30% of these presumed sporadic cases proved to have concealed parenteral contacts in the past. Taken together, the pattern of HCV type distribution might be caused by blood transfusion and (imported) blood(products) [Kinoshita et al., 1993] as well as needle sharing.

Comparison of HCV types with general clinical characteristics revealed the following: (i) no relationship was found with sex; (ii) HCV subtype 1a and types 3 and 4 were most common in young individuals, whereas HCV subtype 1b and types 2 and 5 were predominant in patients of approximately 50 years of age or more. The difference in prevalence of HCV (sub)types by age could be ascribed to the mode of acquisition.

Comparison of HCV type with liver disease revealed that no specific HCV (sub)type was related with a history of jaundice, the activity (i.e. ALT concentration in serum) of liver inflammation or with cirrhosis. Recently, similar results were reported by two Japanese studies with respectively 148 and 251 patients [Yamada et al., 1994; Mita et al., 1994]. In previous small populations the severity of liver disease appeared to be related with either type 1 or type 2. Multivariate analysis as described in chapter 8 revealed that the presence of liver cirrhosis was highly correlated with age and longer duration of infection. This result suggests that the development of liver cirrhosis occurs faster in older patients than in young individuals. Since our study was based on selected populations and HCV carriers without elevated transaminases were not included, a more definite statement can only be made when unselected large populations have been followed for a long period of time.

Remarkably, the HCV type is not related to the severity of liver disease whereas they do affect IFN therapy efficacy [Hino et al., 1994; Kobayashi et al., 1993; Tsubota et al., 1994; Yoshioka et al., 1992]. Also, the level of viremia

seems to be associated with the success of IFN therapy [Hagiwara et al., 1993; Kobayashi et al., 1993; Lau et al., 1993; Yoshioka et al., 1992]. Recent reports using multivariate analysis revealed several prognostic markers for sustained response. The most important markers were HCV type 2 and low concentrations of HCV RNA. Similar results were obtained for the patients described in this thesis. The HCV type and HCV RNA at 4 weeks of treatment had relevant prognostic value. In patients with high virus concentrations prior to treatment, it is less likely that HCV RNA becomes undetectable after 4 weeks of therapy when compared to those with low concentrations. Therefore, it could be speculated that the HCV RNA level at the onset of treatment is, in addition to the HCV type, an important predictive marker for the success of therapy.

In conclusion, this study revealed that HCV viremia was reliably determined by the "in-house" HCV RNA PCR assay. The presence of HCV RNA after 4 weeks of interferon alpha therapy was highly predictive for non-response to treatment. Direct sequencing of the 5'UTR as well as the coding regions of core and E1 revealed that HCV isolates could be reliably separated into the major HCV types by LiPA. No relationship was found between a specific HCV (sub)type and the severity of liver disease. HCV typing appears to have its greatest value in the epidemiology of infection.

REFERENCES

Andonov A, Chaudhary RK (1994): Genotyping of Canadian hepatitis C virus isolates by PCR. Journal of Clinical Microbiology 32:2031-2034.

Brown EA, Zhang H, Ping L-H, Lemon SM (1992): Secondary structure of the 5' nontranslated regions of hepatitis C virus and Pestivirus genomic RNAs. Nucleic Acids Research 20:5041-5045.

Bukh J, Purcell RH, Miller RH (1992): Importance of primer selection for the detection of hepatitis C virus RNA with the polymerase chain reaction assay. Proceedings of the National Academy of Sciences USA 89:187-191.

Chayama K, Saitoh S, Arase Y, Ikeda K, Matsumoto T, Sakai Y, Kobayash M, Unakami M, Morinaga T, Kumada H (1991): Effect of interferon administration on serum hepatitis C virus

RNA in patients with chronic hepatitis. Hepatology 13: 1040-1043.

Cristiano K, Di Bisceglie AM, Hoofnagle JH, Feinstone S (1991): Hepatitis C viral RNA in serum of patients with chronic non-A, non-B hepatitis: detection by the polymerase chain reaction using multiple primersets. Hepatology 14:51-55.

Cuypers HTM, Bresters D, Winkel IN, Reesink HW, Weiner AJ, Houghton M, van der Poel CL, Lelie PN (1992): Storage conditions of blood samples and primer selection affect the yield of cDNA polymerase chain reaction products of hepatitis C virus. Journal of Clinical Microbiology 30:3220-3224.

