THE DEVELOPMENT AND APPLICATION OF QUANTITATIVE PCR-BASED ASSAYS FOR THE DETECTION OF HEPATITIS C AND RELATED VIRUSES

BY

KEVIN PETER WHITBY

Department of Virology,

Royal Free and University College Medical School,

London W1P 6DB.

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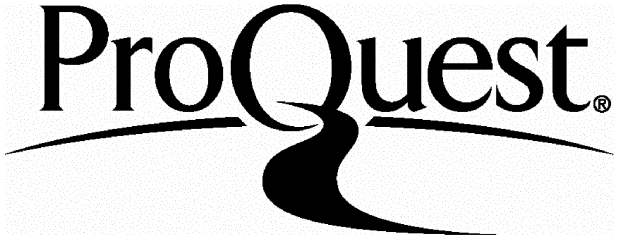

Abstract

PCR has previously been used to measure hepatitis C virus (HCV) levels during antiviral chemotherapy, in viral cultures and in diverse clinical samples. The use of this technique led to a rapid expansion of our understanding of this clinically important virus. However, the majority of methods previously used to quantify HCV were laborious and relatively insensitive. This thesis describes the development of PCR-based quantitative methods for HCV RNA and their application in a number of research settings. Significant advances in assay speed and throughput are demonstrated, as well as important improvements in sensitivity. The aim of the project was to develop and optimise quantitative PCR methods which could conveniently be applied to serum or plasma and also to cells and cell culture supernatants from *in vitro* culture experiments.

The PCR method developed was shown to be effective in providing quantitative viral monitoring data from several trials of antiviral chemotherapy. Viral RNA levels were also monitored in the serum of a patient infected during pregnancy, in a cultured hepatocyte line and the method was also applied to the study of a potential small primate model. The quantity of virus in cultured hepatocytes and in plasma pools prior to fractionation was demonstrated to be very low. In addition a novel single tube 'hot-start' RT-PCR method (RT-HS-PCR) is described. This method was shown to increase the sensitivity of RT-PCR and to provide quantitative data. The discovery of a new flavivirus (GBV-C), closely related to HCV, provided an opportunity to demonstrate the flexibility of this quantitative PCR method. The technique has now been applied to a wide range of DNA and RNA viruses in several laboratories within the UK.

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Figures and Tables

Abbreviations

3TC	2',3'-dideoxy-3'-thiacytidine
A (nucleotide)	adenosine
ABTS	2, 2'-azinobis (3-ethylbenzthiazoline-sulphonic acid)
aa	amino acid
ALT	alanine transaminase
AMV	avian myoblastoma virus
ANOVA	analysis of variance
AP or Alk. Phos.	alkaline phosphatase
AST	aspartate transaminase
bp	base pairs
BSA	bovine serum albumin
C (Flaviviral Structural Protein)	core or nucleocapsid
CAH	chronic active hepatitis
cDNA	complementary deoxy-ribonucleic acid
CMV	cytomegalovirus
CPMP	Committee for Proprietary Medicinal Products
CV%	coefficient of variation
dATP	deoxy-adenosine triphosphate
dCTP	deoxy-cytosine triphosphate
DFO	desferrioxamine
dGTP	deoxy-guanosine triphosphate
dl	decilitre
DNA	deoxy-ribonucleic acid
DNP	dinitrophenol
dNTP	deoxy-ribonucleoside tri-phosphates
DTT	dithiothreitol
dTTP	deoxy-thymidine triphosphate
E (Flaviviral Structural Protein)	envelope
EBV	epstein barr virus
EDTA	ethylene diamine tetra-acetic acid
ELISA	enzyme linked immuno-sorbant assay
ELONA	enzyme linked oligonucleotide assay
ET-NANBH	enterically transmitted non-A, non-B hepatitis
FCS	foetal calf serum
FDA	Food and Drug Administration
g	grammes
GBV-A	GB virus A
GBV-B	GB virus B
GBV-C	GB virus C
GGT	gamma glutamyl transaminase
GOT	glutamate-oxaloacetate transaminase
GPT	glutamate-pyruvate transaminase
Hae III	Haemophilus aegyptii isoenzyme III
HAI	histological activity index

HAV	hepatitis A virus
HBcAg	hepatitis B virus core antigen
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HCV	hepatitis C virus
HDV	hepatitis delta virus
HEV	hepatitis E virus
HFV	hepatitis F (French) virus
HGV	hepatitis G virus
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
HTLV	human T-cell lymphotropic virus
IFN-α	interferon α
Ig M	immunoglobulin subclass M
Ig G	immunoglobulin subclass G
IU	international units
LCR	ligase chain reaction
LPSR	low protein serum replacement
MAb	monoclonal antibody
mg	milligrams
Mg^{2+}	magnesium ions
min (unit of time)	minute(s)
MIU	million international units
ml	millilitres
µl	microlitre
MMLV-RT	Maloney murine leukaemia virus reverse transcriptase
µmol	micromoles
Mn^{2+}	manganese ions
mo. (unit of time)	month
n	number
NANBH	non-A, non-B hepatitis
NASBA	nucleic acid sequence based amplification
NBA	National Blood Authority
NCR	non-coding region
NIBSC	National Institute of Biological Standards and Controls
NIH	National Insititutes of Health
nm	nanometers
NR	non-responder
NS	non-structural
NS (statistics)	not significant
NTP	ribonucleoside tri-phosphate
ORF	open reading frame
p (Statistical Analysis)	probability of null hypothesis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pfu	*Pyrococcus furiosis*

pl	picolitre (10^{-12} of a litre)
Pre-M	pre-matrix
PT-NANBHV	post transfusion non-A, non-B hepatitis virus
QRT-PCR	quantitative reverse transcriptase polymerase chain reaction
rA	adenosine ribonucleoside
RDA	representational difference analysis
RFLP	restriction fragment length polymorphism
Ribavirin	1-β-D-ribofuranosyl 1,2,4-triazole-3-carboxamide
RIBA	recombinant immunoblot assay
RNA	ribonucleic acid
RNAse H	ribonuclease H
RR	responder with relapse
RT	reverse transcriptase
RT-HS-PCR	reverse transcriptase 'hot-start' polymerase chain reaction
rU	uracil ribonucleoside
Rx	treatment
s (unit of time)	second(s)
SD	standard deviation
SDS	sodium dodecyl sulphate
SE	standard error
SFM	serum free medium
SPC	single photon counting
SR	sustained responder
SSC	salt and sodium citrate buffer
Taq	*Thermus aquaticus*
Tfl	*Thermus flavus*
TMB	3, 3', 5, 5' -tetramethylbenzidine
TR	transient responder
Tris	tris(hydroxyethyl) aminomethane
TTA	tris tween azide
Tth	*Thermus thermophilus*
UCL	University College London
UCLMS	University College London Medical School
UK	United Kingdom
XO	xanthine oxidase
YFV	yellow fever virus
yr	year

1. Introduction

1.1 Viral Causes of Hepatitis

Viral hepatitis, apparent as jaundice, was recorded as far back as the fifth century BC by Hippocrates. Outbreaks of 'infectious' hepatitis were recognised from the eighth century AD onwards and numerous reports of jaundice, probably 'infectious' hepatitis, have been made since the seventeenth century up to the present day. Until the 1840's hepatitis was thought to be caused by an obstruction in the bilary tract and an acceptance of a viral aetiology of hepatitis took a further 100 years. Immunisation of soldiers with yellow fever vaccine resulted in many becoming jaundiced. The stabiliser for this vaccine was human plasma and after careful exclusion of alternative explanations it was suggested that serum contained a virus causing jaundice (Findlay and MacCallum, 1938). However, it is only since the late 1960's that rapid progress in our understanding of the hepatitis viruses has occurred.

Further studies of naturally acquired viral hepatitis and experimentally induced hepatitis in volunteers (MacCallum and Bauer, 1944) suggested that two types of viral hepatitis existed with short and long incubation periods. On the basis of the different incubation periods the terms hepatitis A (short incubation period) and hepatitis B (long incubation period) were suggested (MacCallum, 1947). Further evidence for the existence of two forms of hepatitis virus was collected during the 1950's by Krugman and Giles at Willowbrook State School for the mentally retarded (Krugman *et al.,* 1967). An illness resembling hepatitis A with its short incubation period was induced by a serum labelled MS-1, this agent could be transmitted by parenteral or oral routes and was more contagious than serum MS-2. The disease induced by MS-2 resembled hepatitis B in that it typically had an incubation period of 60 days and usually required deliberate transfusion for its transmission. These experiments were terminated following the deaths of several subjects apparently as a direct result of their hepatitis.

1.1.1 Hepatitis A Virus (HAV)

The aetiological agent responsible for hepatitis A was first identified by immune electron microscopy in the faeces of volunteers injected with the serum MS-1. (Feinstone *et al.,* 1973). The virus particles were 27nm in diameter, appeared 5 days before the onset of jaundice and tended to disappear just before the onset of jaundice. The size and morphology of the virus led to its classification within the *Picornaviridae* (Siegl and Frösner, 1978).

Culture of hepatitis A virus in cell lines initially proved unsuccessful as did transmission of the virus to other vertebrate species. However marmosets were shown to be susceptible to hepatitis A virus infection in 1967 (Deinhardt *et al.,* 1967) and by the late '70s Provost and Hilleman had grown the virus in culture (Provost and Hilleman, 1979). Once large quantities of viral antigen could be prepared from viral cultures serological tests were developed, leading to the development of reliable serological tests for the detection of IgM and IgG reactive against the virus. Formalin fixed cultured virus is now licensed as a vaccine and is reported to provide safe and effective immunisation against hepatitis A (British Medical Association and Royal Pharmaceutical Society of Great Britain, 1996).

1.1.2 Hepatitis B Virus (HBV)

In 1965, during a study to identify genetic differences in serum albumins, an antigen was discovered in the serum of an Australian aborigine that precipitated following Ouchterlony double diffusion with serum from an American haemophiliac. Following careful observation and deduction this antigen was eventually associated with post-transfusion hepatitis (Blumberg *et al.,* 1967, 1968; Prince, 1968). This antigen was also demonstrated to be present in the serum sample MS-2. The discovery of this "Australia antigen" allowed the collection of sera from cases of hepatitis B, leading to the first identification of the hepatitis B virus (HBV) by immune electron microscopy (Dane *et al.,* 1970). This virus is 42nm in diameter and was initially often referred to as the Dane particle. HBV was found to contain an

electron dense core surrounded by Australia antigen (subsequently renamed hepatitis B surface antigen (HBsAg)). The virus produces excess HBsAg and this process may act to aid immune evasion by the virus.

In 1971 chimpanzees were shown to be susceptible to infection with HBV (Maynard *et al.,* 1971), but other small primates were resistant to infection. Antigens from HBV have been expressed in cultured cells (McNab *et al.,* 1976) and reliable systems have now been described for the *in vitro* propagation of the virus (Bchini et al., 1990). Immunisation against hepatitis B has been achieved with purified HBsAg derived either from chronically infected patients or by the expression of recombinant protein. Viruses similar to HBV have been discovered in woodchucks (Summers *et al.,* 1978), ground squirrels (Marion *et al.,*1980) and Pekin ducks (Manson *et al.,* 1980). These hepatitis B like viruses cause a chronic infection of the liver, share some limited antigenic and nucleotide similarity with HBV and are more convenient model systems than infected chimpanzees.

1.1.3 Hepatitis C Virus (HCV)

As serological tests were developed for the aetiological agents of hepatitis A and hepatitis B it became clear that a significant proportion of post transfusion hepatitis was not due to either virus (Prince *et al.,* 1974; Feinstone *et al.,* 1975). The virus responsible was referred to as 'post transfusion or parenterally transmitted non-A, non-B hepatitis virus (PT-NANBHV). Attempts to isolate the aetiological agent, either directly in culture or through the identification of specific markers of infection, led to the production of a wide variety of diagnostic tests for non-A, non-B hepatitis (NANBH). However, none of these tests proved sufficiently specific and the US blood transfusion service chose to continue to rely on the detection of antibodies against hepatitis B virus core antigen (HBcAg) as a surrogate marker of infection with PT-NANBHV.

This decision did not deter a large number of laboratories from continuing with attempts to identify virus specific antigens or viral nucleic acid. The revolution in

molecular biology techniques provided new tools to apply to these experiments. The difficulty in isolating the virus was assumed to be due to low levels in patient sera. Choo and co-workers therefore chose to analyse acute phase serum from an experimentally infected chimpanzee, because higher levels of viraemia might be expected to exist before an immune response had become established.

During the work by Choo and co-workers, total nucleic acid was isolated from this serum and was denatured and reverse transcribed to produce a library of cDNA molecules representing both DNA and RNA. The advantage of isolating nucleic acid from serum rather than the viruses' target organ, the liver, is that carefully prepared serum contains much less cellular nucleic acid, thus providing a relatively low complexity library. The cDNA library was cloned into the bacteriophage expression vector λgt11. Infection of susceptible bacteria with this vector causes them to express the cloned sequence as a protein. The majority of protein expressed is not of use, either because the cDNA has been expressed out of the correct reading frame, or because it is not from the aetiological agent. A clone expressing a specific cDNA was identified by screening the bacteriophage library with a well characterised panel of sera from patients with NANBH and controls (Choo *et al.,* 1989). The cDNA clone identified was called 5-1-1 and was shown to hybridise with a positive sense RNA 10,000 nucleotides in length that was found in infected chimpanzee livers, but not in controls.

The expressed peptide formed the basis of the first specific serological assay (Kuo *et al.*, 1989). Studies using assays based on 5-1-1 rapidly showed that the agent associated with antibodies against this marker was responsible for the vast majority of post-transfusion NANBH. The agent identified was therefore named hepatitis C virus (HCV). This virus is described in detail in Section 1.2.

1.1.4 The Delta Agent (HDV)

In the mid-70's Mario Rizzetto used immunofluorescence to identify a 'new' antigen in the nuclei of HBV infected hepatocytes. Subsequently, during work at the National Institutes of Health (NIH), he deduced that the so called 'delta' antigen was in fact a defective virus requiring HBV surface antigen for efficient replication (Rizzetto *et al.,* 1980). This virus is now known as hepatitis delta virus (HDV) or the delta agent.

HDV has a single stranded 1.7kb RNA genome contained within a central core surrounded by HBsAg. The viral particles are 36nm in diameter. Infection may coincide with HBV infection or be a super-infection acquired later by HBV carriers (Rizzetto and Verme, 1985). Super-infection with HDV results in reduced replication of HBV and an increase in disease severity (Smedile *et al.,* 1982). Although there is currently no specific immunisation for HDV infection, vaccination strategies using HBsAg may either directly or indirectly prevent infection with HDV.

1.1.5 Hepatitis E Virus (HEV)

Surrounding the Yamuna River, which flows through Delhi in India, there were 35,000 cases of jaundice during the winter of 1955-56 (Vrati *et al.*, 1992). This remains one of the most intensively studied outbreaks of infectious jaundice to date. It was initially assumed that the causal agent was HAV and that the outbreak resulted from waning immunity. We now know however that previous infection with HAV confers long term immunity. Samples from the Yamuna river outbreak and a similar waterborne outbreak in Kashmir were shown to be serologically distinct from hepatitis A. In 1983 Mikhail Balayan ingested acute-phase stool suspensions collected during an outbreak in Central Asia. He subsequently demonstrated 27-30 nm particles in his own faeces which could be transmitted to cynomologous monkeys (Balayan *et al.,*1983). The disease was referred to as ET-NANBH. The molecular identification of the virus by Reyes and co-workers showed that it was a calicivirus, a

result consistent with the electron microscopy results (Reyes *et al.*, 1990). This virus is now known as HEV.

1.1.6 Hepatitis F Virus

Hepatitis F virus (HFV) has been described in only a handful of cases, all from France, with subsequent experimental transmission to primates. The virology, epidemiology, hepatotropism and clinical importance of HFV are quite uncertain.

An agent responsible for sporadic NANBH in humans was serially transmitted in rhesus monkeys by intravenous inoculation of a stool extract from a patient with hepatitis (Deka *et al.*, 1994). A novel agent called HFV (hepatitis French [origin] virus) was present as 27 to 37nm particles in the infectious stool extract. Hepatic lesions were noticed in infected monkeys during the acute phase of illness. The purified viral particles consist of a double-stranded DNA of approximately 20 kb and were found in infected monkey liver. The viral particles were able to protect monkeys challenged with infectious stool extract, thus providing a candidate vaccine. However, no further reports of hepatitis caused by this virus have been reported in the literature.

1.1.7 Hepatitis G Virus (HGV) or GB Virus C (GBV-C)

Three new members of the *Flaviviridae* have recently been described. Serum from a surgeon (GB) with hepatitis was inoculated into a tamarin (*Saguinus labiatus*; Simons *et al.*, 1995b) for further investigation and the tamarin developed mild hepatitis. Sequences present in the serum of the tamarin following inoculation, but absent prior to inoculation were selectively amplified using a technique called representational difference analysis (RDA).

RDA was performed by preparing two 'pools' of nucleic acid from the same patient, one infected and one uninfected. The infected pool was ligated to a priming sequence

and hybridised with an excess of nucleic acid from the unligated, uninfected pool. Sequences contained in both pools hybridised to each other producing double stranded DNA which was unamplifiable because it contained only one amplification primer. Sequences unique to the infected material were only able to hybridise to other sequences from the same pool, the double stranded DNA thus produced contained a primer attached to both strands and was therefore amplification competent (Lisitsyn *et al.,* 1992). The RDA technique is particularly applicable to transfusion transmissible infectious agents as pre- and post transfusion samples can readily be used to prepare the uninfected and infected nucleic acid pools.

Sequences from two novel flaviviruses were identified and the viruses were described as GB virus-A and GB virus-B (Muerhoff *et al.*, 1995). However further study revealed that neither virus is commonly found in man. The third virus was discovered independently by research groups at Abbott laboratories and by a Genelabs/Boehringer Mannheim collaboration. Workers at Abbott named the virus GB virus-C (GBV-C; Leary *et al.*, 1996), whereas those at Genelabs named it hepatitis G virus (HGV; Linnen *et al.*, 1996). Both these names are used interchangably in the literature and in this thesis the virus will be referred to as GBV-C. All three viruses are closely related to HCV, but are sufficiently distinct not to be referred to as new genotypes.

More recently it has been demonstrated that GBV-A and GBV-C both possess an internal ribosome entry site similar to HCV (Simons *et al.*, 1996). GBV-C infection can be identified through PCR testing, which indicates current infection. An antibody test for GBV-C has recently been developed (Pilot-Matias *et al.,* 1996) and should help elucidate the epidemiology of GBV-C infection more fully than GBV-C RNA testing alone. It appears that once antibodies are detectable GBV-C RNA is usually no longer present (Hassoba *et al.*, 1997).

The nature and frequency of GBV-C infection are unclear; there is also uncertainty about risk factors and means of prevention. Transmission through blood transfusion has been documented and transmission from mother to child in the perinatal period occurs at a high frequency (30%; Viazov *et al.,* 1997). There is an increased

prevalence of GBV-C RNA among groups with frequent exposure to blood or blood products (e.g., people with haemophilia or thalassaemia, patients on haemodialysis and injecting drug users; Linnen *et al.,* 1996). Other modes of transmission (e.g., sexual) are possible but have not been well documented. Coinfection with HBV, HCV or both is common, presumably because of similar modes of transmission. It remains unclear what disease state GBV-C infection causes acutely or in the long term. Although caution and vigilance must be maintained, there is a growing consensus that GBV-C is "a virus looking for a disease" and may in fact prove not to be a cause of viral hepatitis (Alter *et al.*, 1997a). Although GBV-C RNA can be detected for years after infection in perhaps a minority of people who have been infected, there is no compelling evidence that GBV-C infection has important sequelae. GBV-C infection does not seem to worsen coinfection with HBV or HCV. There is no proven treatment for GBV-C infection; at this point, guidelines for its investigation and management cannot therefore be developed.