Cuypers HTM, Damen M, Zaayer HL, Reesink HW, Niesters B, Lelie PN (1994): Quality control of HCV detection methods: interim analysis of the second Eurohep HCV-RNA proficiency panel. EASL meeting, Athens, September 7-10, 1994.

Garson JA, Ring C, Tuke P, Tedder RS (1990): Enhanced detection by PCR of hepatitis C virus RNA. Lancet 336:878-879

Garson JA, Ring CJA, Tuke PW (1991): Improvement of HCV genome detection with "short" PCR products. Lancet 338:1466-1467.

Gianinni, G., Thiers, V., Nousbaum, J.B., Stuyver, L., Maertens, G., & Brèchot, C. (1994). Comparative evaluation of type-specific primers and type-specific probes based assays for hepatitis c virus (HCV) genotyping. Hepatology 20:243A.

Hagiwara H, Hayashi N, Mita E, Takehara T, Kasahara A, Fusamoto H, Kamada T (1993): Quantitative analysis of hepatitis C virus RNA in serum during interferon alfa therapy. Gastroenterology 104:877-883.

Hino K, Sainokami S, Shimoda K, Iino S, Wang Y, Okamoto H, Miyakawa Y, Mayumi M (1994): Genotypes and titers of hepatitis C virus for predicting the response to interferon in patients with chronic hepatitis C. Journal of Medical Virology 42:299-305.

Inchauspe I, Abe K, Zebedee S, Nasoff M, Prince AM (1991): Use of conserved sequences from the hepatitis C virus for the detection of viral RNA in infected sera by polymerase chain reaction. Hepatology 14:595-600.

Kinoshita T, Miyake K, Okamoto H, Mishiro S (1993): Imported hepatitis C virus genotypes in Japanese haemophiliacs. Journal of Infectious Diseases 168:249-250.

Kobayashi Y, Watanabe S, Konishi M, Yokoi M, Kakehashi R, Kaito M, Kondo M, Hayashi Y, Jomori T, Suzuki S (1993): Quantitation and typing of serum hepatitis C virus RNA in patients with chronic hepatitis C treated with interferon-ß. Hepatology 18:1319-1325.

Lau JYN, Davis GL, Kniffen J, Qian K-P, Urdea MS, Chan CS, Mizokami M, Neuwald PD, Wilber JC (1993): Significance of serum hepatitis C virus RNA levels in chronic hepatitis C. Lancet 341:1501-1504.

Machida A, Ohnuma H, Tsuda F, Munekata E, Tanaka T, Akahane Y, Okamoto H, Mishiro S

(1992): Two distinct subtypes of hepatitis C virus defined by antibodies directed to the putative core protein. Hepatology 16:886-891.

Mahony JB, Luinstra KE, Waner J, McNab G, Hobranzska H, Gregson D, Sellors JW, Chernesky MA (1994): Interlaboratory agreement study of a double set of PCR plasmid primers for detection of chlamidia trachomatis in a variety of genitourrinary specimens. Journal of Clinical Microbiology 32:87-91.

Mita E, Kanazawa Y, Hagiwara H, Ueda K, Kasahara A, Fusamoto H, Kamada T (1994): hepatitis C virus genotype and RNA titer in the progression of type C chronic liver disease. Journal of Hepatology 21:468-473.

Okamoto H, Tokita H, Sakamoto M, Horikita M, Kojima M, Iizuka H, Mishiro S (1993): Characterization of the genomic sequence of type V (or 3a) hepatitis C virus isolates and PCR primers for specific detection. Journal of General Virology 74:2385-2390.

Shindo M, DiBisegli A, Cheung L, Shih WK, Christiano K, Feinston S, Hoofnagle J (1991): Decrease in serum hepatitis C viral RNA during alpha-interferon therapy for chronic hepatitis C. Annals of Internal Medicine 115:700-704.

Silini E, Bono F, Cerino A, Piazza V, Solicia E, Mondelli MU (1993): Virological features of hepatitis C virus infection in hemodialysis patients. Journal of Clinical Microbiology 31:2913-2917.

Simmonds P, Holmes EC, Cha T-C, Chan S-W, McOmish F, Irvine B, Beall E, Yap PL, Kolberg J, Urdea MS (1993a). 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 74:2391-2399.

Simmonds P, Rose KA, Graham S, Chan SW, McOmish F, Dow BC, Follett EAC, Yap PL, Marsden H (1993b): Mapping of serotype-specific, immunodominant epitopes in the NS-4 region of hepatitis C virus (HCV): Use of type-specific peptides to serologically differentiate infections with HCV types 1, 2, and 3. Journal of Clinical Microbiology 31:1493-1503.

Simmonds P, Alberti A, Alter H, Bonino F, Bradley DW, Brechot C, Brouwer JT, Chan S-W, Chayama K, Chen D-S, Choo Q-l, Colombo M, Cuypers HTM, Date T, Dusheiko GM, Esteban JI, Fay O, Hadziyannis SJ, Han J, Hatzakis A, Holmes EC, Hotta H, Houghton M, Irvine B, Kohara M, Kolberg JA, Kuo G, Lau JYN, Lelie PN, Maertens G, McOmish F, Miyamura T, Mizokami M, Nomoto A, Prince AM, Reesink HW, Rice C, Roggendorf M, Schalm SW, Shikata T, Shimotohno K, Stuyver L, Trepo C, Weiner A, Yap PL, Urdea MS (1994): A proposed system for nomenclature of hepatitis C viral genotypes. Hepatology 19:1321-1324.

Stuyver L, van Arnhem W, Wyseur A, DeLeys R, Maertens G (1993): Analysis of the putative E1 envelope and NS4a epitope regions of the HCV type 3. Biochemical and Biophysical Research Communications 192:635-641.

Stuyver L, van Arnhem W, Wyseur A, Hernandez F, Delaporte E, Maertens G (1994): Classification of hepatitis C viruses based on phylogenetic analysis of the envelope 1 and nonstructural 5B regions and identifation of additional subtypes. Proceedings of the National Academy of

Sciences USA 91:10134-10138.

Stuyver L, Wyseur A, van Arnhem W, Lunel F, Laurent-Puig P, Nkengasong, van doorn L-J, Kleter B, Maertens G (1995): Hepatits C virus genotyping by means of 5'UR/core line probe assays and molecular analysis of untypeable samples reveals existence of at least 9 types and 52 subtypes. (submitted for publication).

Tokita H, Okamoto H, Tsuda, F, Song P, Nakata S, Chosa T, Iizuka H, Mishiro S, Miyakawa Y, Mayumi M (1994): Hepatitis C virus variants from Vietnam are classifiable into the seventh, eighth and ninth major genetic groups. Proceedings of the National Academy of Sciences of the USA 91:11022-11026.

Tsubota A, Chayama K, Ikeda K, Yasuji A, Koida I, Saitoh S, Hashimoto M, Iwasaki S, Kobayashi M, Hiromitsu K (1994): Factors predictive of response to interferon-α therapy in hepatitis C infection. Hepatology 19:1088-1094.

Tsukiyama-Kohara K, Liauka N, Kohara M, Nomoto A (1992): Internal ribosomal entry site within hepatitis C virus RNA. Journal of Virology 66:1476-1483.

Widell A, Shev S, Mansson S, Zhang Y-Y, Foberg U, Norkrans G, Fryden A, Weiland O, Kurkus J, Nordfelt E (1994): Genotyping of hepatitis C virus isolates by a modified polymerase chain reaction assay using type specific primers: Epidemiological applications. Journal of Medical Virology 44:272-279.

Xu L-Z, Larzul D, Delaporte E, Brechot C, Kremsdorf D (1994): Hepatits C virus genotype 4 is highly prevalent in central Africa (Gabon). Journal of general Virology 75:23903-2398.

Yamada M, Kakumu S, Yoshioka K, Higashi Y, Tanaka K, Ishikawa T, Takayanagi M (1994): Hepatitis C virus genotypes are not responsible for development of serious liver disease. Digestive Diseases and Sciences 39:234-239.

Yohsioka K, Kakumu K, Wakita T, Ishikawa T, Itoh Y, Takayanagi M, Higashi Y, Shibata M, Morishima T (1992). Detection of hepatitis C virus by polymerase chain reaction and response to interferon-α therapy: relationship to genotypes of hepatitis c virus. Hepatology 16:293-299.