1.1.8 Other Viruses Causing Hepatitis

Other viruses such as human cytomegalovirus (CMV), Epstein Barr virus (EBV), parvovirus B19 (Hillingsø *et al.,* 1998) and yellow fever virus (YFV) have been shown to cause hepatitis, however these viruses are not classified amongst the hepatitis viruses because jaundice is not the primary feature of infection. A significant amount of hepatitis still occurs without an obvious cause and virologists are likely to remain keen to find infectious aetiologies. This is especially the case when the hepatitis appears to be triggered by a particular event such as blood transfusion. PCR based techniques, such as RDA and amplification with degenerate oligonucleotides, provide extremely powerful tools for the identification of new viruses. It is plausible that many of the viruses discovered in this way will not be involved in the aetiopathogenesis of the disease state from which they are isolated. Special care needs to be taken to establish clear criteria that establish a minimum level of evidence required to define causality. Many such schemes have already been proposed since Koch's postulates (Hill, 1965; Fredricks and Relman, 1996) and

refinement of these ideas will undoubtedly be required as new technological developments yet further stretch these definitions.

1.2 Hepatitis C Virus

Hepatitis C virus (HCV), formerly known as "the agent of post transfusion non-A, non-B hepatitis (PT-NANBH)," was cloned in 1989 (Choo *et al.*, 1989). The virus was shown to contain a single-stranded, positive sense RNA genome with a single open reading frame (ORF). This ORF encodes a polypeptide of approximately 3000 amino acids and comprises almost the entire length of the viral genome (Choo *et al.*, 1991; Takamizawa *et al.*, 1991). Flanking the ORF are two non-coding regions, the 5' non-coding region (NCR) is 324-347 nucleotides in length, but the length of the 3' NCR is considerably more variable and may contain a poly uracil ribonucleotide (rU) or a poly adenosine ribonucleotide (rA) tail (Choo *et al.*, 1991; Han *et al.*, 1991).

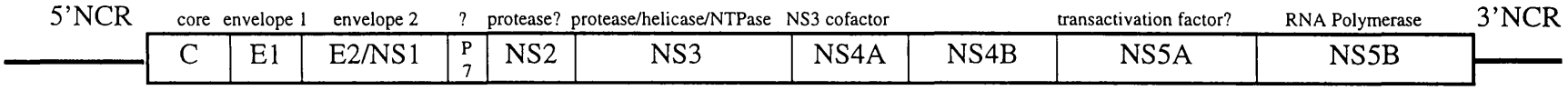
1.2.1 Organisation of the HCV Genome

A large number of chimpanzee and human derived HCV isolates have now been sequenced in their entirety by several groups (Choo *et al.*, 1991; Chen *et al.*, 1992; Okamoto *et al.*, 1991, 1992a; Takamizawa *et al.*, 1991). The genome size and structure show remarkable similarity to members of the flavivirus family (*Flaviviridae*) and so virally encoded proteins and glycoproteins were named using the same nomenclature as the classical flavivirus yellow fever virus. Post-translational cleavage of the polyprotein encoded by the flaviviruses produces three structural proteins (the core or nucleocapsid [C], the prematrix [pre-M] and the envelope [E] protein) and seven non-structural (NS) proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5).

The structural proteins form the virus particle, whereas the non-structural proteins are involved in the replication of the virus. The structural proteins are found towards the N-terminus of the polyprotein and the non-structural proteins towards the C-terminus. HCV does not possess a prematrix protein unlike other members of the *Flaviviridae* and the region corresponding to the NS1 protein of the flavi- and pestiviruses is glycosylated, forming the major envelope glycoprotein E2. Although HCV shows remarkable similarities with the other members of the *Flaviviridae* in

terms of amino acid hydrophobicity profile, nucleic acid and amino acid sequence homology are less obvious (Miller and Purcell, 1990). The function of many of the proteins has been elucidated and functional similarities seem to correlate with areas of amino acid homology. Two regions of homology have been found in the non-structural protein NS3: the first from aa 1230-1500, has been shown to act as an NTP binding helicase; the second, just upstream from the helicase, possesses trypsin-like serine protease activity very similar to the analogous region in both the flavi- and pestiviruses. The NS5 protein also contains a region of homology (aa 2703-2739) including the GDD motif (glycine-aspartic acid-aspartic acid) common to all RNA-dependent RNA polymerases (Choo *et al.*, 1991; Koonin, 1991; Miller and Purcell, 1990). This protein has been expressed and shown to contain the expected enzymatic activity. Based on these data HCV is currently classified as a separate genus within the *Flaviviridae*.

Figure 1: Schematic Diagram of the HCV Genome Showing the Location of Functional Genes and Non-Coding Regions



The hepatitis C virus possesses a single stranded, positive sense RNA genome. Its single open reading frame is flanked by two non-coding regions. The 5'-end of the open reading frame encodes the viruses 3 structural proteins (C represents core, E1 and E2 are envelope glycoproteins) and the 3'-end encodes non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, NS5B).

1.2.2 Genetic Variation and Genotypes

DNA and RNA polymerases incorporate nucleotide bases complementary to those found on the template strand and the genetic stability of an organism is dependent upon the fidelity of this process. Eukaryotic and prokaryotic DNA polymerases contain proof-reading domains that reduce the frequency of misincorporation of nucleotide bases. However, RNA-dependent RNA polymerases such as those encoded by the NS5 region of flaviviral genomes have no proof-reading capability. During nucleic acid replication changes in nucleotide sequence occur, some of which may hinder or enhance viral fitness and so through the process of Darwinian selection (Darwin, 1859) new genetic variants evolve. The coexistence of several closely related sequences or quasispecies in a single patient has been observed. The average rate of mutation of the HCV genome has been estimated to be approximately 10^{-3} base substitutions per site per year due to misincorporation of nucleotides by the RNA polymerase (Mizokami *et al.*, 1994). The distribution of the observed mutations along the genome is not random, a hypervariable region has been demonstrated at the 5' end of the envelope glycoprotein E2, whereas the 5' NCR is remarkably well conserved (Simmonds, 1995). The relative level of sequence conservation may reflect the functional importance of the region. Mutations in the 5' NCR presumably have a detrimental effect on its function as a ribosomal binding site and consequently on the replication rate of the virus. Envelope proteins are exposed to the immune system and highly variable epitopes may help the virus to evade the immune system (Kato *et al.*, 1993). This concept of immune escape has been proposed as a mechanism of viral persistence, however it is difficult to explain why persistence is achieved by HCV whereas other single stranded RNA viruses such as influenza virus cause transient infections.

As several quasispecies can develop within an individual (Tanaka *et al.*, 1992; Murakawa *et al.*, 1992; Oshima *et al.*, 1991), it would not be surprising to find more substantial variation between populations of infected individuals separated temporally, geographically, or by their lifestyles. Based upon the sequence data available, several groups have proposed essentially similar phylogenetic trees

(Okamoto *et al.*, 1992b; Simmonds *et al.*, 1993), but rather confusingly have proposed different systems of nomenclature. For the sake of simplicity the nomenclature proposed by Simmonds *et al.*, (1993) will be used in this thesis. The construction of phylogenetic trees by the comparison of 5' NCR, core, NS3, or NS5 nucleotide sequence shows the existence of at least six major genotypes with a much larger number of subtypes. The major genotypes have been designated a number from 1 to 6; a further three have been proposed (7, 8, and 9; Tokita *et al.*, 1994), although it is as yet undecided whether these genotypes are sufficiently dissimilar to genotype 6 to warrant separate classification. Subtypes have been allocated a letter (1a, 1b, *etc.*), however as subtypes are being discovered faster than the sequences are being published (Pers. Comm., Dr. C Howard, Royal Veterinary College, London), the system of sequential classification is currently difficult to maintain, leading to confusion in the literature.

There are a number of methods to determine the genotype of an HCV isolate. The 'gold standard' is sequencing of the entire genome (Okamoto *et al.*, 1992a), but this method is too laborious to be used routinely. Because chimeric HCV genomes have not been demonstrated it is possible to deduce the genotype by analysing the sequence of a single genomic fragment. This can be done in several ways; sequencing of a PCR product (Simmonds *et al.*, 1993), restriction fragment length polymorphism (RFLP; Mellor *et al.*, 1996), hybridisation with genotype specific oligonucleotide probes (Stuyver *et al.*, 1993), PCR amplification with genotype specific primers (Holland *et al.*, 1996) and the detection of antibodies reactive to serotype (and genotype) specific epitopes (Dixit *et al.*, 1995).

1.2.3 Morphology

Relatively little is known about the morphology of HCV. Immune capture onto electron microscope grids coated with anti-core monoclonal antibody allowed the visualisation of 33nm icosahedral particles. However, buoyant density gradient experiments support the theory that the complete virus particle possesses an envelope studded with glycoprotein (Bradley *et al.*, 1991). More recently, electron

micrographs of flavivirus-like particles have been generated from plasma samples with high HCV RNA titre (Kaito *et al.*, 1994; Li *et al.*, 1995) and confirmed to be HCV by indirect immunogold labelling. The unlabelled particles were 55 to 66nm in diameter with fine 6nm spike-like projections and a buoyant density of 1.16g/ml. This buoyant density is lower than typically found in members of the *Flaviviridae* and is believed to be due to virus interaction with low density plasma components including lipoproteins (Kaito *et al.*, 1994).

1.2.4 Clinical Features of Hepatitis C

The incubation period of the hepatitis C virus is intermediate between that of hepatitis A and hepatitis B viruses and is typically around 6 and 9 weeks. The incubation period is followed by a peak serum aminotransferase level, symptoms at this stage are often mild or imperceptible. HCV infected patients however may present clinically with fatigue, nausea, loss of appetite, cholestatic symptoms with or without icterus, hepatomegaly and splenomegaly (Dienstag, 1983). Once infected the majority of patients become chronic carriers. The course of infection is variable, HCV may cause a benign and asymptomatic disorder with an indolent course, but it may also cause progressive liver disease, with 20% of those chronically infected developing cirrhosis with its associated risk of hepatocellular carcinoma (Di Bisceglie *et al.*, 1991; Tsukuma *et al.*, 1993). Morbidity is effected by many interactive factors including age at acquisition (Bell *et al.*, 1997), concomitant alcohol abuse (Brechot *et al.*, 1996), co-infection with other viruses (Chuang *et al.*, 1993) and the host immune response (Wright *et al.*, 1994). Iron, when present in Kupffer cells or hepatocytes, may be another factor affecting the natural history of HCV infection (Di Bisceglie *et al.*, 1992a). Serological screening of blood donations has led to a dramatic reduction in the rate of post transfusion hepatitis from 0.19 to 0.03%, thus confirming HCV's role as the major aetiological agent (Donahue *et al.*, 1992).

HCV infection has been associated with a wide variety of diseases, including autoimmune hepatitis characterised by a reactivity to liver kidney microsomal

antigen (LKM; Garson *et al.,* 1991b; Meyer zum Büschenfelde *et al.,* 1995), mixed cryoglobulinaemia (Bloch, 1992), porphyria cutanea tarda (English *et al.*, 1996) and Sjögrens syndrome (Rodriguez Cuartero *et al.*, 1994). The strength of the evidence implicating HCV as the aetiological agent in each of these clinical conditions varies but they may represent rare complications of hepatitis C.

1.2.5 Biochemical Markers of Liver Damage

The primary manifestation of HCV infection is liver damage and *in vivo* studies of HCV infection are generally incomplete without monitoring the effect on the liver. The type and severity of liver damage can often be determined by measuring a combination of biochemical markers of liver damage.

Markers of Necrosis

Two transaminases are commonly used diagnostically to demonstrate liver damage; these are aspartate transaminase (AST, also known as glutamate-oxaloacetate transaminase, GOT); and alanine transaminase (ALT, or glutamate-pyruvate transaminase, GPT). AST is present in large amounts in normal liver and damage to the liver causes release of this enzyme into serum. However the enzyme is also present in heart, skeletal muscle, brain and red blood cells, so although serum levels may be raised in liver disease, levels are also elevated in myocardial infarction, myopathies and muscular disease or trauma. ALT is present in large concentrations in the liver and in lesser amounts in kidney heart and skeletal muscle and is therefore a more specific marker for liver disease than AST.

Although hepatitis is often due to infection of the liver with viruses, it is important to remember that other causes of raised transaminase levels include bacterial toxins and chemical toxins such as alcohol, paracetamol and carbon tetrachloride. These tests are very sensitive, transaminases levels often become elevated before symptoms occur, but they are also non-specific, detecting only that the liver is damaged, but providing little information about the cause of the liver disease.

Markers of Cholestasis

Alkaline phosphatase (normal values 85-240 IU/l) are a group of enzymes present in bile duct cells, intestine, placenta, bone and kidney. Serum levels of these enzymes are elevated following bile duct obstruction which may be caused by gallstones, sclerosing cholangitis, primary biliary cirrhosis or by a tumour. Elevated levels of these enzymes also occur due to bone disorders such as osteomalacia, bone metastases and Paget's disease.

It is possible to distinguish between skeletal and hepatic disease by measuring serum levels of gamma glutamyl transpeptidase (GGT; normal values 5-40 IU/l) as this marker is raised in hepatic disease but not in skeletal disease.

Markers of Synthetic Ability

Damage to the liver is characterised by reduced synthesis of albumin (normal serum levels 3.5-5g/dl) and blood clotting factors resulting in increased clotting times, this may also occur in advanced liver disease as a result of reduced bile production which is vital for the absorption of vitamin K. The liver also serves to catabolise haem groups produced during the breakdown of red blood cells leading to the production of bilirubin. A normal liver secretes bilirubin into the bile duct from where it is excreted via the gut. Liver damage results in an accumulation of bilirubin in serum.

Histology

Biochemical monitoring of liver damage provides an indirect measure of liver inflammation. A more direct measure is obtained by histopathology following a liver biopsy. A numerical scoring system for assessing histological activity was described by Knodell and co-workers (1981). This scoring system is divided into two parts, an inflammatory score and a fibrosis score. The inflammatory score tends to fall significantly once the agent of hepatic damage is removed and can be considered to reflect the current rate of liver damage. The level of fibrosis rises in a cumulative manner during hepatitis and does not resolve significantly once the cause of the liver damage is removed, it can therefore be considered as a measure of the total amount of liver damage.

1.2.6 Epidemiology

The development of reliable serological assays for HCV has made the study of the epidemiology of hepatitis C possible. The presence of antibodies reactive against HCV can be confirmed in 0.02-0.05% of United Kingdom (UK) blood donors (Garson *et al.,* 1992; Mutimer *et al.*, 1995) . The seroconversion rate amongst UK blood donors is approximately 1-2 donors per 100,000 per year (Atrah *et al.,* 1996). The UK operates a policy of voluntary donation without donor remuneration. Potential donors are asked to exclude themselves from donation if they are unwell or fall into clearly defined risk groups. The aim of this policy is to improve the safety of blood transfusion by excluding donors who have an elevated risk of being carriers of blood transmissible agents. UK blood donors therefore have a lower seroprevelance and incidence compared with the general population, where the prevalence is 0.7% (Sallie *et al.,* 1994). Prevalence rates vary from country to country and rates as high as 20% have been reported in some parts of the world (Saeed *et al.,* 1991; El Zayadi *et al.,* 1996).

HCV is transmitted primarily through contaminated blood and has also been detected in other body fluids including saliva, urine and semen, but the infectivity of these

fluids is less clearly defined. Although the infection rate is raised in sexually promiscuous groups, sexual partners of HCV antibody positive people are typically seronegative (Dusheiko *et al.,* 1996a). Other risk factors for HCV infection include intravenous drug use, transfusion of blood products (Roberts, 1996), haemodialysis, organ transplantation (Wreghitt *et al.,* 1994), tattooing (Abildgaard and Peterslund, 1991; Ko *et al.,* 1992), babies born to HCV infected mothers (Inoue *et al.,* 1991; Ohto *et al.,* 1994) and healthcare workers receiving sharps injuries (Okamoto *et al.,* 1992b). The proportion of patients with 'unexplained community acquired' infection varies widely between studies. It has been suggested that the majority of these patients would admit to high risk behaviour, particularly intravenous drug use, following more thorough questioning (Osmond *et al.,* 1993).

Implementation of HCV sceening reduces the risk of HCV infection from blood transfusion. In the United States the risk of transfusion transmission of HCV decreased from 0.19 to 0.03% per unit transfused following the introduction of screening (Donahue *et al.,* 1992). However transfusion of blood accounts for less than 10% of cases of chronic hepatitis C; intravenous drug use accounts for 40-50% of cases. Transmission of HCV from contaminated lots of intravenous immune globulins has been demonstrated (Bjoro *et al.,* 1994; Power et al.,1995) and the American Food and Drug Administration (FDA) as well as the Committee for Proprietary Medicinal Products (CPMP) now require that all immune globulin products manufactured through a process not including a specific viral inactivation step must be tested for HCV RNA.

1.2.7 Antiviral Therapy

Currently the only licensed therapy for hepatitis C infection is interferon α (IFN-α). Fifty percent of patients treated with interferon have normalised ALT (alanine aminotransferase) levels and are negative for HCV RNA during therapy (Di Bisceglie *et al.*, 1989; Davis *et al.,* 1989; Davis, 1990). However ALT and viraemia levels rise again either at the end of therapy (relapse) or during therapy (breakthrough) in approximately half of the patients who responded (Ryff, 1995).

IFN-α therapy may cause side effects including flu like symptoms, fatigue, thrombocytopenia, leucopenia and autoimmune reactions (Meyer zum Büschenfelde *et al.,* 1995; Okanoue *et al.,* 1996). In light of the poor response rate, side effects and the high cost of therapy, it would be useful to establish clear factors that predict a successful outcome, thus allowing more effective targeting of therapy.

In the absence of an established cell culture model of HCV infection the evaluation of novel antivirals has been severely hampered. Currently, candidate antivirals are screened by assessing the inhibition of recombinant enzymes. Novel antivirals are being developed targeted against the serine protease and the helicase function of NS3 and also against the RNA-dependent RNA polymerase function of NS5 (Clarke, 1995). Developing antiviral monotherapy against RNA viruses has proven to be difficult because of the rapid mutation rate of these viruses. Experimental strategies using monotherapy with established antivirals such as Ribavirin (1-β-D-ribofuranosyl 1,2,4-triazole-3-carboxamide) appear to have little impact on viraemia levels in hepatitis C (Hoofnagle *et al.,* 1996). Trials currently being conducted to find effective therapy for human immunodeficiency virus type 1 (HIV-1) infection use multiple antiviral agents that act at several points in the virus life cycle (Delta Co-ordinating Committee. 1996; Sharp, 1996). This approach may also be effective in HCV treatment, especially as HCV appears to have no classical latent state. The mechanisms by which HCV persists are poorly understood, it has been suggested that the rapid mutation rate of the virus allows the repeated development of neutralisation resistance (Wiener *et al.*, 1992).

1.2.8 Vaccine Development

The ideal preventative strategy would be to develop a vaccine against HCV. However early experiments by Choo *et al.* (1994) proved particularly discouraging; chimpanzees infected with strain H77 of the virus were inoculated with a homotypic inoculum and the immune response to this challenge was monitored. Each challenge with the infectious human blood sample containing strain H77 of HCV produced a response bearing all the hallmarks of the primary immune response and reinfection

occurred. More recent work by the same group was more encouraging; following vaccination with vaccinia expressed envelope glycoprotein, 5/7 chimpanzees were protected against low doses of virus (Choo *et al.*, 1994). The weak humoral immune response to viral infection perhaps suggests that more success may be achieved using vaccines designed to stimulate the T lymphocyte response (Zuckerman and Zuckerman, 1995).

1.2.9 Detection of Hepatitis C Virus

HCV was identified when several laboratories including Kuo *et al.* (1989) at Chiron Corporation demonstrated that antibodies from the sera of infected patients reacted with recombinant antigens expressed from viral RNA (Kuo *et al.*, 1989). Serology still remains the major method for diagnosing HCV infection. The first antigen expressed by Chiron was 5-1-1 derived from the NS3/NS4 region. This was used to develop a radioimmunoassay (RIA) and subsequently an enzyme immunoassay (EIA). This first generation assay produced both false positive results, due to non-specific reactions and false negative results, due to a lack of sensitivity. Immunoassays now contain antigens representing both structural and non-structural proteins, which has greatly improved both the sensitivity and specificity of these tests. However, false positives may still occur and confirmatory or 'supplemental' testing is required to verify results, especially in populations where the prevalence is low such as among blood donors (Sakugawa *et al.*, 1995). The most widely used supplemental test is the recombinant immunoblot assay (RIBA), in which the structural and non-structural antigens are bound to different positions on a nitro-cellulose strip. Reactivity to antigens representing two or more regions of the genome is considered to be indicative of a true positive.

It is however important to remember that antibody detection as a method of diagnosis has limitations. False negative results occur in patients due to immunosuppression and in recently infected patients during the seronegative 'window' period (Alter *et al.*, 1989). The presence of anti-HCV antibodies usually indicates infectivity, however, approximately 10-20% of patients infected with HCV

produce an immune response which clears the virus. Sensitive methods which directly detect the presence of the virus should therefore provide a better predictive marker of both current infection and infectivity.

In situations where serological tests are suspected to have yielded either false positive, false negative or indeterminate results the polymerase chain reaction (PCR) is often used as a confirmatory tool. PCR involves the exponential amplification of specific regions of the genome to levels such that the nucleic acid can be readily detected thus allowing the direct detection of the virus. The PCR process is described in more detail below (Section 1.3.1).

1.3 Nucleic Acid Amplification

There are three widely used methods of sequence specific nucleic acid amplification, polymerase chain reaction (PCR), ligase chain reaction (LCR) and nucleic acid sequence based amplification (NASBA). Two other methods have also been described; transcription mediated amplification is sufficiently similar to NASBA to be considered the same method (Pasternack *et al.,* 1997) and strand displacement amplification (SDA Walker *et al.,* 1992) is rarely used. It is technically possible to adapt any of these methods for quantification.

1.3.1 Polymerase Chain Reaction

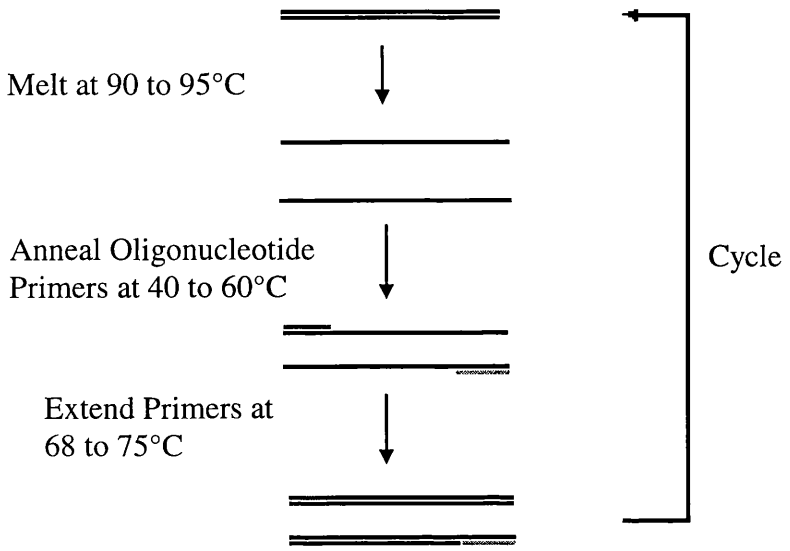
The polymerase chain reaction (PCR) was devised and developed by Kary Mullis at the Cetus Corporation (Mullis *et al.*, 1986). Its first practical application was to amplify the human β-globin gene to detect the mutation leading to sickle-cell anaemia (Saiki *et al.,* 1985). The purification of a thermostable polymerase from the eubacteria *Thermus aquaticus* (*Taq* polymerase) has allowed the practical application of this process to large areas of molecular biology.

PCR is an *in vitro* reaction involving the thermal separation of the two strands of DNA, the annealing of short oligonucleotide primers complementary to each of the DNA strands and the extension of these primers by a DNA-dependent DNA polymerase (Figure 2).

The consequence of this process is a doubling of the original number of DNA copies. If this doubling is repeated a number of times the resulting two-fold geometric increase in DNA copy number leads to a huge amplification of the number of DNA copies identical to the original target sequence (Figure 3).

Figure 2: The Polymerase Chain Reaction

PCR involves a repeated process of melting double stranded DNA, annealing of short oligonucleotide primers and the extension of these primers with a DNA dependent DNA polymerase. Each reaction cycle potentially leads to a doubling of the initial number of DNA strands.



The basic steps involved in a PCR are as follows:

Strand Separation: Incubating DNA at temperatures in excess of 90°C results in the breakage of the hydrogen bonds that hold the two strands together leading to their separation.

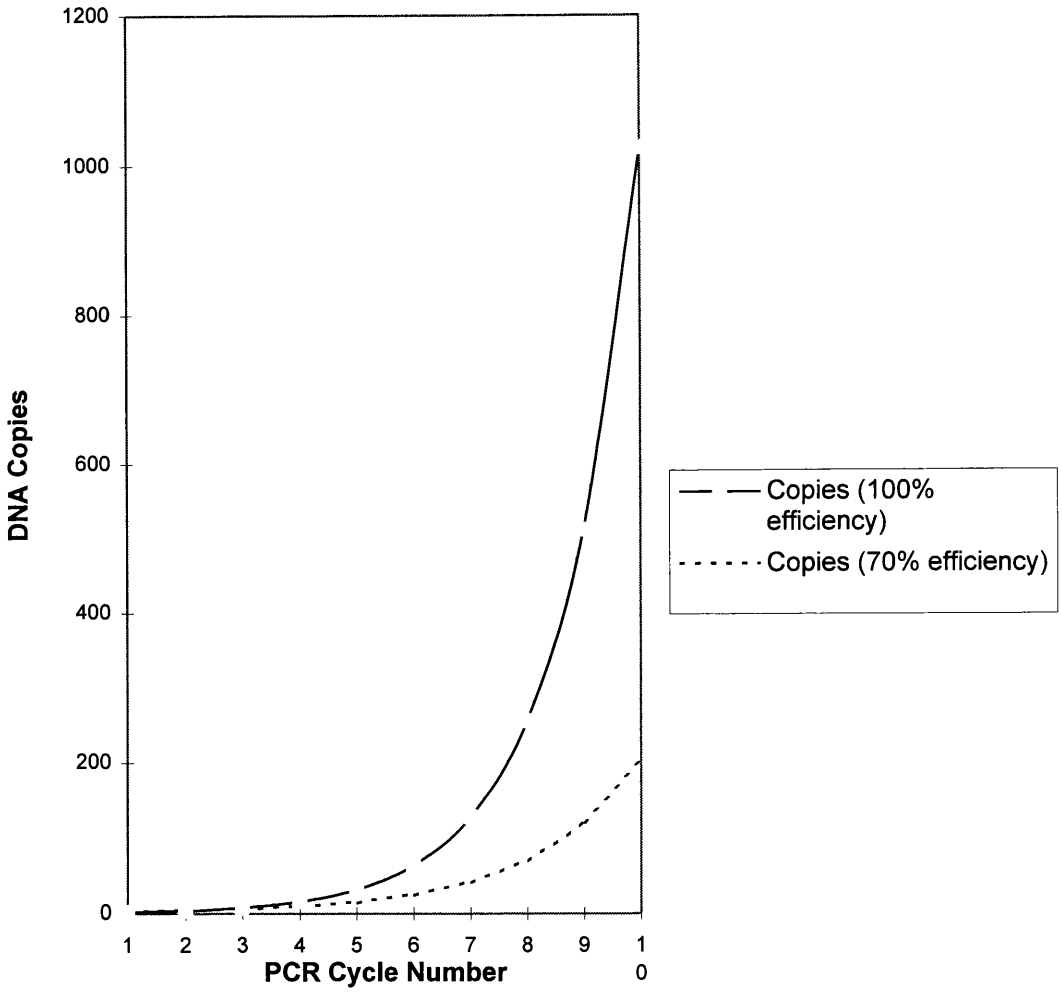
Priming: Once the strands of DNA are separated the reaction mix is allowed to cool thus allowing two synthetic oligonucleotides, usually between 15 and 40 bases in length, to bind to the target sequence. One oligonucleotide binds to the sense strand and one to the antisense strand at a predetermined distance apart. The distance between the 5' ends of the two oligonucleotides, typically between 50 and 10,000 bases determines the size of the PCR product. The exact location that the oligonucleotides bind is dictated by their sequence. Optimised conditions for PCR allow only one binding location for each primer, limited by the annealing temperature of the reaction and the laws of thermodynamics.

Strand Extension: The annealed primers are extended to complete the second strand by heating the reaction to a temperature optimal for the incorporation of deoxynucleotide tri-phosphates (dNTP's) by the thermostable polymerase, usually 68-75°C. During the first cycle of replication the strand extension proceeds beyond the binding site of the second primer, but on subsequent cycles the number of molecules with their 3'-end determined by the binding site of the second primer increases logarithmically. The final size of PCR product after many cycles of replication is predominantly that determined by the distance between the binding sites of the two primers. The number of product molecules generated from a single target molecule assuming a theoretical two-fold increase at each cycle or a 1.7 fold increase are shown in Figure 3.

In reality it is unlikely that the efficiency of PCR is constant from cycle to cycle, because the enzyme loses activity, either because of its thermal inactivation or because it remains attached to the PCR product. Furthermore depletion of reaction

substrates such as nucleotides or primers will become limiting as the reaction progresses. As is clearly illustrated in Figure 3 small differences in reaction efficiency produce dramatic differences in the rate of accumulation of PCR products. Careful optimisation of the factors influencing reaction efficiency are therefore important in the amplification of the target sequence.

Figure 3: Number of DNA Copies Generated Assuming PCR is 70% or 100% Efficient



In practice PCR requires the optimisation not only of the parameters described above but of several procedures peripheral to the reaction itself. The optimisation of these parameters and procedures as well as their application to the study of HCV and its close relatives, form the subject of this thesis. In general the practical application of PCR to HCV can be divided into five stages:

1 Sample handling

The stage from phlebotomy to nucleic acid extraction is often a major source of variation. Serum samples stored or transported at room temperature or above suffer a rapid loss of viral RNA (Busch *et al.,* 1992).

2 Nucleic acid preparation

The efficiency and reproducibility of stages 3-5 are dependent upon the yield and purity of the viral nucleic acid isolated. RNA is inherently less stable than DNA being more thermolabile and readily digested by cellular enzymes. The following stages are essential to the efficiency of extraction:

- *Inactivation of ribonucleases present in all living tissues.* This is achieved either by proteinase digestion of the enzymes or by chemical denaturation by chaotropic agents. Unlike deoxyribonucleases, ribonucleases by virtue of highly cross linked secondary structure readily reform following heat or chemical denaturation. Meticulous preparation of reaction vessels and buffers to remove contaminating ribonucleases is necessary to ensure a high yield.
- *Lysis of cells and viral particles.* This is usually achieved using detergents such as N-lauryl sarcosine, Triton X-100, or NP40.
- *Physical separation of RNA from potential inhibitors.* PCR is particularly susceptible to inhibition by proteins containing porphyrin rings such as haemoglobin (Higuchi, 1989) and the anticoagulant heparin. Avoidance of these inhibitors is achieved by limiting analysis to that of fresh, or fresh frozen, serum or heparin free plasma. The viral RNA can be separated from small amounts of contaminating haemoglobin during phase separation using organic solvents, or by

selective binding to either silica (Boom, 1990) or oligonucleotides (Van Doorn, 1992).

- *Concentration.* This is achieved either by precipitation with an alcohol, or during the selective binding described above, followed by elution or resuspension in water or appropriate buffers.

3 cDNA Synthesis by Reverse Transcription of Viral RNA

PCR is a method for the amplification of DNA, and amplification of RNA targets such as the HCV genomes first require the RNA to be reverse transcribed into DNA. As this process is never 100% efficient (typically 5-20%; Simmonds *et al.,* 1990b) the detection of RNA targets is invariably less sensitive than the detection of DNA targets.

4 cDNA Amplification by PCR

Practical considerations for PCR optimisation include the following:

- *The concentrations of reaction components.* Concentrations of the following must be optimised: oligonucleotide primers, dNTPs and thermostable DNA polymerase (*Taq, Tth, Tfl, Pfu, etc.*). Thermostable polymerases are now supplied with pre-optimised reaction buffers containing the following: divalent cations (magnesium ions [Mg^{2+}], or manganese ions [Mn^{2+}] which can be buffered with bicine), hydrogen ions (pH buffer), potassium or sodium ions (these serve to alter the stringency) and a variety of detergents, carriers and reducing agents which improve reaction efficiency, but are not essential components.
- *Primer design:* Oligonucleotide primers used in PCR are chemically synthesised. Their design is based primarily on the known sequence of the target nucleic acid. Ideally their sequence should be 100% homologous to the target sequence, however, due to the inherent variability of the genome of RNA viruses such as HCV (Section 1.2.2), this is not always possible. The primers used to amplify HCV sequences in this project were designed by members of the Department of Virology, and were selected on the basis of their proven track record as has been described elsewhere (Garson *et al.,* 1991a).

- *Cycling parameters:* PCR is now invariably performed using an automated programmable heating block (PCR machine) instead of repetetive transfer between waterbaths at different temperatures. Reaction vessels are placed in the PCR machine and each of the three stages of PCR occur during predetermined incubation periods and at predetermined temperatures. Denaturation occurs at temperatures above 90°C; too low a temperature or too short an incubation time leads to a loss of sensitivity due to incomplete denaturation; too high a temperature or too long an incubation time leads to a loss of sensitivity due to thermal inactivation of the polymerase. Annealing temperature is the most variable parameter between primer sets, if the temperature is not allowed to drop sufficiently, little or no primer will hybridise to its target, but if the annealing temperature is too low sequences other than the target may be amplified. The optimum annealing temperature is dependent upon the nucleotide composition of the primer (% GC and length), the variability of the target sequence and the monovalent cation concentration of the reaction buffer. Primer extension occurs at a temperature near the optimum for the polymerase used, for example the optimum for *Taq* polymerase is 72°C and for *Pfu*, 75°C. The optimum extension time is dependent upon the length of the PCR product generated. For short products (<100bp) extension times need only be a few seconds. The number of cycles performed is limited by the amount and stability of polymerase added, but usually does not exceed 40 cycles.
- *Number of rounds of cycling ("nested" PCR):* For the reasons outlined above, the number of PCR products that can be accumulated in a single reaction is finite (Mullis, 1991). The sensitivity and specificity of PCR can be greatly increased by the use of 'nested' or double reactions (Mullis and Falloona, 1987). In this method the products of a PCR are used as the input sample in a second PCR with primers internal to the primer pair used in the first PCR. The use of four PCR primers in this way allows the specific detection of even a single initial target molecule (Garson *et al.,* 1990; Simmonds *et al.,* 1990a).
- *Contamination avoidance:* Due to the extreme sensitivity of PCR false negative results are much less likely than with other nucleic acid detection techniques. However, as outlined previously PCR results in the production of large numbers

of PCR products, typically $>10^9$ molecules, in a small reaction volume, usually <100µl, thus contaminating volumes of less than 1pl (picolitre) from a previous reaction using the same primers may result in a false positive result. PCR contamination is therefore a major potential problem with PCR, which can only be avoided by adherence to stringent precautions such as those outlined by Kwok and Higuchi (Kwok and Higuchi, 1989). In addition a variety of contamination prevention strategies have been designed, in which reaction products are selectively destroyed (Abravaya *et al.*, 1997).

5 Analysis of the PCR Product

The nature of the analysis performed on PCR products depends primarily on the initial purpose of the PCR. PCR has been applied to a bewildering array of different uses within molecular biology and an outline of each adaptation is outside the scope of this thesis. Briefly, the analysis of PCR products falls into 3 broad categories:

- *The qualitative detection of PCR products* (i.e. has a specific product of the expected size been generated). This is most commonly achieved by electrophoresis using ethidium bromide stained agarose gels.
- *Quantitative PCR.* The use of PCR to quantify HCV is the focus of this thesis and is described in detail in Section 1.4. Quantification using PCR is dependent upon the optimisation of the reaction conditions to produce a range of product concentrations which can be related to the amount of virus in the starting material.
- *Sequence analysis.* PCR is a convenient method of generating large quantities of DNA for sequence analysis. This analysis may be performed by direct sequence determination of the PCR product, or following cloning of the products. The sequence can be confirmed using chain termination methods, hybridisation or by restriction fragment analysis.

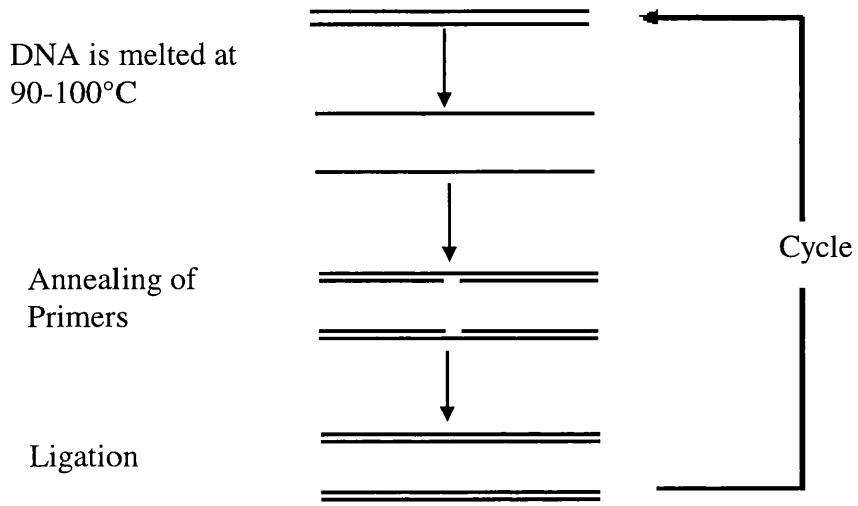
1.3.2 Ligase Chain Reaction (LCR)

The ligase chain reaction is composed of three steps analogous to those of PCR (Zebala and Barany, 1993). The DNA is converted into the single stranded form by

heating (90-100°C), the reaction temperature is reduced to allow the annealing of four primers with two pairs of primers annealed to adjacent primer binding sites on each DNA. Unlike DNA polymerase, DNA ligase is unable to incorporate nucleotides, so the "extension" phase of LCR involves both primer pairs being joined by a thermostable DNA ligase to form two molecules of double stranded DNA. Repeated doubling reactions can occur allowing the amplification of target DNA in a similar way as is seen in PCR (Figure 4).