Zaaijer HL, Cuypers HTM, Reesink HW, Winkel IN, Gerken G, Lelie PN (1993: Reliability of the polymerase chain reaction for detection of hepatitis C virus. Lancet 341:722-724.

Samenvatting en discussie

Dit proefschrift beschrijft in 3 delen de karakterisering van hepatitis C virus (HCV) isolaten die verkregen zijn van chronisch geïnfecteerde patiënten. In hoofdstuk 1 is beknopt de algemene kennis van hepatitis C beschreven. In 1987 werd door Houghton en zijn medewerkers vastgesteld dat HCV de veroorzaker was van het ziektebeeld dat bekend is als post transfusie non-A, non-B hepatitis. Deze lange naam geeft aan dat de diagnose voorheen gebaseerd was op uitsluiting van virale hepatitis infecties. Na de belangrijke ontdekking van HCV zijn er testen ontwikkeld voor specifieke diagnose van dit virus. De eerste diagnostische testen waren gericht op het aantonen van HCV anti-lichamen omdat de concentratie van HCV antigenen, de virus eiwitten, in serum te laag zijn voor detectie met conventionele test systemen. Het nadeel van anti-lichaam detectie is echter dat er geen onderscheid gemaakt kan worden tussen een actieve HCV infectie en een HCV infectie die reeds is opgelost. Nog een nadeel is dat anti-lichamen pas kunnen worden aangetoond na een immunologische reactie. Detectie van het HCV RNA genoom is tot nu toe de enige toepasbare methode voor het vaststellen van actieve virus replicatie (=viremie). Voor een adequate diagnose is een betrouwbare HCV RNA test uitslag absoluut noodzakelijk omdat HCV viremie maar met één parameter kan worden vastgesteld.

Het eerste deel van dit proefschrift, hoofdstuk 2 en 3 zijn gericht op detectie van HCV RNA. In hoofdstuk 2 wordt de ontwikkeling van een universele HCV RNA PCR test beschreven. Het virale RNA genoom wordt gedetecteerd door toepassing van de zogeheten reverse transcription polymerase chain reaction (RT-PCR) techniek met primers die gericht zijn tegen het sterk geconserveerde 5'onvertaalde gebied (5'UTR). Met de PCR techniek worden kwalitatieve data geproduceerd. Een negatief test resultaat, beter gezegd HCV RNA niet aantoonbaar, krijgt meer betekenis als de detectie grens van de RT-PCR test bekend is. Na analyse van een verdunnings reeks met de branched DNA test (bDNA, Chiron) bleek dat de limiet van onze PCR test rond de 500-1000 HCV genoom equivalenten per milliliter zit.

Vals positiviteit is een zeer bekend probleem bij de toepassing van de PCR

techniek. Daarom is de "in-house" ontwikkelde HCV RNA PCR test geëvalueerd in 2 kwaliteit controle studies. Onze test is zeer gevoelig en specifiek. Vals positieve resultaten werden niet geproduceerd door toepassing van een strategie. HCV plasma monsters werden geanalyseerd in 2 onafhankelijke experimenten. Gelijke resultaten werden beschouwd als geldig en vormden tevens het uiteindelijke test resultaat. Indien het resultaat ongelijk was dan werd het monster opnieuw geanalyseerd in 2 onafhankelijke experimenten. Test resultaten die weer ongelijk waren werden beschouwd als 'indeterminate'. Een interessant gegeven uit de laatste kwaliteit controle studie was dat de toepassing van een semi-automatische PCR test (HCV amplicor, Roche) geen garantie was voor het vermijden van vals positieve resultaten. De kwaliteit van de HCV Amplicor resultaten waren gecorreleerd aan een goede uitvoering van de "in-house" ontwikkelde PCR test. Deze resultaten geven aan dat naast een goede strategie alleen goed getraind laboratorium personeel in staat is om betrouwbaar PCR test resultaten te produceren.