Figure 4: Ligase Chain Reaction

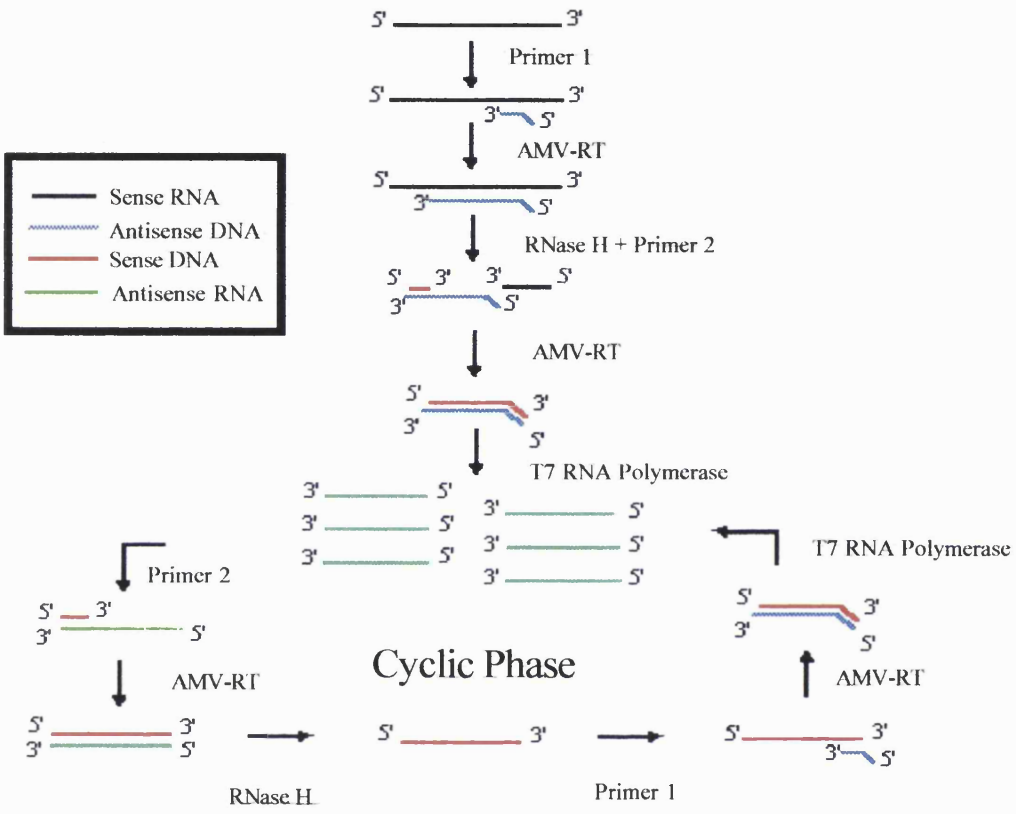
During LCR the DNA is rendered single stranded by heating, then cooled to allow four oligonucleotide probes to bind. Provided each pair of probes (one pair per DNA strand) bind next to each other the probes will then ligate in the presence of DNA ligase.



1.3.3 Nucleic Acid Sequence Based Amplification (NASBA)

NASBA is able to amplify either RNA or DNA using a combination of three enzymes (Keivits *et al.*, 1991). Amplification begins with the extension of an oligonucleotide primer that incorporates the T7 promoter by AMV reverse transcriptase on a single stranded DNA or RNA template. If the initial template strand was RNA this strand is degraded by the ribonuclease RNase H, and a second DNA strand is synthesised by extension from a second primer. Finally, a new RNA template is synthesised by T7 RNA polymerase allowing the cycle to begin again. All three enzymes have an optimum temperature of approximately 37°C thus allowing the whole process to occur at constant temperature (Figure 5). This system has also been refered to as self-sustained sequence replication (3SR; Gingeras *et al.*, 1991), and is identical in all but minor detail to an assay called transcription mediated amplification (TMA).

Figure 5: Nucleic Acid Sequenced Based Amplification



1.3.4 Strand Displacement Amplification (SDA)

Strand Displacement Amplification (SDA) is an isothermal, *in vitro* nucleic acid amplification technique based upon the ability of *Hinc*II to nick the unmodified strand of a hemiphosphorothioate form of its recognition site, and the ability of the Klenow fragment of DNA polymerase to extend the 3'-end at the nick and displace the downstream DNA strand. Exponential amplification results from coupling sense and antisense reactions in which strands displaced from a sense reaction serve as target for an antisense reaction and vice versa (Walker *et al.*, 1992).

1.3.5 Other Methods for the Direct Detection of Infectious Agents

Qβ Replicase Amplified Assay

This assay system differs from the target amplification systems described above in that this stratagy is based on probe amplification. *Qβ* replicase is a tetrameric enzyme complex capable of inducing the amplification of single stranded RNA molecules containing a specific recognition sequence and a terminal nucleotide sequence required for polymerase recognition. One of the four subunits of the enzyme complex is derived from the *Qβ* bacteriophage, the other three are produced by its host *Escherichia coli*. Probes for *Qβ* replicase based assays are generated by modifying the single stranded RNA genome of the *Qβ* bacteriophage MDV-1. The target specific sequence is inserted into the phage genome to generate a hybridisation probe which can then be used to bind viral sequences. Once unbound probe is removed by careful washing the enzyme complex is added leading to the exponential amplification of the probe (Olive, 1997). Although this system has largely been applied to the detection of bacterial and Chlamydia targets its adaptation to the detection of viral nucleic assays should be possible. The sensitivity of this assay is limited largely by the level of background signal generated as a result of non-specific hybridisation. This can be overcome at least in part by dividing the sequence specific probe into two parts, with each half containing only one of the sequence elements

required for amplification. A ligase dependent step would only proceed if both sections of the probe were perfectly aligned (Carrino *et al.,* 1997).

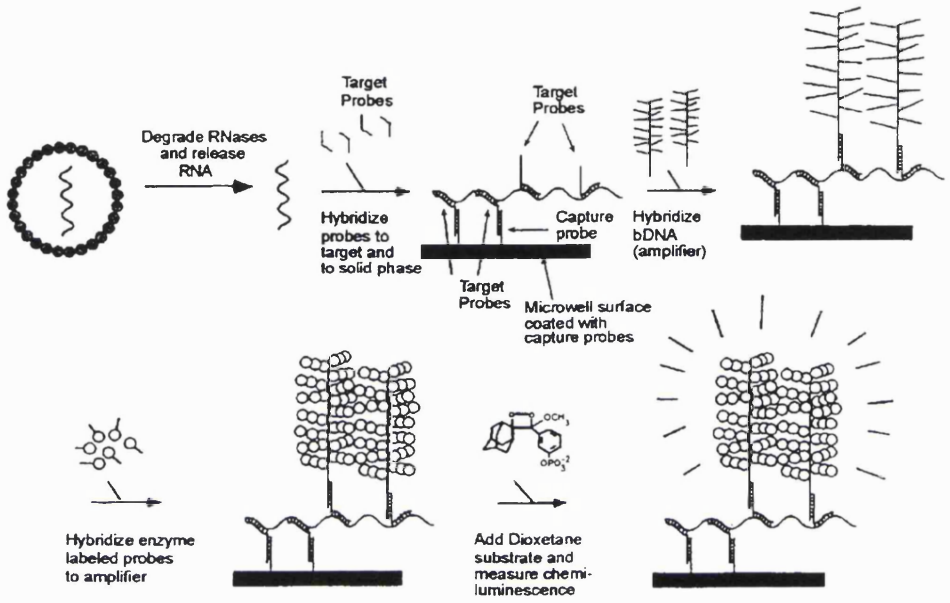
Chiron Quantiplex Assay (bDNA) and the Ampliprobe System

The Chiron Quantiplex assay uses oligonucleotide probes complementary to the 5'NCR and core regions to capture HCV RNA (Urdea *et al.,* 1988). Modification of some of the nucleotide bases allows the construction of DNA probes with a branched 'tree-like' structure. Alkaline phosphatase labelled oligonucleotides are bound to the branches of the 'tree-like' DNA probes, thereby allowing amplification of the signal produced on addition of a dioxetane substrate. The detection limit of this assay is 3.5 x 10^5 HCV genomes/ml, a sensitivity somewhat lower than comparable nucleic acid amplification methods. This method is illustrated in Figure 6.

This assay, although less sensitive than nucleic acid amplification methods, is both rapid and reproducible. The technique is considerably less prone to contamination than PCR and does not require modification to yield quantitative results. The method has been widely used for the quantification of HCV RNA during trials of antiviral therapy, and further discussion of the strengths and weaknesses of this assay is reserved for the General Discussion (Section 4).

Figure 6: Chiron Quantiplex (bDNA) Assay

Reproduced with permission of Chiron Corporation.



The principle of signal amplification is also utilised by the Ampliprobe system (ImClone Systems, New York, U.S.A), however the chemistry used to construct probes is considerably less complicated. The single stranded RNA genome of the bacteriophage M13 is modified to include a region complementary to the target sequence (e.g. viral RNA fixed to a solid support). Secondary probes bind to multiple sites in the M13 genome and these secondary probes either directly or indirectly incorporate alkaline phosphatase.

Direct Detection of Core Antigen

In addition to methods for the detection and quantification of HCV RNA a method has been described for the direct detection of the core antigen. This method requires the concentration of core antigen by ultracentrifugation of infectious samples. Ultracentrifugation cannot always be performed in routine diagnostic laboratories and greatly limits the number of samples that can be analysed in parallel. Quantitative data generated using this method have been shown be similar to those produced using quantitative PCR (Tanaka *et al.*, 1996). Further protocol simplification and evaluation will probably be required before this assay is more widely used.

1.4 Quantitative PCR

Several methods exist in which PCR can be used to quantify either the DNA or RNA level in a sample and each has advantages and disadvantages.

Limit Dilution ('Quantification of the Fewest')

The three methods use a 'nested' format and allow the PCR reaction to continue until one of the reaction components is used up. This approach results in a constant PCR product level irrespective of the number of target molecules in the reaction vessel. This qualitative PCR method can be used for quantifying DNA provided it is able to detect single molecules of DNA. This is assumed to be the case with 'nested' PCR because the results are digital ('all or nothing') in nature (Figure 7) and the proportion of positive and negative results generated occur at frequencies predicted by statistical methods.

The concentration of a sample can be estimated by preparing a 10 fold dilution series of DNA from each sample. The last dilution to test positive can be assumed to contain approximately one molecule. At low concentrations the presence or absence of a detectable copy of DNA will be determined by the Poisson distribution (Simmonds *et al.*, 1990b). An accurate determination of concentration can be obtained by repeat testing at a dilution that gives a mixture of positive and negative PCR results and using statistical tables to predict the mean concentration of DNA molecules at that dilution (Poisson distribution). This method has proved reliable and sensitive for the quantification of HCV (Garson *et al.*, 1990c), but large numbers of PCRs must be performed to obtain accurate data making it inappropriate for applications where a high throughput is required.

Competitive Template PCR

A second approach relies on the principle that competitor templates (also called internal calibrators or internal controls) can be co-amplified with the target and that the ratio of target to competitor will remain constant throughout the reaction. Competitive template methods use a synthetic template that includes primer binding sites identical to the target sequence. The sequence internal to the primer binding sites must be altered in some way (*e.g.* made larger, or have a unique restriction site included) so that the products generated from the target and competitor template can easily be distinguished. A series of PCRs is performed with different concentrations of competitor template; the concentration of the target template is determined by comparison between the signals from the target and the competitor. If the signals are the same it is assumed that both the target and the competitor templates started at the same concentration. This principle should remain true even if the efficiency of the PCR is altered by inhibitors or by changes in the reaction conditions. This approach should therefore have the advantage of controlling for tube to tube variation in amplification efficiency (Kaneko *et al.*, 1992). The fact that competitor templates are added to each reaction reduces the risk of false negative results caused by reaction inhibition because a true negative result will always produce a signal from the competitor even in the absence of target templates. However, because the target template concentration is unknown several reactions must be performed for each sample to be certain of finding signals of comparable intensity. Some sensitivity may be lost because of the competition for reagents.

Using a non-competitive internal standard, where the synthetic template is amplified using primers different from those used to amplify the target sequence, has been shown to produce accurate quantification of HIV-1 target sequences (Ferré *et al.*, 1994). This method presumably retains the ability to control for tube to tube variation without competing with the target template for primers. However competition for other reaction components still occurs (Zachar *et al.*, 1993).

Quantitative Generation of PCR Products

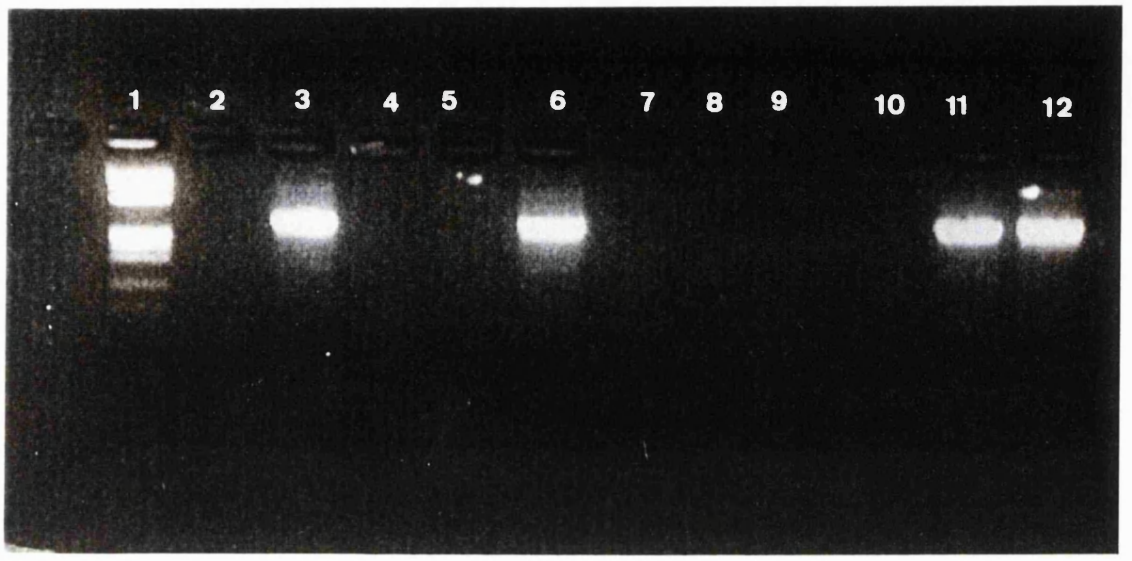
As described above "nested" PCR is optimised to give uniform band intensity on an ethidium bromide stained agarose gel, irrespective of the DNA input concentration (Figure 7). Interrupting the PCR before it has reached completion should lead to the production of PCR product concentrations proportional to the initial concentration of template molecules. The concentration of PCR products can be determined by the incorporation of fluorescent or radioactive labelled nucleotides (Semple *et al*, 1993), by hybridisation of the PCR product with labelled oligonucleotide probes (Mallet et al., 1993) or by the specific detection of double stranded DNA using DNA binding proteins (Kemp *et al.*, 1989) or antibodies (Payan *et al.*, 1995). The major disadvantage of incorporation based methods is that they produce signals for both specific and non-specific products such as 'primer dimers'. This problem can be overcome by confirming the products size on an agarose gel or, somewhat more conveniently, confirming the product's sequence by introducing a short hybridisation step.

'Real-Time' PCR

Quantification of PCR products as described above is performed once cycling has been completed. However, it is possible to intercalate fluorescent dyes, such as ethidium bromide, into the PCR products as they are being produced and measure their incorporation at the same time (Higuchi *et al.*, 1993) The approach has been called kinetic or 'real-time' PCR. Using real time PCR it is possible to measure the cycle number at which the PCR products become detectable. Samples containing high concentrations of template will produce detectable levels of PCR product at lower cycle numbers than samples containing lower template concentrations. Samples can be quantified by comparing the cycle number at which PCR products are detectable with a series of external controls. Both samples and controls should yield PCR products at similar rates and it is possible to identify samples which contain inhibitors by comparing the relative increase in fluorescence per cycle number with that of the controls.

Measuring the production of PCR products by incorporating fluorescent dyes has the disadvantage that it might also be expected to produce signals from non-specific products. Eubacterial polymerases such as *Taq* DNA polymerase possess a 5'-3' exonuclease activity which can be used to release dye from a hybridisation probe. By attaching a fluorescent dye to one end of the probe and a quencher molecule to the other, *Taq* polymerase will digest the probe separating the fluorescent dye from the quencher causing an increase in fluorescence. These probes, known as Taqman probes, have successfully been used for the quantification of HCV (Martell *et al.*, 1999).

Figure 7: "Nested" PCR Products.



3% Agarose Gel: Lane 1: Markers- pBR322 *Hae* III; Lanes 3, 6, 11 and 12 contain 402bp 'nested' PCR products generated from the NS5 region of GB virus C using primers YK 874, YK 877, YK 1183 and YK 876. Lanes 2, 4, 5 and 7-10 contain amplification products from samples which were negative.

1.5 Aims of the Thesis

Prior to the commencement of this project the methods available for HCV quantification were highly laborious, involving multiple PCRs for each sample, making it difficult to apply quantitative techniques to large scale trials of antiviral therapy for HCV. The major aim of this project was to overcome this problem by developing a method of quantification that is rapid and inexpensive without compromising sensitivity, specificity or accuracy. Such a method should also be sufficiently flexible that it could be applied to other viral targets. The ongoing HCV projects to which the techniques developed in this thesis have been applied include:

- Attempts to identify pre-treatment markers predictive of interferon response.
- The development of novel treatment strategies for HCV infection.
- The development and optimisation of protocols for the *in vitro* propagation of HCV.
- The evaluation of new animal models of HCV infection.
- HCV RNA detection in blood, blood fractions and blood products.
- HCV infection during pregnancy.
- GBV-C RNA detection in blood donors, transfusion recipients and patients undergoing maintenance haemodialysis.

2. Development and Evaluation of Methods

As outlined in Section 1.3.1 of the Introduction the practice of PCR requires the optimisation of a series of methodologies peripheral to PCR as well as those parameters directly effecting the PCR itself. This section of the thesis describes the development of optimal methods for each stage of the viral quantification process from the handling and storage of blood samples to the estimation of viraemia levels.

2.1 Sample Storage

2.1.1 Storage of Citrate Plasma Samples at -20°C

Introduction

Previous studies have demonstrated that specimen handling and storage can have a significant effect on the diagnostic sensitivity of PCR for HCV RNA (Busch *et al.*, 1992). Many early studies of PCR detection of HCV were performed following routine serological testing where samples may be stored refrigerated or at room temperature for periods of days to weeks. These studies reported that only 50-70% of RIBA confirmed HCV positive specimens were also PCR positive (Van der Poel *et al.*, 1991; Cuypers *et al.*, 1992). This contrasts with other reports that almost 100% of these samples were PCR positive (Ulrich *et al.*, 1990) where care was taken to store samples for PCR at -20°C. It is now established that HCV RNA levels will decline in serum stored at room temperature at a rate of 99% per week (Busch *et al.*, 1992).

Although HCV contained in serum or plasma has been reported to be most stable when stored at temperatures below -70°C, storage space at this temperature is often a limited commodity in laboratories. We investigated the storage of HCV containing citrate plasma at -20°C.

Methods

Citrate plasma from an HCV infected blood donor was quantified by the limit dilution/ Poisson distribution method of Simmonds *et al.* (1990b). This standard, referred to as 'Plasma A', was stored at -20°C for a period of 35 months before being re-quantified by the same limit dilution/ Poisson distribution method. Plasma A was stored in the same freezer as serum and plasma samples for quantification. This freezer is an upright freezer which is typically accessed to retrieve samples at least once per day.

Results

In April 1994 'Plasma A' was shown to contain 3.2×10^7 HCV genomes/ml (11 of 20 nested PCR reactions scored positive when a 10^{-5} dilution of cDNA was tested). Following storage at -20°C for 35 months 'Plasma A' was shown to contain 4×10^6 HCV genomes/ml(13 of 20 nested PCR reactions scored positive when a 10^{-4} dilution of cDNA was tested). The loss per year can therefore be estimated at approximately 50%.

Conclusions

The samples described in this thesis are serum or heparin free plasma unless otherwise stated. These samples are typically separated within a few hours of being taken, they are frozen at -20°C and transported frozen (on dry ice) before being thawed immediately prior to RNA extraction.

The decline in HCV RNA titre reported here is insufficient to effect the routine handling of samples as storage times are not usually more than a few weeks. It does however have important implications for comparative quantification using citrate plasma standards. Citrate plasma taken from blood donors shown to have HCV reactive antibodies remains the most practical source of large volume, single donor samples suitable for use as quantification standards. It is therefore important that

these standards are recalibrated using the limit dilution/ Poisson distribution technique at intervals of approximately six months. National and international quantification standards are currently being evaluated, clearly these need to be more stable than the citrate plasma standards described here. More stable storage might be achieved at a lower temperature (-70°C), in a freezer that is less frquently accessed or following lyophilization.

2.1.2 Storage of Samples Diluted in RNAzol A Solution

Introduction

The storage of HCV contaminated serum or plasma at temperatures at or above ambient results in the rapid loss of viral RNA (Busch *et al.*, 1992). Samples transported for PCR testing at reference centres such as UCLMS Department of Virology, are typically transported frozen using dry ice. When large numbers of samples are transported as part of international collaborative trials, such as those described in this thesis (Sections 3.1, 3.2 and 3.3), the transportation costs can be large. Samples transported in this way present two problems; firstly, the viral particles remain intact and infectious and therefore represent a biohazard; secondly, the reduction in pressure that occurs in the luggage hold of aircraft would result in the rapid sublimation of the dry ice, therefore these hazardous samples must be transported at cabin pressure. It would therefore be desirable to be able to store samples containing HCV RNA in a transport medium which would allow storage without using dry ice, preferably at ambient temperatures. The purpose of this study was to determine if the acid guanidinium thiocyanate lysis buffer RNAzol A solution (Chomczynski and Sacchi, 1987) could also be used as a transport medium for samples containing HCV.

Methods

A citrate plasma standard containing approximately 4 x 10^6 HCV genomes/ml was diluted 1:100 in HCV RNA negative citrate plasma and divided into 24 200µl aliquots. 400µl of RNAzol A solution was added to each aliquot and vortexed. The samples were then stored for 48 hours as follows:

1. Eight aliquots were snap frozen in liquid nitrogen and stored at -20°C.
2. Eight aliquots were stored at room temperature (18-25°C).
3. Eight aliquots were stored in a waterbath at 37°C.

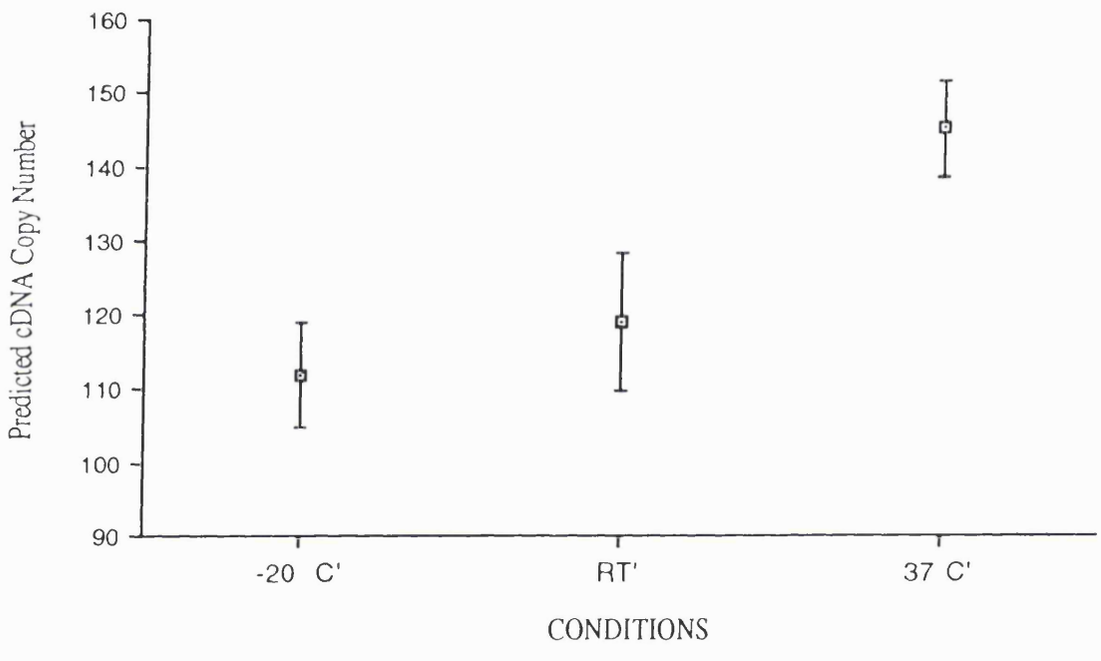
Following storage each aliquot was returned to room temperature and the remaining components of the RNAzol solution were added (400µl of water saturated phenol and 40µl of sodium acetate). HCV RNA was extracted using chloroform : iso amyl alcohol (24:1) and alcohol precipitation as described in Appendix I and Appendix II. cDNA was generated using MMLV-RT and quantified using the QRT-PCR method described in Appendix II.

Results

Citrate plasma containing HCV RNA diluted in RNAzol A was shown to contain similar levels of viral RNA when stored for 48 hours at -20°C and room temperature. Samples stored at 37°C appeared to contain higher levels of RNA but this difference was only slight (Figure 8).

Figure 8: Stability of HCV Stored in RNAzol A Solution

Eight replicates of 200μl of Citrate plasma diluted in two volumes of RNAzol A stored frozen, at ambient temperature and at 37°C. Data is expressed as mean predicted cDNA copy number per ml ± standard error. One cDNA copy number/ml equates to 400 HCV RNA genomes/ml. The starting concentration of HCV RNA was 4 x 10^4 (equivalent to 100 cDNA molecules).



Conclusions

Previous reports have described the rapid decay of HCV RNA levels in samples stored without freezing or refrigeration (Busch *et al.,* 1992). These results indicate that plasma samples for HCV RNA quantification by PCR can be readily transported at ambient temperatures once diluted in RNAzol A solution. On the basis of these results it is reasonable to conclude that transportation of samples involved in international collaborative studies could be achieved without special precautions for infectious materials or for the preservation of dry ice, thereby dramatically reducing costs. However, it is important to remember that a significant chemical hazard would remain.

2.2 Evaluation of Methods for RNA Extraction.

2.2.1 Methods of RNA Extraction

Introduction

A wide variety of methods have now been developed for the extraction of RNA from biological specimens. This section of the thesis describes their comparative evaluation as applied to the extraction of HCV RNA from serum or plasma.

The following criteria are desirable when preparing RNA for quantitative analysis: the method should be rapid and allow a high sample throughput, it should remove potential inhibitors of either reverse transcription or polymerase chain reaction (PCR) to gain sensitive and reproducible results and must efficiently remove or inactivate ribonucleases (RNase) to produce undegraded RNA, thereby producing a high yield of RNA in a reproducible manner.

Methods

A wide range of methods for RNA extraction has been evaluated, each of which is detailed below. All the results were evaluated relative to the efficiency of the RNAzol A extraction method by comparing the number of complementary DNA (cDNA) molecules generated from 5µl of extracted RNA, as measured by QRT-PCR (Appendix II). Plasma A was used as the starting material for each method. Each protocol was performed as described in the relevant publication or according to the manufacturers' instructions unless otherwise stated.

RNAzol A
(Chomczynski and Sacchi, 1987)

This is a single tube method which was performed as follows: a solution of guanidinium isothiocyanate (4M), N-lauryl sarcosine (0.034M) and sodium citrate (0.025M) was prepared (RNAzol A) to which 360µl of β mercaptoethanol is added before dilution (1:1) in water saturated phenol. The pH of this solution was lowered by adding 40µl of 2M sodium acetate (pH 4.0) so as to preferentially dissolve RNA rather than DNA. 800µl of this RNAzol solution was added to 200µl of serum or heparin free plasma and vortexed for 15 seconds bfore being incubated on ice for 15 minutes. Phase separation occured as a result of adding 100µl of a chloroform iso-amyl alcohol mixture (24:1) and centrifugation at 15,000 rpm at 4°C for 15 minutes. RNA in the aqueous phase (600µl) was precipitated with an equal volume of ice cold propan-2-ol using glycogen as a co-precipitant. The precipitated RNA was pelleted by centrifugation at 15,000 rpm at 4°C for 15 minutes. Pellets were washed twice in ice cold 75% ethanol before being dissolved in 10µl of nuclease free water.

Simple Guanidinium Method
Young et al. (1993)

This method uses a less complex lysis buffer (5.75M guanidinium isothiocyanate buffered with Tris.Cl (pH 7.5) plus 0.36% β mercaptoethanol) than the RNAzol A method described above (and Appendix II). Once the sample was lysed (60°C 10 minutes) the RNA was left to precipitate in propan-2-ol. As there is no phase separation both protein and lipid were co-precipitated, rendering the pellet very difficult to resuspend. The pellet was eventually resuspended in 1ml of water. In order to reduce the high level of dilution of the RNA attempts were made to remove the protein with proteinase K and the lipid with chloroform. Heating at 80°C was required to remove the proteinase K.

Prince's Method
(per. comm. Dr. A.M. Prince, New York Blood Centre, USA)

2µl of serum was diluted in 10µl of foetal calf serum and 5µl of this mixture was added directly to the cDNA reaction mix containing random hexamers in place of the RNA (Appendix I).

Silica Adsorption:

This method of viral RNA purification was first described by Boom *et al.* (1990) and has since been modified by several groups. Two versions were evaluated. The HIV NASBA kit from Organon Teknika uses a method very close to the original, here the serum (200µl) was denatured in acid guanidinium lysis buffer, bound to silica particles, washed twice and then eluted into 50µl of distilled water. The modified method (QIAamp Viral RNA Kit, Qiagen) uses a similar extraction buffer, but the silica is in the form of the porous membrane of a spin column and only 140µl of serum or plasma is added. The RNA is again eluted in 50µl of distilled water.

Ultrafiltration

Virus can be concentrated by ultrafiltration using micropore filters that retain molecules larger than 30 kilodaltons. This method has been shown to be effective using tissue culture supernatants (data not shown), but plasma tend to contain high molecular weight components which block the filters and make this method too slow to remain practicable. Neither prefiltering with 0.2µm filters nor pre-centrifugation of the plasma samples improved the efficiency. Virus was concentrated from 200µl of plasma lysed in 0.01% Triton X-100, precipitated in propan-2-ol, washed twice with 75% ethanol and resuspended in 10µl of nuclease free water.

Oligonucleotide Capture
(Van Doorn, 1992)

Serum was added to a LiCl-SDS based lysis buffer and the HCV RNA was extracted using a positive strand-specific oligonucleotide (PT2; Table 5) bound via streptavidin to paramagnetic particles.

Catrimox 14
(tetra-decyl-trimethyl-ammonium oxalate; Iowa Biotechnologies, Iowa):

This novel detergent theoretically precipitates total nucleic acid whilst solubilising proteins. 200µl of serum was mixed with 1ml of Catrimox 14, incubated at room temperature for 15 minutes and spun at 15,000 rpm for 15 minutes. The pellet was re-extracted by adding 200µl of RNAzol A solution, 200µl of water saturated phenol and 20µl of chloroform. The aqueous phase was removed and placed in an equal volume of propan-2-ol and left to precipitate for 1 hour. Following centrifugation at 15,000 rpm for 15 minutes at 4°C the pellets were washed twice in 75% ethanol and resuspended in 20µl of nuclease free water.

Simple Caesium Chloride Method:

This method was shown to be effective when detecting picornavirus RNA (Afzal and Minor; 1994). The method described uses a buffer similar to the lysis buffer described by Young *et al.* (1993; 4M guanidinium isothiocyanate, sodium citrate [pH 7.0], 0.5% sodium laurylsarcosine, 300µl/100ml antifoam solution [Sigma], 700µl/100ml 2-mercaptoethanol), but phase separation is achieved using a caesium chloride cushion (100µl of 5.7M caesium chloride [refractive index 1.4] added through viral lysis buffer). RNA being denser than the caesium chloride is concentrated at the bottom of the tube. The method described in the paper appears to have no mechanism for RNA precipitation so the same method was repeated adding an equal volume of propan-2-ol to the lysis mix.

Results

In total ten different methods of RNA extraction were evaluated. None of the methods resulted in the production of as many cDNA molecules as the RNAzol method. However, three methods yielded equal total amounts of HCV RNA. The RNAzol A method of Chomczynski and Sacchi (1987) and the two commercially available methods based on adsorption to silica (RNA extraction reagents from the HIV-1 NASBA kit, Organon Teknika; and QIAamp Viral RNA Kit, Qiagen). Although the reverse transcription of 5µl of RNA extracted using RNAzol A resulted in the production of five times as many cDNA molecules as the silica based methods this discrepancy could easily be explained because the optimal elution volume was 50µl compared with the 10µl of water used to resuspend the RNA following the RNAzol A method. The elution volume of the oligonucleotide capture protocol was also high (50µl) but the amount of RNA that this method yielded varied suggesting that it may be unsuitable for use in accurate quantification assays. Most of the methods described were more rapid than the RNAzol A protocol, with the direct addition of serum as described by Prince being the most rapid (15-30 minutes) and none of the methods required more than 3 hours of 'hands on' time. The results of these experiments are summarised in Table 1.

Table 1: A Summary of RNA Extraction Protocols

Method	Efficiency Relative to RNAzol A[1]	Time Taken	Comments
RNAzol A	100%	3 hours 'hands on' plus overnight precipitation	Current 'in house' method
Simple Guanidinium Method Young *et al. (1993)*	5-10%	3 hours	Large volume required to resuspend RNA (1ml).
Prince's Method	0-0.1%	15-30 minutes	Efficiency improved by boiling serum mixture.
Silica Adsorption (Organon Teknika)	20%	2 hours	Large volume required for elution, because of space taken by silica.
Silica Adsorption (QIAamp Viral RNA Kit)	1-20%	2 hours	Elution inefficient in small volumes.
Ultrafiltration	0-1%	>2 hours	Rates of filtration vary from serum to serum.
Oligonucleotide Capture	1-10%	2 hours	Method from van Doorn
Caesium Chloride Method (Original)	1%	1 hour	Longer spin would cause deformation of CsCl cushion
Caesium Chloride Method (with precipitation)	10%	2 hours	Precipitation increases efficiency.
Catrimox 14	0.1%	2 hours	

[1] Efficiencies based on the signal generated by QRT-PCR, relative to RNAzol A, when 5µl of RNA solution was added to the reverse transcription reaction.

Conclusions

Sensitive and reproducible RT-PCR assays are dependent upon the efficient extraction of highly purified RNA. A wide variety of RNA extraction protocols were performed and were compared with reference to the ice cold acid guanidinium thiocyanate protocol (RNAzol A) of Chomczynski and Sacchi (1987) on the basis of efficiency, reproducibility and potential throughput. None of the methods evaluated produced as high a concentration of extracted RNA as the RNAzol A method and although this method requires a long precipitation stage this can be performed overnight. It has been reported that this method fails to remove heparin from plasma samples which can inhibit the PCR. Methods based upon silica particles (Boom *et al.*, 1990; Organon Teknika HIV-1 NASBA Kit) may be more suitable when heparinised plasma is the analyte. The loss of sensitivity observed here resulting from elution volumes in excess of 10µl, could perhaps be corrected by adapting the reverse transcription protocol to allow for a higher volume of RNA to be added.

2.3 Reverse Transcription Methods

2.3.1 Moloney Murine Leukaemia Virus Reverse Transcriptase (MMLV-RT).

Introduction

PCR is a method for DNA amplification, in order to assay for RNA it must first be converted to cDNA. This process is called reverse transcription and can be performed using enzymes purified from retroviruses. MMLV reverse transcriptase has been widely used as a reverse transcriptase for RT-PCR and is reported to have an efficiency of approximately 10% (Simmonds *et al.,* 1990b). This section of the thesis describes attempts to calculate reverse transcription efficiency and to assess the effect of using antisense oligonucleotide PT2 (Table 5) to prime this process in place of random hexamers.

Methods

Reverse transcription was performed in a 20µl reaction containing 5µl of RNA solution, 9.06µl H_2O, 1µl 100mM HEPES (pH 6.9), 0.5µl of 200µM random hexamers (Pharmacia), 1µl 1M Tris.Cl (pH 7.5), 1.5µl 1M KCl, 0.24µl 250mM $MgCl_2$, 0.2µl 1M DTT, 0.4µl dNTP mix (25mM each dATP, dCTP, dGTP, dTTP), 0.6µl RNAguard (40 units/µl Pharmacia) and 0.5µl Moloney murine leukaemia virus reverse transcriptase (MMLV-RT, RNase H minus, 200 units/µl, Promega). After incubating for 90 min at 37°C the reaction mix was boiled for 5 min then cooled rapidly on ice. The concentration of cDNA was measured using QRT-PCR as described in Appendix II. The efficiency of reverse transcription with random hexamers was estimated by comparing the number of cDNA molecules generated from a known number of SP6 RNA transcripts (kindly provided by Dr. P. Lelie, Netherlands Red Cross Blood Transfusion Service).

Reverse transcription can also be performed using a specific oligonucleotide primer, 0.5µl of 0.75mM primer PT2 (antisense oligonucleotide complementary to the

5'NCR, see Table 5 for sequence; Garson *et al.*, 1991a) in place of the random hexamers. The relative reverse transcription efficiency with random hexamers or the specific oligonucleotide were compared by measuring the amount of cDNA produced from RNA, extracted from several dilutions of citrate plasma containing HCV using the RNAzol A method. This was achieved by applying the QRT-PCR method described in Appendix II.

Results

1.25×10^4 SP6 transcripts yielded a signal equivalent to 1.08×10^3 molecules of cDNA using random hexamers. This result suggests that this method has a reverse transcription efficiency of 8.6%.

The number of cDNA molecules generated from citrate plasma controls using random hexamers to prime cDNA synthesis was between 2.2 to 4.4 fold lower than was generated using primer PT2 (Table 2). The efficiency of reverse transcription using primer PT2 is presumably between 2.2 and 4.4 times that of random hexamers (i.e. between 19 and 38%).

Table 2: A Comparison of Reverse Transcription Primed Using Oligonucleotide PT2 with Random Hexamers

cDNA Molecules Generated Using Directed Reverse Transcription (PT-2)	cDNA Molecules Generated Using Random Primed Reverse Transcription	Ratio (directed : random)
1132	518	2.2:1
500	209	2.4:1
154	39	4.4:1

Conclusions

'Nested' PCR protocols that have been well optimised are able to detect single intact molecules of DNA (Simmonds *et al.*, 1990a). The maximum sensitivity of RT-PCR methods must therefore be limited by the efficiency with which cDNA is generated

following RNA extraction. Section 2.2 of this thesis describes attempts to select the most efficient method of RNA extraction. The data presented in Table 2 suggest that a 2 to 4 fold increase in reverse transcription efficiency and therefore in assay sensitivity, can be gained by using PT2 in place of random hexamers to prime cDNA synthesis of HCV RNA. The observation that specific priming reduces the level of non-specific amplification may in part explain the increase in sensitivity (Baier *et al.*, 1993). The variation in efficiency observed may be due to inaccuracies in measurement, but apparently show an increase in efficiency with reduction in RNA concentration. The QRT-PCR used to estimate the cDNA concentrations has been shown to be able to resolve differences of as little as 3 fold. It is also possible that an increasing ratio of specific primer to target might provide a greater increase in reverse transcription efficiency. Alternatively, an increase in the ratio of random hexamers to target might result in the binding of increasing numbers of primers to the template RNA, thereby increasing the chance that more than one hexamer will bind within the target sequence, resulting in the production of cDNA molecules shorter than the distance between the PCR primers and therefore unamplifiable. Therefore the relative efficiencies of random hexamer primed and PT2 primed cDNA synthesis may vary with the primer concentrations.