Voor behandeling van hepatitis C wordt al jaren interferon alfa aan de patiënt gegeven. Het effect van dit medicijn werd vroeger bepaald door het monitoren van de concentraties alanine aminotransferase (ALT), een specifiek lever enzym, in serum. Met deze parameter werd in eerdere studies vastgesteld dat ongeveer 50% van de patiënten die een normale ALT hadden bereikt tijdens de behandeling weer een verhoging van ALT vertoonden na interferon therapie. Door het volgen van de parameter HCV RNA, zoals beschreven in hoofdstuk 3, werd aangetoond dat interferon alfa de virale replicatie onderdrukt in bijna alle patiënten met een biochemische response (normale ALT) tijdens de behandeling. Een relapse, verhoogde ALT waarden na behandeling, werd gevonden in een paar patiënten met niet aantoonbaar HCV RNA en in bijna alle patiënten met aantoonbare hoeveelheden HCV RNA aan het einde van de therapie. Deze resultaten geven aan dat het volgen van ALT concentraties tijdens de behandeling onvoldoende is voor het vaststellen van de uitkomst van interferon therapie. Een betere definitie voor een blijvende response is de combinatie van een normale ALT concentratie en niet aantoonbaar HCV RNA. De afwezigheid van aantoonbaar HCV RNA voor een lange periode na behandeling suggereerde dat

de HCV infectie is geklaard. Interessant om te vermelden is dat HCV RNA na 2-3 jaar na therapie nog steeds niet aantoonbaar is in alle 4 patiënten met een biochemische en virologische response.

Om het uiteindelijke effect van interferon op de virale replicatie al in een vroeg stadium van de behandeling te voorspellen werd HCV RNA bepaald na 4 weken therapie. Uit deze studie kon worden vastgesteld dat de HCV RNA bepaling na 4 weken van behandeling prognostische waarden heeft en daardoor klinisch relevant is. Patiënten die na 4 weken therapie nog steeds HCV RNA positief zijn hebben een zeer kleine kans op een blijvende response.

Het tweede deel van dit proefschrift, hoofdstukken 4 t/m 7, is gericht op de karakterisering van HCV isolaten. Sequentievariatie tussen de HCV isolaten die verkregen waren van chronisch geïnfecteerde patiënten werden geanalyseerd om voorspellende markers te identificeren voor de uitkomst van interferon therapie en om de klinische relevantie vast te stellen van de verschillende HCV (sub)typen.

Eerdere onderzoeken waarbij complete genoom sequenties werden vergeleken, toonden aan dat er verschillende HCV typen bestaan. Door een groot aantal virus isolaten te vergelijken werd vastgesteld dat sequentievariatie over het hele genoom op verschillende niveaus voorkomt. Zo zijn het 5'UTR en core geconserveerd; de E1 en NS5 regios zijn variabel en de N-terminus van het E2 gebied is hypervariabel. Sequentie analyse van complete HCV genomen is de meest betrouwbare manier om virus isolaten te classificeren in verschillende (sub)typen. Het is echter niet haalbaar om grote patiënt populaties op deze manier te analyseren. Daarom worden kleine delen van het HCV genoom geanalyseerd om het isolaat te typeren. Recentelijk is er voor de karakterisering van HCV isolaten een classificatie systeem geïntroduceerd die gebaseerd is op verschillende niveaus van sequentie homologie tussen isolaten. Voor dit systeem werd een klein fragment, 222bp, in het NS5 gebied als model gebruikt. Van een nieuw HCV type is sprake als de sequentie homologie minder is dan 72% met reeds bekende sequenties, sequentie homologie tussen de 75% en 86% is een mogelijk bewijs voor een nieuw subtype. De uiteindelijke classificatie van

nieuwe HCV (sub)typen moet bevestigd worden door analyse van en andere regio, bijvoorbeeld E1.

In hoofdstuk 4 werden 5'UTR sequenties bepaald om de verschillende HCV typen en subtypen te onderscheiden. De sequentie data lieten zien dat het 5'UTR sterk geconserveerd is en dat de beperkte sequentievariatie hoofdzakelijk voorkomt in 2 motieven. Deze sequentie motieven zijn van belang voor de vorming van de secundaire RNA structuur die een functioneel onderdeel bevat in de levens cyclus van het virus. De sequentievariaties in deze motieven waren meestal gepaarde mutaties (covariatie) die type-specifiek zijn. Hierdoor kan het 5'UTR op een snelle manier gebruikt worden om de verschillende typen te identificeren.

Hoofdstuk 5 beschrijft de evaluatie van een 'reverse hybridisatie' test, line probe assay (LiPA). De gevonden variaties zoals beschreven in hoofdstuk 4 vormde de basis van deze test. De typerings resultaten van 54 isolaten die waren verkregen met LiPA correleerden zeer goed met de sequentie data. De enige discrepant was na herhaling met de LiPA gelijk aan de sequentie analyse.

Om aan te tonen dat het 5'UTR betrouwbaar gebruikt kan worden voor HCV genotypering werd een groep van meer dan 300 isolaten met behulp van de LiPA gekarakteriseerd. Voor deze studie, beschreven in hoofdstuk 6, werden na 5'UTR genotypering een 40 tal monsters geselecteerd die representant zijn voor de verschillende (sub)typen. Sequentie analyse werd verricht op core en core/E1, en vervolgens werden de data geanalyseerd op hun phylogenetische relatie door iedere sequentie met alle andere te vergelijken. Deze gepaarde vergelijkingen leveren een phylogenetische boom met verschillende takken en zij takken. De typering van isolaten op basis van 5'UTR analyse correleerde zeer goed met de indeling gebaseerd op core en core/E1 sequenties. De core sequenties zijn relatief goed geconserveerd en de core/E1 sequenties vertonen veel meer variatie. Daarom is deze regio beter geschikt om onderscheid te maken tussen HCV subtypen. HCV isolaten van het type 2 en 4 vertonen meer intratypische variatie dan de andere typen. Aangetoond is dat isolaten die behoren tot deze 2 groepen bestaan uit een aantal verschillende subtypen die niet allemaal herkend kunnen worden op 5'UTR.

Identificatie van verschillende HCV subtypen binnen de typen 1 en 2 door toepassing van snelle typerings testen is beschreven in hoofdstuk 7. Snelle genotyperings methoden zijn gebaseerd op herkenning van een klein stukje (± 20bp) van het HCV genoom. Om een indruk te krijgen in de betrouwbaarheid van subtypering op basis van 5'UTR analyse werden 58 isolaten, 37 type 1 en 21 type 2, geanalyseerd met LiPA en subtype-specifieke core amplificatie. De verkregen typerings resultaten werden geëvalueerd door sequentie analyse in beide regios. Deze studie toonde aan dat zowel LiPA als subtype-specifieke core amplificatie hun verschillende sterktes en zwaktes hebben. Een praktisch voordeel van genotypering op 5'UTR is dat deze regio routine matig wordt gebruikt voor HCV diagnostiek. De verkregen PCR producten kunnen direct gebruikt worden voor genotypering. Het 5'UTR heeft als nadeel dat de gelimiteerde nucleotiden variatie wel voldoende is om onderscheid te maken tussen de verschillende typen maar soms onvoldoende is voor het aantonen van een subtype. Hier tegenover staat dat subtype-specifieke core amplificatie goed in staat is om te discrimineren tussen verschillende subtypen vanwege de hogere graad in sequentievariatie. Echter in onze populatie konden 22 van de 58 (38%) isolaten niet gekarakteriseerd worden met dit systeem dat gericht is op herkenning van de typen 1a, 1b, 2a en 2b. Onlangs zijn er diverse modificaties gerapporteerd die tot een verbetering hebben geleid. Technisch is subtype-specifieke core amplificatie een elegante en accurate test. Praktisch gezien zal dit systeem geen efficiënte methode zijn om heterogene populaties te karakteriseren. Voor een meer nauwkeurige en efficiënte manier om HCV isolaten te typeren is het zinvol om de sterkte van het LiPA test format te combineren met de sequentievariatie in zowel 5'UTR als core.

Het derde deel van dit proefschrift, hoofdstuk 8, beschrijft de relatie tussen de HCV (sub)typen en regio van infectie, route van transmissie, klinische karakteristieken en lever ziekten. In deze studie werden 292 HCV isolaten geanalyseerd van chronisch geïnfecteerde patiënten. De eerste karakterisering studies van het HCV genoom suggereerden een relatie tussen een HCV type en de geografische verspreiding. Dit had tot gevolg dat isolaten al snel werden bestempeld als het