2.3.2 Detection of Negative Strand HCV RNA

Introduction

Single-stranded RNA viruses whose genomes serve as messenger RNAs are known, by convention, as positive (or plus) strand RNA viruses. These positive strand RNA viruses are then sub-divided into viruses coding for a single genome-sized mRNA (which is translated into a single polyprotein) and viruses that are able to make subgenomic mRNAs. Members of the picorna- and flaviviruses, including HCV, fall into the former category. Their genomic RNAs act as templates for the synthesis of complementary full-length minus (-) strand RNAs by the virally encoded polymerase and these minus strand RNAs in turn serve as templates for the production of progeny genomic RNAs. It has therefore been considered reasonable to rely on the

detection of minus strand HCV RNA as a marker of viral replication. This does however depend upon two assumptions being correct. Firstly, the virus must package only positive strand HCV RNA and secondly the method used for detection must be entirely specific and not indicate the presence of minus strand RNA in its absence. Much of the technology designed to specifically detect minus strand RNA has relied on the stringent hybridisation of an oligonucleotide primer complimentary to the negative strand of HCV genomic RNA and its exclusive use by a reverse transcriptase to synthesise the appropriate cDNA.

This section of the thesis describes attempts to determine whether the QRT-PCR, as described in Appendix II, can be adapted to specifically detect negative strand RNA in cultured cells by simply switching the oligonucleotide used to prime cDNA synthesis from the antisense primer PT2 to the sense primer PT1 (Table 5).

The lymphocyte and human embryonic lung cells were grown by the diagnostic virology laboratory (UCLMS) and supplied by G. Patel. The hepatoma cell line was cultured by D. Lubach (also UCLMS).

Methods

Cell Culture and RNA Extraction

Hepatoma cell line HUH7 was grown as a monolayer in RPMI 1640 medium (Gibco BRL) containing 10% heat-inactivated foetal calf serum (FCS). Suspension cultures of the lymphocyte line J-JHAN were grown in RPMI 1640 medium (Gibco BRL) containing 10% heat-inactivated FCS. A human embryonic lung cell line (HEL) was grown as a monolayer in MEM (Gibco BRL) containing 10% heat-inactivated FCS. All media contained 100 units/ml penicillin G, 100 µg/ml streptomycin and 2.5µg/ml fungizone to inhibit bacterial and fungal growth. Cultures were maintained at 37°C in a 95% air/5% CO_2 atmosphere in 25 cm^2 tissue culture flasks. The culture flasks for growing the hepatocyte line were coated with collagen type 1 and fibronectin (Sigma).

The lymphocytes were grown to a density of 10^6 cells per ml, and 1ml centrifuged at 1500 rpm, the supernatant was removed and stored and the cells were lysed in 400µl of RNAzol A solution. HEL cells and hepatocytes were grown to confluence, the supernatant was removed and stored and 10^6 cells were lysed in 400µl of RNAzol A solution.

200µl of HCV RNA negative human citrate plasma, negative human serum and the cell culture supernatants, were each added to 400µl of RNAzol A solution. All the cell pellets, culture supernatants, plasma and serum samples were spiked with 10µl of citrate plasma containing approximately 7 x 10^3 HCV RNA genomes. RNA was extracted by the method of Chomczynski and Sacchi.

cDNA Synthesis, PCR Amplification and Quantification

Each RNA sample was divided into two 5µl aliquots which were reverse transcribed either using the specific primer PT2, as described in Appendix II, or with the same reverse transcription conditions except that an equivalent volume of water replaced primer PT2. The reverse transcription reaction products were amplified by PCR using *Pfu* DNA polymerase as described in Section 2.4.1. Quantification of PCR products was achieved using the ELONA technique (Appendix II).

In addition to the samples described above, a high titre quantification standard was also extracted along with 4 negative plasma controls. The quantification standard was used to generate a standard curve against which the titre of all other samples were compared.

Results

The estimated number of cDNA molecules generated during reverse transcription with and without PT2 is shown in Table 3. Rather surprisingly cDNA synthesis progressed in the absence of the reverse transcription primer in all but one of the samples tested. The level of cDNA produced in the absence of PT2 (mean 69.5, SD 37.4) was lower than that produced using the specific primer (mean 174, SD 64). The

smallest amount of cDNA generated in the absence of PT2 was produced from spiked serum (0 cDNA molecules/sample) and the largest from the supernatant taken from the cultured hepatocytes (112 cDNA molecules/sample).

Table 3: A Comparison of cDNA Synthesis in the Presence and Absence of a Specific Primer

	Reverse Transcription with PT2	Reverse Transcription without PT2
Negative Plasma	100	48
Negative Serum	144	0
HUH 7 Cells	308	48
HUH 7 Supernatant	204	112
HEL Cells	180	100
HEL Supernatant	188	92
J-JHAN Cells	136	60
J-JHAN Supernatant	132	96

Conclusions

The use of negative strand detection as a marker of viral replication has been widely used (Fong *et al.*,1991; Sherker *et al.*, 1993). Demonstrating the specificity of the methods used for detecting this marker is essential in providing convincing results. The fact that HCV RNA could be efficiently reverse transcribed in the absence of a specific cDNA synthesis primer in all cell pellets, supernatants and in negative human citrate plasma clearly demonstrates the unsuitability of this assay in its current format for strand specific PCR.

Several theoretical reasons exist to explain how reverse transcription can occur in the absence of a specific primer. Extracting total RNA from cellular material will result in the co-purification of cellular RNA and degraded DNA along with the HCV RNA; this cellular nucleic acid, in the relatively low stringency conditions used here (37°C and 75mM [K^+] for 90 minutes), may prime reverse transcription non-specifically. Cellular RNAs are known to spontaneously form hairpin structures which can prime cDNA synthesis (Sambrook *et al.*, 1989). The 5' NCR of the HCV genome has a highly folded structure (Brown *et al.*, 1992) it is therefore a reasonable assumption

that the extracted HCV RNA may be able to prime itself. During active viral replication flaviviruses are known to produce excess negative strand RNA, it is possible that HCV is able to package some of this RNA, either in place of its genomic RNA within the viral core, or in addition to genomic RNA between the core and viral envelope (Shindo *et al.,* 1994).

The increased variation in reverse transcription efficiency observed in the absence of a specific primer (with PT2, CV = 36.8%, without PT2 CV = 53.8%) may suggest that degraded cellular nucleic acids are priming the reverse transcription reaction. However, this data could also be interpreted as providing evidence that cellular factors inhibit the formation of hairpin structures to varying degrees. The fact that no detectable HCV specific cDNA synthesis was observed in RNA extracted from spiked serum in the absence of PT2, also suggests that either this nucleic acid does not contain sufficient cellular material for non-specific priming, or that serum in some way inhibits the formation of self priming hairpin structures.

In order for QRT-PCR to be used to detect the presence of negative strand HCV RNA in cellular material, the reverse transcription stage of the protocol requires significant modification. An increase in the stringency of hybridisation could be achieved by performing the reverse transcription reaction at elevated temperatures, although this would lead to the rapid degradation of Moloney murine leukaemia virus reverse transcriptase at least two more thermostable enzymes exist. Avian myoblastoma virus reverse transcriptase is able to synthesise cDNA at 45-50°C thereby providing greater stringency of primer hybridisation, as well as destabilisation of the secondary structure of the 5'NCR. Reverse transcription can be performed at temperatures in excess of 60°C by using *Tth* DNA polymerase which also possesses reverse transcriptase activity. Alternatively, or in addition to the specificity gains achieved using elevated temperatures, even greater specificity has been achieved by using 'tagged' primers (Mellor *et al.,* 1998). This approach uses a specific reverse transcription primer with a 5' oligonucleotide 'tag'. Strand specificity is ensured by performing the PCR with only one virus specific primer, the second primer binds to the oligonucleotide 'tag'.

2.4 Quantitative Reverse Transcription PCR (QRT-PCR)

2.4.1 Optimisation of QRT-PCR Parameters.

Introduction

Although the isolation of *Taq* DNA polymerase represented such a major step in the development of PCR that it soon established itself as the enzyme of choice for all PCR assays, there now exists a wide variety of other thermostable DNA polymerases, each with their own properties and optimal reaction conditions. This section of the thesis describes the development and optimisation of PCR parameters for the purpose of quantifying HCV RNA in clinical samples efficiently and accurately.

Methods

Taq DNA Polymerase

Using the 5' NCR primer set PT1-4 (Table 5; Appendix I; Garson *et al.*, 1991a) it is possible to generate consistent amounts of PCR product (assessed by ethidium bromide stained agarose gel electrophoresis). Using this nested primer set it is possible to detect a single cDNA molecule. In order to make this assay quantitative it was necessary to change the cycling parameters so that the amount of PCR product generated correlated with the amount of cDNA added to the reactions. Instead of generating cDNA, a dilution series of PCR product generated from patient cDNA, using primers NCR1 and NCR2 (Garson *et* al., 1990b), was used. These primers generate a product spanning almost the entire 5'NCR. A ten-fold dilution series of this product was subsequently used as template during the PCR optimisation. Once optimised the PCR reactions could then be performed using cDNA dilution series.

Nested and non-nested formats were tested using primers PT 1-4 (Table 5) either unmodified or modified by 5' end labelling with biotin or dinitrophenol (DNP), or by incorporation of the recognition sequence of the double stranded binding protein

GCN4 (Kemp *et al.*, 1989) to enable product capture. A Techne PHC-3 thermocyler was used to perform these experiments and *Taq* DNA polymerase was obtained from Perkin Elmer. The cycle number was varied from 20-40 in the first round and from 0-35 in the second round. Denaturation was performed at 94 or 95°C for 1 min; annealing was varied from 50 to 55°C for either 1 min or 30 s. Extension was assumed to occur whilst the temperature was rising from the anneal to the denaturation temperature (N.B. the PCR product is only 60 bp in length and therefore requires only a few seconds for extension).

Pfu DNA Polymerase

Pfu DNA polymerase has higher thermostability a higher thermofidelity than *Taq* DNA polymerase and should therefore theoretically be able to produce higher concentrations of PCR product. A ten fold dilution series of cDNA was amplified either by *Taq* or *Pfu* enzymes using the following reaction conditions: initial denaturation 94°C for 4 minute once; then denature at 94°C for 1 minute, anneal at 50°C for 1 minute and extend at 72°C for 10 s, cycling 35 times; a final extension of 72°C for 4 min.

A wide range of PCR conditions were investigated in an attempt to reduce PCR plateau and to maximise sensitivity and dynamic range. The cycle number was varied from 30-40 cycles, the anneal temperature from 50-55°C, the extension time from 0-10 s and the temperature from 72-75°C.

Results

Using 'nested' PCR for HCV quantification produces shallow product concentration gradients. Only faintly visible bands (by ethidium bromide stained agarose gel electrophoresis) were produced following a single round of PCR with *Taq* polymerase.

A single round of PCR with *Pfu* DNA polymerase generated bands visible by ethidium bromide stained agarose gel electrophoresis, even with an initial template input of 10 DNA molecules. Less intense bands were generated by *Taq* DNA polymerase after the same number of reaction cycles (Figure 9).

Figure 9: Single round PCR products using *Taq* or *Pfu* Polymerase.

Pfu polymerase (top row) and *Taq* polymerase (bottom row) performed using an identical cDNA dilution series, 35 cycles of amplification with 2U of enzyme per reaction. Lane 1: Marker Φx174 *Hae* III; Lanes 2-6 contain reaction products using 10,000, 1,000, 100, 10 and 1 molecules of HCV cDNA; Lane 7 is a negative control. The arrows indicate the position of the specific 60bp band.
A = 60bp products generated using *Pfu* DNA polymerase
B = 60bp products generated using *Taq* DNA polymerase

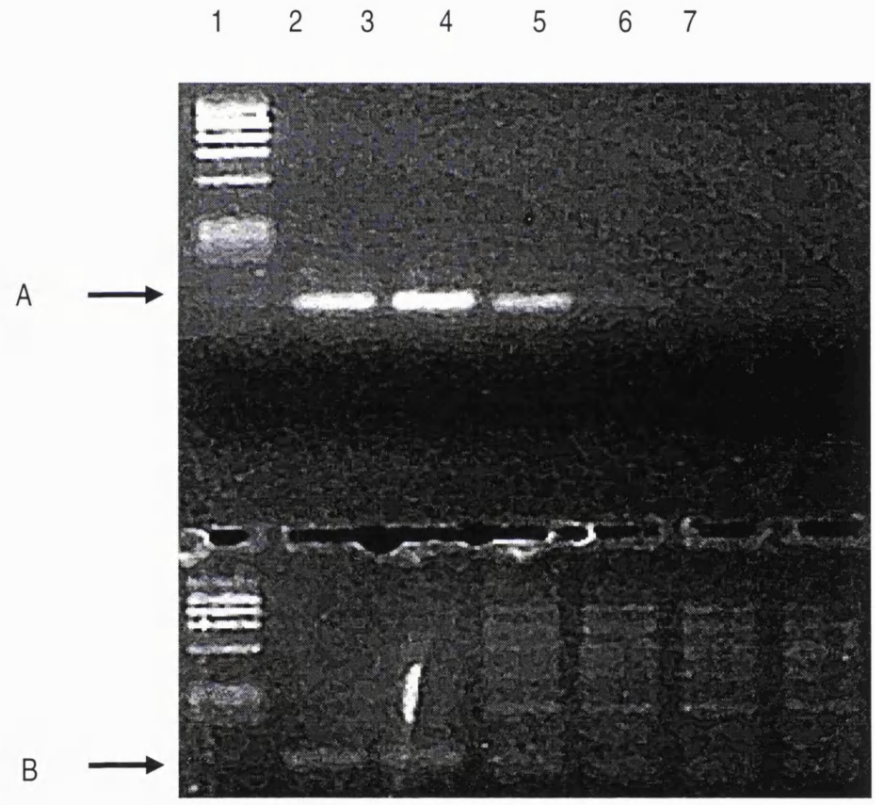
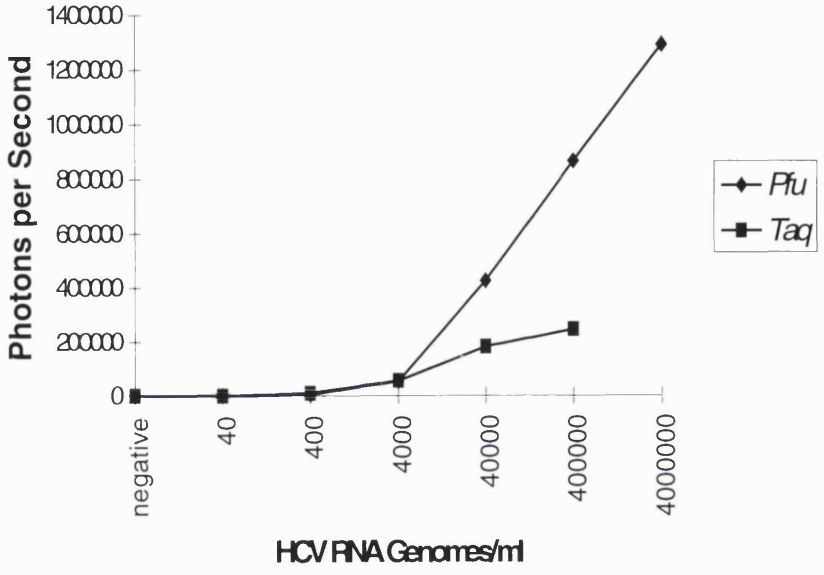


Figure 10: A comparison of signals generated by Enzyme Linked Oligonucleotide Assay (ELONA) from the PCR products shown in Figure 9.

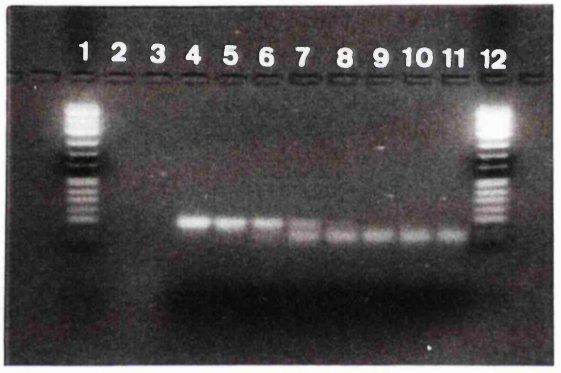
(See Appendix II).



Optimum results were obtained with a single round of 35 cycles (94°C/1min, 53°C/30sec, 75°C/2sec, final 7min extension at 75°C), 100ng each of primers PT3BIO and PT4 (Table 5) per 50µl reaction and 2 units of Exo$^-$ *Pfu* DNA polymerase (Appendix II; and Figure 11).

Figure 11: Standard Curve Dilution Series.

3% agarose gel.
Lanes 1 and 12 Marker pBR322 *Hae* III;
Lanes 2 and 3 are empty;
Lanes 4-8 contain a dilution series (10^{-3}-10^{-9}; equivalent to 10^{6}-1 molecules) of a PCR product generated using primers NCR1 and NCR 2 (Table 5);
Lanes 9-11 contain negative control samples.



NB: Bands visible in the lanes containing negative controls (9-11) are primer artifact. Primer artifacts are also visible in lanes containing products amplified from small numbers of cDNA molecules (lanes 5, 6 and 7).

2.4.2 Quantification of PCR Products.

Methods

PCR Product Capture Techniques

A variety of black and white 96 well microtitre plates from several different manufacturers (Dynatech, Nunc Maxisorb, Labsystems) were tested for binding capacity and for level of non-specific background luminescence. For capture of biotin-labelled PCR products, avidin and streptavidin coatings were compared (Sigma; native and recombinant). The coating buffer used was 0.5M carbonate/bicarbonate buffer (pH 9.6), or 10mM Tris.Cl (pH 7.6) or phosphate buffered saline (PBS). Alternatively, products labelled with dinitrophenol (DNP) were captured on microtitre plates coated with monoclonal anti-DNP antibody (kindly provided by J Grzybowski, Oswel DNA Services). Microtitre plates pre-coated with the double stranded DNA-binding protein GCN4 (British Biotechnology Ltd.) were used for the capture of PCR products generated with primer PT3 with a GCN4 recognition sequence (Table 5; Kemp *et al.*, 1989). Hybridisation capture of DNP-labelled PCR products (generated with PT4DNP) was also investigated, using a biotinylated 20 mer oligonucleotide (INT2BIO). This oligonucleotide (Table 5) was linked to the solid phase via streptavidin, either before or after hybridisation with the DNP-labelled PCR product.

Methods Evaluated for the Detection of Captured PCR Products

Although double stranded PCR products, labelled at one end with biotin or the GCN4 recognition sequence (for capture onto the solid phase) and at the other end with DNP, can be detected directly, greater specificity is obtained by the addition of a hybridisation step. Prior to hybridisation, denaturation of the double stranded product was achieved either by heating (95°C/10 min) or by treatment with sodium hydroxide (100-200mM). Hybridisation was performed using internal oligonucleotide probes (Table 5) labelled at the 5' end with either biotin (INT1BIO), DNP (INT1DNP), xanthine oxidase (INT1XO) or alkaline phosphatase (INT1AP). A

wide range of hybridisation conditions was tested with each of these labelled probes. For detection of INT1BIO after hybridisation, a xanthine oxidase streptavidin conjugate (provided by Dr A Baret) was employed. INT1DNP detection was performed by sequential incubations with monoclonal anti-DNP followed by either rabbit anti-mouse IgG enzyme conjugate [horseradish peroxidase (HRP; Dako), alkaline phosphatase (Sigma), or xanthine oxidase (Dr A Baret)] or staphylococcus protein A alkaline phosphatase conjugate (Sigma). All antibody, protein A and streptavidin conjugates were incubated (at the concentrations recommended by the supplier) for one hour at 37°C in PBS containing 0.1% $Tween_{20}$ and either 1% bovine serum albumin (BSA) or 5% casein (Cambridge Research Biochemicals Ltd.) as blocking agents.