'Amerikaanse type' (type 1a) en het 'Japanse type' (type 1b). Op dit moment zijn er 9 verschillende HCV typen bekend. Onze studie en die van anderen toonden aan dat de prevalentie van de HCV typen nogal sterk verschilt per geografische regio. De HCV typen 1, 2 en 3 zijn wereldwijd verspreid terwijl andere meer verbonden zijn aan een specifieke regio. Type 4 komt het meest voor in Noord en Centraal Afrika. De typen 5 en 6 werden gevonden in respectievelijk Zuid-Afrika en Hong-Kong. Zeer recentelijk werden de typen 7, 8 en 9 geïdentificeerd in Patiënten uit Vietnam. Bij Europeanen is HCV subtype 1b predominant (64%) en de prevalentie van de (sub)typen 1a, 2, 3, 4 en 5 varieerde tussen de 3% en 15% (hoofdstuk 8). Merkwaardig was dat de typen 4 en 5 werden gevonden met een lage frequentie in patiënten afkomstig uit België en Nederland. Misschien heeft dit te maken met onze multi-raciale samenleving.

Kennis over de route van transmissie kan ons beter doen begrijpen hoe de verschillende HCV (sub)typen zich verspreiden. De studie in hoofdstuk toonde aan dat subtype 1b hoofdzakelijk werd verspreid door bloed transfusie terwijl subtype 1a en type 3 waren geassocieerd met transmissie door intraveneus drug gebruik. Tevens werd vast gesteld dat de typen 1 t/m 5 zowel parenteraal als non-parenteraal (=risico factor onbekend) worden over gedragen.

Bij de vergelijking van HCV typen en klinische karakteristieken werd allen een relatie gevonden met de leeftijd. HCV subtype 1a en de typen 3 en 4 werden veelal gevonden bij jongeren terwijl subtype 1b en de typen 2 en 5 predominant waren bij patiënten van ongeveer 50 jaar en ouder. De relatie tussen het HCV type en leeftijd kan worden toegeschreven aan de route van transmissie.

In eerdere studies waarbij kleine patiënt populaties werden bestudeerd vonden de auteurs dat HCV type 1 of type 2 gerelateerd was aan een ernstiger vorm van lever ziekten. In onze populatie vonden we geen relatie tussen een specifiek HCV type en de activiteit van lever ontsteking (verhoogde ALT concentraties in serum) en cirrhose. Overeenkomstige resultaten werden onlangs gerapporteerd in 2 grote studies uit Japan. Omdat de resultaten van onze studie zijn verkregen uit een geselecteerde groep van HCV patiënten met verhoogde

ALT concentraties kan een definitieve uitspraak pas gemaakt worden na analyse van een niet geselecteerde groep die voor een lange periode wordt vervolgt.

Reeds gepubliceerde studies toonden aan dat het HCV type van invloed is op de efficiëntie van interferon therapie. De meest belangrijke prognostische markers voor een blijvende response na interferon therapie zijn de HCV typen 2 en 3 en een lage HCV RNA concentratie. Overeenkomstige resultaten werden gevonden voor de in dit proefschrift beschreven patiënten.

De studies die zijn weergegeven in dit proefschrift hebben een bijdrage geleverd aan een betrouwbare bepaling van HCV viremie en de karakterisering van HCV isolaten die van belang is voor de predictie van de uitkomst van interferon therapie.

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Bernhard

Curriculum Vitae

Bernhard Kleter werd geboren op 28 december 1961 te Wageningen. De tiener jaren werden door gebracht op "het Wagenings Lyceum". Na het behalen van het VWO diploma in 1982 riep de militaire dienst plicht hem naar de Jan van Schaffelaer kazerne te Ermelo. Hij werd opgeleid tot instructeur infanterie en gaf vervolgens les aan nieuwe recruten te Ossendrecht. In 1983 nam hij opnieuw plaats achter de banken en werd een start gemaakt met de studie scheikunde aan de Katholieke Universiteit Nijmegen. Het propedeutisch examen werd in april 1985 afgelegd. Zijn doctoraal studie bestond uit het hoofdvak biochemie dat opgesplitst werd in 2 onderwerpen. De acute fase reactie, een proces dat plaats vindt na een ontsteking in de lever werd bestudeerd op de afdeling maag-, darm- en lever-ziekten van het Sint Radboudziekenhuis te Nijmegen (Dr. S.H. Yap en Prof. Dr. H. Bloemendal). Het tweede onderwerp bestond uit de karakterisering van een kalfooglens membraan eiwit (Dr. J.W.M. Mulders, Dr. W. Hendriks, Dr. W.W. de Jong en Prof. Dr. H. Bloemendal). Tijdens het bijvak virologie heeft hij gewerkt aan de ontwikkeling van een gevoelige hepatitis B virus specifieke capture assay. Dit onderzoek werd uitgevoerd bij Organon Teknika te Oss (Dr. P.J.M. Rijntjes, Dr. J. Logt). Het doctoraal examen werd afgelegd in januari 1990. Vanaf februari 1990 kwam hij in dienst van de Erasmus Universiteit Rotterdam (EUR). De werkzaamheden werden uitgevoerd op de afdeling Opleiding en Ontwikkeling, later Moleculaire Biologie, van het Diagnostisch Centrum SSDZ te Delft en de afdeling Virologie van de EUR. Het in dit proefschrift beschreven onderzoek werd verricht onder begeleiding van Dr. W.G.V. Quint, Dr. R.A. Heijtink en Prof. Dr. S.W. Schalm.