Chemiluminescent substrates appropriate for each of the different detector enzymes were employed. For the horseradish peroxidase system a luminol-based substrate was used (Amersham ECL™ reagent). With xanthine oxidase conjugates the hypoxanthine/luminol/Fe^{++}/EDTA reagent described by Fert and Baret (1990) was selected. Four different dioxetane-based substrates (AMPPD, CSPD, $Lumiphos^{480}$, $Lumiphos^{530}$) were used with alkaline phosphatase conjugates (Beck and Köster, 1990). AMPPD and CSPD were obtained from Tropix Inc. Both $Lumiphos^{480}$ and $Lumiphos^{530}$ (Lumigen Inc.) contain cetyltrimethylammonium bromide miscelles; $Lumiphos^{530}$ uses fluorescein isothiocyanate packaged in miscelles to enhance the luminescent signal. Table 4 summarises the various formats employed for the detection of immobilised PCR products. Light output was measured using an automated microtitre plate luminometer (TopCount, Canberra Packard Inc.) at room temperature, in single photon counting (SPC) mode for 6 s/well.

Table 4: Summary of PCR Product Detection Protocols

No.[1]	Probe	Layer 1	Layer 2	Substrate	Typical[2] S:N Ratio /Single Molecule	Stable Light Output	Assay Duration[6]	Dynamic Range	Comments
1	INT1-DNP	MAb[3] anti-DNP	anti-mouse IgG HRP[4] conjugate	ECL[tm] Amersham	NA[5]	3-4 mins	4 hours	NA	Poor reproducibility due to short duration of stable light output
2	INT1-DNP	MAb[3] anti-DNP	anti-mouse IgG XO conjugate	Hypo-xanthine/ Luminol/ Fe:EDTA	2:1	>24 hours	4 hours	3-4 $\log_{10}$	Reduced dynamic range due to signal variation
3	INT1-BIO	Strept-avidin XO	none	Hypo-xanthine/ Luminol/ Fe:EDTA	NA	>24 hours	3 hours	NA	Unacceptably high backgrounds
4	INT1-DNA	MAb[3] anti-DNP	anti-mouse IgG AP conjugate	AMPPD or CSPD	NA	1 hour	4.5 hours	NA	Without enhancers dioxetane substrates are insufficiently sensitive
5	INT1-DNP	MAb[3] anti-DNP	anti-mouse IgG AP conjugate	Lumiphos-480	2:1	1hour	5 hours	4 $\log_{10}$	High sensitivity, wide dynamic range but two antibody incubations required
6	INT1-DNP	MAb[3] anti-DNP	Protein A Alk Phos conjugate	Lumiphos-480	4:1	1hour	5 hours	4 $\log_{10}$	As protocol 5 but lower backgrounds
7	INT1-AP	none	none	Lumiphos-530	6:1	1hour	2.5 hours	4 $\log_{10}$	High sensitivity, wide dynamic range, short assay duration
8	INT1-XO	none	none	Hypo-xanthine/ Luminol/ Fe:EDTA	NA	>24 hours	2.5 hours	NA	Inadequate sensitivity

[1]Protocol number
[2]Typical signal to noise ratio obtained with single molecule cDNA input.
[3]MAb = monoclonal antibody
[4]HRP = horseradish peroxidase
[5]NA = not applicable
[6]Time from end of PCR to luminometer reading

Table 5: Oligonucleotide Sequences

Designation	Sequence[1]	Location[2]/Orientation	Function
PT1	5'CGTTAGTATGAGTGTCG TGC3'	90 - 109 / sense	amplification
PT2	5'CGGTGTACTCACCGGTT CC3'	171 - 153 / antisense	amplification
PT3	5'AGTGTCGTGCAGCCTCC AGG3'	100 - 119 / sense	amplification
PT3BIO	biotin-5'AGTGTCGTGCAGC CTCCAGG3'	100 - 119 / sense	amplification and capture via biotin moiety
PT3GCN4[3]	5'GGATGACTCAAGTGTCG TGCAGCCTCCAGG3'	100 - 119 / sense	amplification and capture via GCN4 recognition sequence
PT4	5'CGGTTCCGCAGACCACT ATG3'	159 - 140 / antisense	amplification
PT4DNP	DNP-5'CGGTTCCGCAGAC CACTATG3'	159 - 140 / antisense	amplification and detection
NCR1[4]	5'GTAT*CTCGAG*GCGACA CTCCACCATAGAT3'	19 - 38 (from base 10 of primer) / sense	amplification
NCR2[4]	5'ATA*CTCGAG*GTGCACG GTCTACGAGACCT3'	341 - 322 (from base 9 of primer) / antisense	amplification
INT2BIO	biotin-5'CCCCCTCCCGGGA GAGCCAT3'	123 - 142 / sense	hybridisation capture of PT4DNP-generated products
INT1BIO	biotin-5'ATGGCTCTCCCGG GAGGGGG3'	142 - 123 / antisense	hybridisation and detection
INT1DNP	DNP-5'ATGGCTCTCCCGG GAGGGGG3'	142 - 123 / antisense	hybridisation and detection
INT1XO[5]	xanthine oxidase-5'ATGGCT CTCCCGGGAGGGGG3'	142 - 123 / antisense	hybridisation and detection
INT1AP	alkaline phosphatase-5'ATGG CTCTCCCGGGAGGGGG3'	142 - 123 / antisense	hybridisation and detection

[1]Oligonucleotides obtained from Oswel DNA Service, Edinburgh, unless otherwise indicated.
[2]Numbered according to Han *et al.*, [1990].
[3]Underlined portion of primer PT3GCN4 represents the recognition sequence of the DNA-binding protein GCN4 [Kemp *et al.*, 1989].
[4]Artificial restriction sites introduced into primers NCR1 and NCR2 to facilitate cloning of PCR products are shown in italics.
[5]Primer INT1XO provided by G Mock, British Biotechnology Ltd.

Results

Assay Format Optimisation and Details of the Optimised Protocol

Of the various microtitre plates tested, Nunc Maxisorb (black) gave the highest signal to noise (i.e. background) ratio. Optimum performance was achieved with native streptavidin, 100µl per well (5µg/ml), coated overnight at 4°C in 0.05M carbonate/bicarbonate buffer (pH 9.6). Following coating, plates were blocked by incubation for 1 hour at 37°C with 400µl PBS containing 10% casein blocking buffer (casein was found to block more effectively than BSA) and 0.01% thiomersal as a preservative. Blocked plates were stable at 4°C for up to 6 months.

Prior to the addition of 5µl of biotin-labelled PCR products to the streptavidin-coated wells, the blocking buffer was removed and replaced with 95µl of sample diluent buffer (PBS/5% casein/0.5% $Tween_{20}$). After adding the labelled-products the plates were incubated for 1 hour at 37°C. Denaturation of the immobilised double stranded DNA was most efficiently achieved by treatment with sodium hydroxide (150mM) for 2 minutes at room temperature, followed by aspiration and washing with TTA buffer (10 mM Tris.Cl [pH 7.5], 0.05% $Tween_{20}$, 0.1% sodium azide) to remove the non biotinylated second strand. Of the various protocols evaluated for detection of the captured single stranded product, protocol 7 (Table 4) provided the best performance in terms of sensitivity, reproducibility, dynamic range and assay duration. The characteristics of protocol 7 and of the alternative detection protocols are summarised in Table 4 (also Figures 12 and 13).

Detection protocol 7 was performed as follows:- 100µl of INT1AP, diluted 1:500 (optimum dilution determined empirically for each new batch of conjugate) in hybridisation buffer (10 x SSC, 5% casein, 0.5% $Tween_{20}$) were added to each well and incubated for 30 minutes at 37°C. After aspiration of the labelled probe the wells were washed 10 times with TTA buffer. Finally, 100µl of the substrate Lumiphos^{530} was added per well and the plate incubated for 1 hour at room temperature. Light output was measured as described in the Methods (Section 2.4.2).

Figure 12: A Schematic Diagram Illustrating ELONA Protocol 2

Biotinylated PCR products are captured onto streptavidin coated black microtitre plates. The unbiotinylated strand is removed by denaturation in 0.15M NaOH and washing. A DNP labelled oligonucleotide probe is hybridised to the captured strand and then incubated in the presence of a DNP reactive monoclonal antibody. A xanthine oxidase conjugated anti-mouse monoclonal is bound to the first antibody layer and subsequently detected using a chemiluminescent substrate.

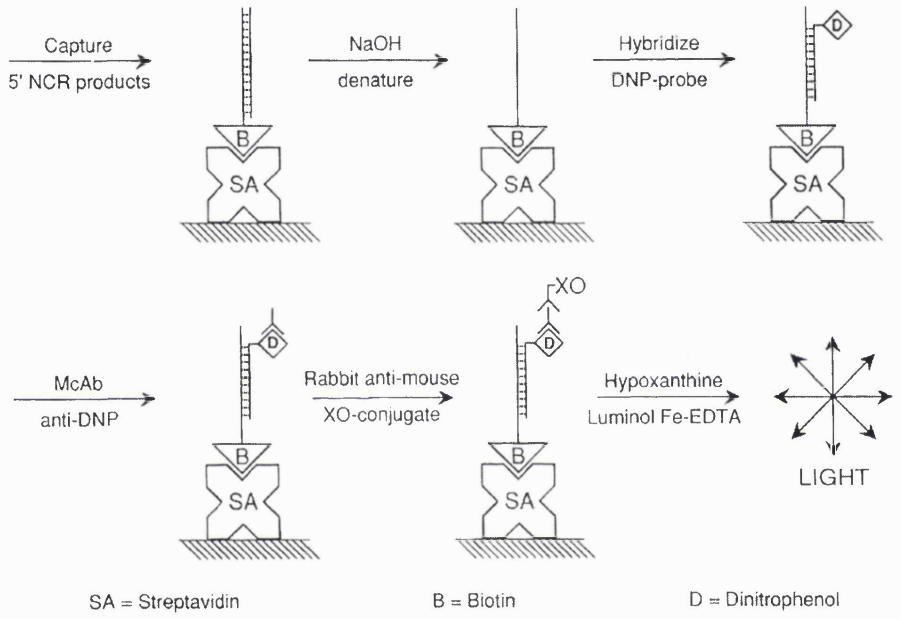
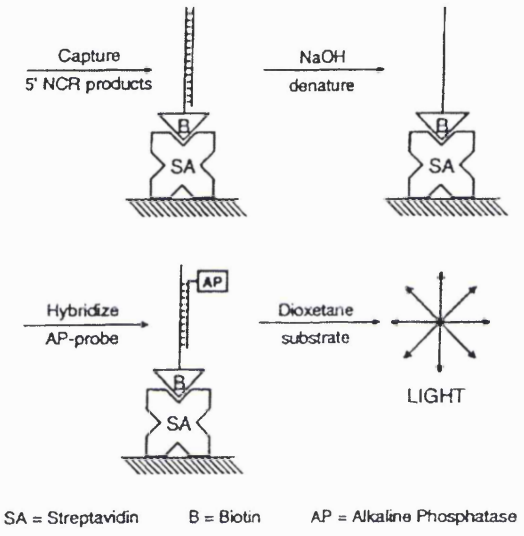


Figure 13: A Schematic Diagram Illustrating ELONA Protocol 7

Biotinylated PCR products are captured onto streptavidin coated black microtitre plates. The unbiotinylated strand is removed by denaturation in 0.15M NaOH and washing. A alkaline phosphatase conjugated oligonucleotide probe is hybridised to the captured strand and then incubated in the presence of chemiluminescent substrate.



Results Obtained with the Optimised Protocol

i) Sensitivity, Specificity and Dynamic Range

Samples of high dilutions of HCV cDNA which had been shown by the Poisson distribution method to contain (on average) a single molecule, were detectable using the optimised protocol at the predicted frequency. The signal to noise ratio generated by such samples, when positive, was typically around 6:1 (range 3:1 - 18:1). The ability to detect single molecules of HCV cDNA implies a sensitivity equivalent to approximately 100 - 400 HCV genomes/ml of serum. This figure is derived by taking into account the volume of sample analysed, the proportion of the RNA used for the cDNA synthesis, the proportion of the cDNA used for the PCR and the 9 - 38% efficiency of the reverse transcription reaction. All 128 anti-HCV positive serum samples (from the diagnostic virology laboratory, UCLMS) found to contain HCV RNA by non-quantitative nested PCR (Garson *et al.*, 1991a) were also PCR positive when analysed by the quantitative assay.

Table 6: 141 Anti-HCV Positive Sera Analysed by Both Nested PCR and QRT-PCR Assays

		Non-quantitative "nested" PCR	
		+	−
Quantitative PCR (protocol 7)	+	128	0
	−	0	13

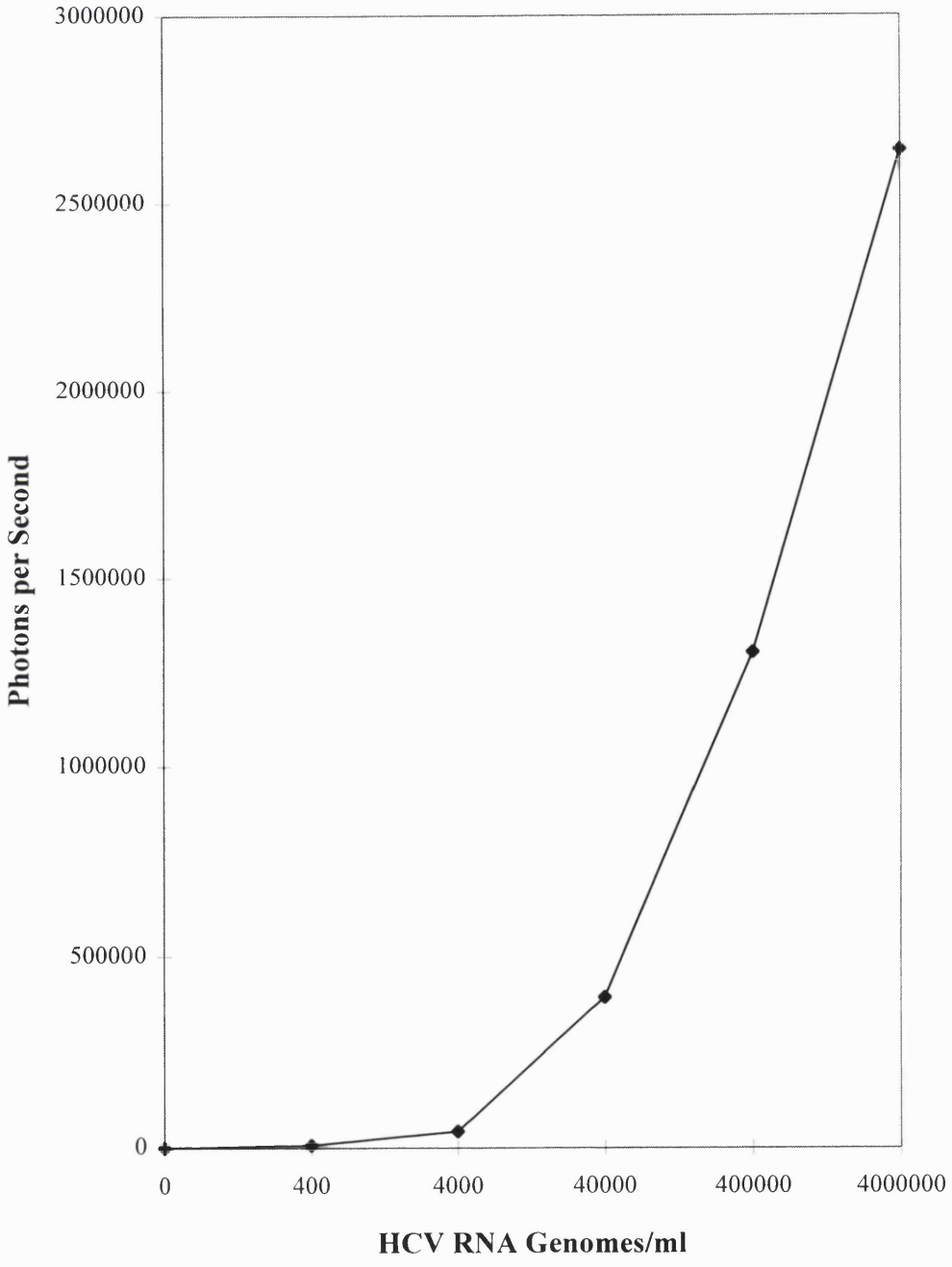
Sera from normal blood donors, from HIV-infected and from HBV infected individuals failed to generate any signal in the assay. Table 6 shows the results of testing 141 anti-HCV positive sera by both the non-quantitative nested PCR and the quantitative chemiluminescent system described here. There was complete concordance between the results of the two assays. The specificity of the detection system for HCV sequences was also tested by adding biotin-labelled HIV PCR

products to the streptavidin coated plates. Specificity was maintained even in the presence of high levels of such non-complementary biotin-labelled DNA.

The dynamic range of the quantitative assay using the optimised detection protocol was approximately 4 $\log_{10}$. A typical standard curve is illustrated (Figure 14) and shows the relationship between light output and HCV RNA input concentration. The size and shape of the standard curve was essentially the same irrespective of whether the ten fold dilution series was made with serum, with RNA, with cDNA or with NCR1/NCR2 generated PCR product.

Figure 14: A Typical Standard Curve Generated by RT-PCR and ELONA

This figure shows the relationship between light output generated by the ELONA and the initial HCV RNA concentration in a 10 fold dilution series following amplification using the QRT-PCR protocol (Appendix II).



Assay Reproducibility

Coefficients of variation (cv) were calculated for the product detection stage alone, for the PCR with the product detection stage and for the entire assay including cDNA synthesis and RNA isolation. Calculations were performed after conversion of the light output data into cDNA equivalents by use of MultiCalc interpolation software (Labsystems, Wallac Oy, Finland) and the standard calibration curve. Repeat testing (n = 6) of the same biotinylated PCR product gave a CV of 12% for the detection stage alone (mean 35.8 cDNA molecules, standard deviation 4.3). Repeat testing (n = 6) of the same cDNA preparation gave a CV of 19% (mean 38.6, SD 7.5). To test the reproducibility of the entire assay a 2µl aliquot of an anti-HCV positive serum was spiked into 198µl aliquots of serum from nine different anti-HCV negative blood donors. The results after conversion into cDNA molecule equivalents were 35, 41, 45, 21, 34, 42, 50, 31, 30 (CV 24%, mean 36.5, SD 8.9).

Discussion

PCR conditions were optimised to produce an assay capable of quantifying HCV RNA over a wide dynamic range without compromising sensitivity. A single round of 35 PCR cycles generated a detectable product with even a single cDNA molecule input, providing a level of sensitivity comparable with 'nested' PCR. These conditions produced increasing concentrations of product over a five $\log_{10}$ range of cDNA molecules, covering most of the 10^3-10^8 HCV genomes/ml seen in patient samples. Performing this reaction in a Perkin Elmer 9600 thermal cycler significantly increases the speed with which the assay can be performed and introduces the potential for automation because of its 96 well microtitre format.

Using chemiluminescent detection of an alkaline phosphatase conjugated oligonucleotide allows the quantification of biotinylated PCR products in a microtitre format hybridisation assay (enzyme linked oligonucleotide assay [ELONA]). Although classical hybridisation assays such as northern and Southern blotting take several days to perform, this method can be completed in 2-3 hours. ELONA has also been shown to add specificity to the PCR assays described, only

detecting the specific product, even in the presence of non-specific 'cold start' products, or large quantities of irrelevant target (e.g. biotinylated HIV-1 PCR product). The ELONA technique developed here has subsequently been successfully used in several other laboratories both in the United Kingdom and abroad. The technique has been used to quantify PCR products generated from HIV-1, HIV-2, Cytomegalovirus, HBV and hepatitis A virus as well as HCV and GBV-C.