Publicaties

1 Moshage HJ, Kleter GEM, van Pelt JF, Roelofs HMJ, Kleuskens JAGM, Yap SH (1988): Transcriptional regulation of fibrinogen and albumin synthesis during the acute phase response. J. Hepatology 7: suppl. 1, S120.

2 Rijntjes J, Kleter B, Kuypers L (1989): A HBV-DNA test by DNA amplification after Dane particle capture using solid phase anti-Pre-S1. 2nd Joint Meeting of the European Society against virus diseases and the European Group for rapid diagnosis. Groningen, The Netherlands, june 18-22.

3 Falcinelli C, Claas E, Kleter B, Quint W (1992): Detection of the human papilloma virus type 16 mRNA-transcripts in cytological abnormal scrapings. J. Med. Virol. 37:93-98.

4 Claas ECJ, Sprenger MJW, **Kleter GEM**, van Beek R, Quint WGV, Masurel N (1992): Type-specific identification of influenza viruses A, B and C by the polymerase chain reaction. J. Virol. Meth. 39:1-13.

5 Kleter GEM, Brouwer JT, Heijtink RA, Schalm SW, Quint WGV (1993): Detection of hepatitis C virus RNA in patients with chronic hepatitis C virus infections during and after therapy with alpha interferon. Antimicrob. Agents and Chemother. 37:595-597.

6 Kleter GEM, van Doorn L-J, Brouwer JT, Schalm SW, Heijtink RA, Quint WGV (1994): Sequence analysis of the 5' untranslated region of at least four genotypes of hepatitis C virus in the Netherlands. J. Clin. Microbiol. 32:306-310.

7 van Doorn L-J, Kleter GEM, Stuyver L, Maertens G, Brouwer JT, Schalm SW, Heijtink RA, Quint WGV (1994): Analysis of hepatitis C virus geno-

types by a line probe assay (LiPA) and the correlation with antibody profiles. J. Hepatology 21:122-129.

8 van Doorn L-J, Kleter B, Voermans J, Maertens G, Brouwer H, Heijtink R, Quint W (1994): Rapid detection of hepatitis C virus RNA by direct capture from blood. J. Med. Virol. 42:22-28.

9 van Doorn L-J, Shyamala V, Han JH, Kleter GEM (1994): HCV RNA detection in heparinized blood by direct genomic RNA capture onto paramagnetic particles. J. Virol. Methods 48:339-341.

10 Kleter GEM, van Doorn L-J, Stuyver L, Maertens G, Brouwer JT, Schalm SW, Heijtink RA, Quint WGV (1995): Experiences with rapid genotyping of hepatitis C virus RNA-isolates obtained from patients residing in Western Europe. J. Med. Virol. 47:35-42.

11 van Doorn L-J, Kleter GEM, Stuyver L, Maertens G, Brouwer JT, Schalm SW, Heijtink RA, Quint WGV (1995): Sequence analysis of hepatitis C virus genotypes 1 to 5 reveals multiple novel subtypes in the Benelux countries. J. Gen. virol. 76:1871-1876.

12 Stuyver L, Wyseur A, van Arnhem W, Lunel F, Laurent-Puig P, Nkenga-song, van Doorn L-J, Kleter B, Maertens G (1995): Hepatitis C virus genotyping by means of 5'UTR/core line probe assays and molecular analysis of untypeable samples reveals existence of at least 9 types and 52 subtypes. Virus Research, in press.