In addition, the generation of results as numerical data allows the use of objective criteria for the definition of 'positive' and 'negative' results and simplifies the assessment of assay performance. This represents an advantage over the essentially subjective assessment of the presence or absence of bands on an ethidium bromide stained agarose gel used in qualitative PCR. The data presented in the preceeding section has been published in the Journal of Virological Methods (Whitby and Garson, 1995).

2.4.3 Quantification of PCR Products Using a Colourimetric ELONA.

Introduction

In order to expand the number of laboratories able to perform the ELONA technique beyond those in possession of a luminometer, it was thought helpful to develop a colourimetric version of this assay. The majority of diagnostic virology laboratories possess a spectrophotometer capable of reading mictotitre plates.

Methods

Transparent microtitre plates (Nunc Maxisorb) were coated with streptavidin (5µg/ml recombinant streptavidin [Sigma] in carbonate/bicarbonate Buffer pH 9.6) overnight at 4°C and then blocked for 1 hour at 37°C with 0.1% BSA, PBS and 0.01% sodium azide. Blocked plates were stored at 4°C with the wells filled with blocking buffer. PCR products were added to these plates, the second strand was removed with NaOH and the products were probed, all as per the standard ELONA protocol. The alkaline phosphatase labelled probe was detected using amplified chromogenic substrate as described by Self (1985).

A ten fold dilution series of HCV RNA was amplified using the reverse transcription 'hot start' PCR (RT-HS-PCR) method described in Section 2.5. The products generated were quantified by the standard chemiluminescent ELONA as described in Section 2.4.2 and Appendix II, or using the colourimetric method described above.

Results

Both the colourimetric ELONA and the chemiluminescent ELONA methods described above were able to detect PCR products from samples containing 40 HCV genomes/ml amplified using the RT-HS-PCR technique. In addition to the sensitive detection offered by these methods both produced signals that increased with the level of HCV RNA in the analyte, such that both methods are suitable as quantitative assays (Table 7).

Table 7: A Comparison of the Signals Generated by RT-HS-PCR Using Either Colourimetric or Chemiluminescent ELONA

HCV RNA genomes/ml	Colourimetric Substrate (Signal: Noise)	Chemiluminescent Substrate (Signal: Noise)
40	2.5:1	200:1
400	5.0:1	600:1
4000	10.0:1	1500:1
40,000	12.0:1	2100:1
400,000	15.0:1	2900:1

Conclusions

Routine diagnostic virology laboratories use microtitre format spectrophotometers to perform the majority of diagnostic tests. The adaptation of the ELONA described in Section 2.4.2 (protocol 7) from a chemiluminescent assay to a colourimetric assay would therefore allow many more laboratories to use the technique. The colourimetric version of the ELONA is able to detect PCR products at a concentration which produces a 200:1 signal to noise ratio using the standard ELONA technique. This does not result in a loss of sensitivity when the products are generated using the RT-HS-PCR because HCV RNA concentrations as low as 40 HCV genomes/ml can be amplified to levels detectable by the colourimetric ELONA. If colourimetric methods are to be used to detect products amplified using the QRT-PCR described in Section 2.4.1 then this method would require re-optimisation, as single molecules of cDNA typically yield only 6 times the background signal using chemiluminescence.

2.5 Increased Detection Sensitivity for Hepatitis C Virus: A Quantitative Single Tube Reverse Transcription 'Hot Start' PCR (RT-HS-PCR)

Introduction

Since the development of early serological assays to detect antibodies against HCV, the level of post transfusion hepatitis has decreased substantially. Following infection with HCV a 'window period' exists before seroconversion (Alter, 1989), during which time serologically negative but viraemic blood donations could be transfused. Although a specific antigen test for HCV has now been developed (Tanaka *et al.*, 1996) and alternative signal and nucleic acid amplification tests exist (Section 1.3.2, 1.3.3 and 1.3.4) RT-PCR remains the method of choice for the sensitive detection of HCV. RT-PCR has been shown to be a valuable method for detecting viral contamination of blood products purified from pooled plasma donations (Garson *et al.*, 1990c; Saldanha and Minor, 1994).

Due to the success of serological screening the chances of even a single contaminated donation being present in a plasma pool for fractionation is small and pooling often thousands of donations presents problems for RNA detection, even for a method as sensitive as RT-PCR. The range of viraemia seen in patient samples is 10^3 to 10^8 HCV genomes/ml with a median viraemia of 8.5 x 10^5 HCV genomes/ml (Brillanti *et al.*, 1991). Clotting factor concentrates are typically made using 10,000-20,000 units of plasma. A commercially available method for the detection of HCV RNA (Roche Amplicor, Hoffman La Roche) has a detection limit of 10^3 HCV genomes/ml. Extrapolating from this data, if only a single unit of plasma was contaminated in each plasma pool less than half would have detectable RNA levels by Roche Amplicor. It can be argued that an increase in the sensitivity of RT-PCR therefore has the potential to increase the safety of plasma products.

Methods

RNA Extraction

RNA was extracted from 200µl of plasma using the acid guanidinium thiocyanate method (Chomczynski and Sacchi, 1987; Appendix I and II) as described in Section 2.2. The RNA pellet was resuspended in 20µl of RNase free water. A standard curve was prepared by making serial ten-fold dilutions of RNA extracted from an 'in-house' quantification standard (Plasma A) of known titre (4 x 10^6 HCV genomes/ml). A ten-fold dilution series was also made of a proposed international standard supplied by the National Institute of Biological Standards and Controls (NIBSC, South Mimms, Hertfordshire, UK; Saldanha and Minor, 1996) containing 4 x 10^3 HCV genomes/ml.

Reverse Transcription 'Hot Start' PCR (RT-HS-PCR)

The reaction mix was prepared in two parts as follows: mix 1 contained 0.5µl of primer PT3BIO (Table 5;100ng/ml, Oswel DNA Services), 3µl 5x AMV/*Tfl* reaction buffer (Promega), 1µl of *Tfl* DNA polymerase (Promega) and was made up to 15µl with 10.5µl of water; mix 2 contained 0.5µl of primer PT4 (100ng/ml, Oswel DNA Services), 7µl 5x AMV/*Tfl* reaction buffer, 2µl $MgSO_4$, 1µl dNTP mix (Promega) , 1µl AMV reverse transcriptase (Promega) and was made up to 25µl with 13.5µl of water. 15µl of mix 1 was added to each 0.5ml PCR tube (Alphalabs), along with one wax bead (Ampliwax Gem 50, Perkin Elmer). The tubes were heated to 80°C for 5 minutes to melt the wax and the wax was allowed to set at room temperature for 5 minutes. 25µl of mix 2 was layered on top of the wax and covered with two drops of mineral oil to prevent evaporation and 10µl of RNA solution was added to the upper compartment.

The following reaction conditions were performed in a Techne PHC-3 thermal cycler: 48°C for 45 min for reverse transcription, 95°C for 2 min; and then 40 cycles of 30 s denaturation at 90°C, 30 s anneal at 53°C and 2 s extension at 68°C; the

cycling was followed by a final 7 min extension 68°C. The wax barrier between the two compartments melts at 70-80°C during the first cycle of PCR allowing the reaction to commence under 'hot start' conditions thereby minimising false priming and the generation of spurious amplification products (Chou *et al.*, 1992).

QRT-PCR

The same samples reverse transcribed and amplified using the RT-HS-PCR were also reverse transcribed, using MMLV-RT and primer PT2, and amplified using the optimised QRT-PCR described above (Appendix II).

Quantification of PCR Products

The products of the RT-HS-PCR and the QRT-PCR were quantified using an enzyme linked oligonucleotide assay (ELONA) as previously described (Appendix II). This assay involves the capture of biotinylated PCR products onto streptavidin coated microtitre plates, followed by hybridisation with an alkaline phosphatase labelled probe (INT1AP; Table 5), which was detected using the chemiluminescent substrate Lumiphos^{530} (Lumigen). The light signals obtained from the samples were interpolated to give viral titres by comparison with the signals generated from their respective standard curves.

Results

The relative sensitivity of each method was estimated by comparing the frequency with which dilutions of RNA prepared from plasma A (4 x 10^6 HCV genomes/ml) and the NIBSC standard were detected. The limit of detection was defined as the concentration of predicted HCV RNA genomes/ml that yields a single detectable unit as defined by the Poisson distribution of positive and negative results. The reproducibility of each method at predicting the concentration of a given sample was assessed by repeat testing of a single dilution of plasma A. The RT-HS-PCR was able to detect a 10^{-4} dilution of plasma A 13 times out of 13, a 10^{-5} dilution 10 times out of 13 and a 10^{-2} dilution of the NIBSC standard 11 times out of 20 repeats,

giving a limit of detection of approximately 40 HCV genomes/ml. This method predicted the concentration of a dilution of plasma A with a coefficient of variation of 9.1% (mean 61 cDNA molecules, SD 5.58, n=10). This level of variation is lower than that previously reported for the ELONA stage only, presumably as a result of improvements in technique rather than technology.

As can be seen from Figure 15, the signals produced by the RT-HS-PCR have a greater signal to noise ratio when compared with the QRT-PCR described above (Section 2.4 and Appendix II), even when the sample is diluted to the limit of detection. The discrimination between positive and negative results in samples containing very low levels of HCV RNA is thus improved using the RT-HS-PCR method.

Figure 15: A Comparison of Standard Curves Using QRT-PCR and RT-HS-PCR

This figure shows the relationship between light output generated by the ELONA and the initial HCV RNA concentration in a 10 fold dilution series following amplification using the RT-HS-PCR and the QRT-PCR.

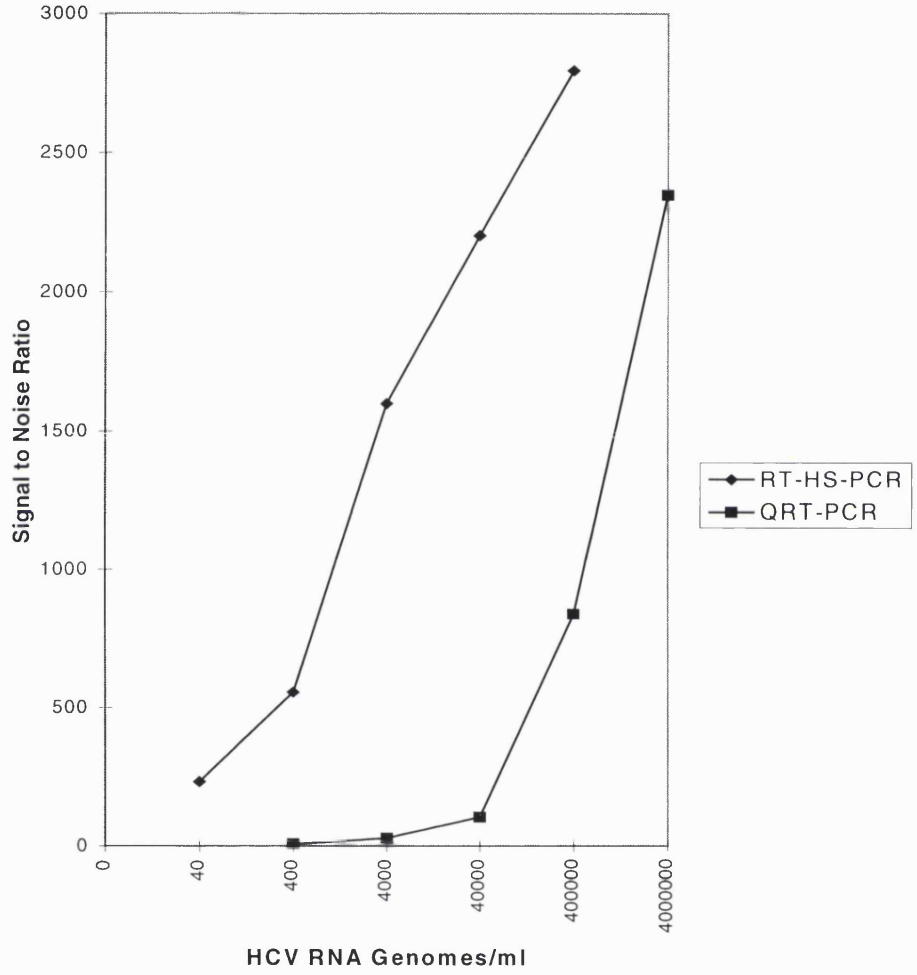
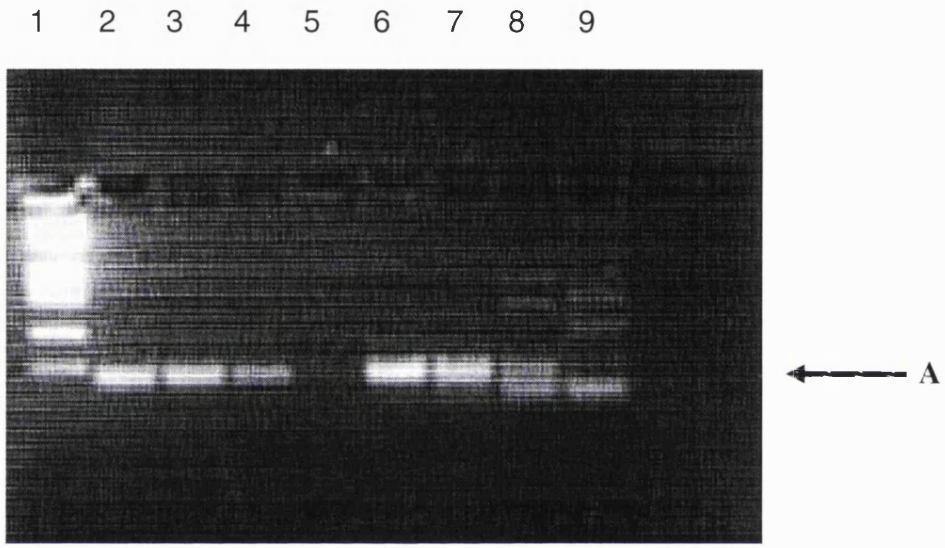


Figure 16: The Effect of a Wax 'Hot Start' on AMV/*Tfl* RT-PCR

A 4% Metaphor gel (NuSieve): lane 1 contains markers (ΦX174 *Hae* III), lanes 2-4 contain RT-HS-PCR products from an RNA dilution series (10^{-2} to 10^{-4}) and lane 5 contains the corresponding negative control. Lanes 6-9 contain the same series amplified using the same RT-PCR protocol without the wax layer. Note the non-specific products visible in lanes 8 and 9.
A = 60bp specific product.



Conclusions

Studies in 1990 showed that anti-HCV antibody was undetectable in plasma pools from the United Kingdom non-commercial donors, suggesting a low prevalence of HCV positive donations (Minor *et al.*, 1990). The subsequent introduction of routine screening of individual plasma donations has further reduced the risk of HCV contamination of plasma pools. Individual donations of plasma may still find their way into plasma pools, either because the patient was in the seronegative window period, or because of a procedural error in the preparation of the pool. However, the levels of HCV RNA in plasma pools are much lower than those observed in patient serum because of the enormous dilution factors involved.

An RT-PCR was optimised to detect HCV RNA with the greatest possible sensitivity without generating ambiguous results. The single tube RT-'Hot Start'-PCR described here is able to detect HCV RNA at concentrations as low as 40 HCV genomes/ml, equivalent to 4-5 RNA genomes per reaction. This is approximately 10 fold more sensitive than QRT-PCR (Section 2.4 and Appendix II) and 100 fold more sensitive than commercially available tests. Combining the reverse transcription and the PCR stages of the assay may also reduce the risk of false positives caused by contamination.

The ultimate limit of detection of any PCR is a single molecule of cDNA. Therefore the efficiency with which intact cDNA molecules are produced fundamentally affects the sensitivity of the assay and this in turn is dependent on a number of different factors. The efficient extraction of high purity intact RNA from dilute samples allows a large proportion of the RNA to be added to the reverse transcription reaction. We can speculate that higher temperature (48°C) reverse transcription may reduce the secondary structure that has been reported in the 5' non-coding region of HCV (Brown *et al.*, 1992; Smith *et al.*, 1995) and may therefore increase the hybridisation rate of the primers to this region of the genome. Reducing the size of the PCR target sequence has been shown to increase the sensitivity of RT-PCR by reducing the length of intact cDNA required for PCR (Garson *et al.*, 1991a). As the

primers PT3BIO and PT4 amplify a region only 60bp in length this would further be expected to increase efficiency.

The accepted reasoning has been that for sensitive, specific PCR, a 'nested' format is required and that only using two primers for the cDNA synthesis and the PCR might be expected to introduce false primed products. Separating the reverse transcription reaction from both the DNA polymerase and the sense primer appeared to reduce the amount of false primed or cold start products (Figure 16). The post amplification hybridisation used in the ELONA also adds an extra level of specificity.

The standard curve generated using the RT-HS-PCR shows that this method is able to accurately quantify (coefficient of variation 9.1%) HCV over a 4 $\log_{10}$ dynamic range. The viraemia range of clinical samples is typically 10^3 to 10^8 HCV genomes/ml this method is capable of detecting the majority of these samples, although it is more applicable to samples where a lower than average viraemia is expected. The increase in sensitivity provided by the new RT-HS-PCR should allow a larger proportion of HCV contaminated plasma pools to be detected.

3. Method Applications

The development of methods is always an ongoing process, new technical and methodological developments continuously provide opportunities to improve techniques. This section of the thesis describes the application of the methods, for the quantification of HCV RNA, that have been developed during the course of the study. It thereby demonstrates the strengths and limitations of each method as they apply in the clinical and research applications for which they were developed.

Sections 3.1 - 3.4, 3.7 and 3.9 of this chapter have been published as full papers, sections 3.6 and 3.10 have been presented at scientific meetings and their abstracts published (see Section entitled "Publications Arising from this Thesis") and in each publication co-authorship is appropriately acknowledged. My technical and intellectual contribution to the published and unpublished sections of this chapter has chiefly been focused on viral quantification and HCV serotyping, although the studies on GBV-C described in section 3.10 were largely designed and performed by myself.

3.1 Analysis of Clinical and Virological Factors Associated with Response to Interferon α Therapy in Chronic Hepatitis C

Background

The first study has been published in the *Journal of Medical Virology* in 1995 (Garson *et al.*; 1995) and describes the use of QRT-PCR to quantify pre-treatment samples from 30 chronically infected Italian patients. At this stage of method development the directly labelled oligonucleotide probe had not yet been introduced and a DNP labelled probe was detected using sequential incubations with anti-DNP monoclonal antibody and alkaline phosphatase labelled staphylococcal protein A. A signal was generated with a dioxetane based chemiluminescent substrate (Protocol 6, Table 4; Section 2.4.2).

Introduction

Hepatitis C virus infection often progresses to chronic liver disease, with its associated morbidity and risk of cirrhosis and hepatocellular carcinoma (Di Bisceglie *et al.*, 1991) as described in detail in Section 1.2.4. Interferon α is currently the only licensed therapy for chronic HCV infection and has been shown to be effective in normalising liver function tests, reducing the level of hepatic inflammation and lowering the risk of progression to hepatocellular carcinoma (Nishiguchi *et al.*, 1995). However, complete long term clearance of the virus is only seen in approximately a 15-20% of patients treated (Jouet *et al.*, 1994). Therapy is expensive and frequently associated with unpleasant side effects, so the ability to predict which patients would have a sustained response to this treatment would be highly advantageous.

Methods

Patients

Thirty Italian patients with chronic HCV infection were treated with 3 MIU of human leukocyte interferon α (Alfaferone, Alpha-Wassermann, Alanno, Italy), intramuscularly, three times per week for six months. All the patients in the study had persistently raised liver enzyme levels (ALT and AST), chronic viral hepatitis confirmed by the histological assessment of a liver biopsy and antibodies against HCV detected by ELISA (second generation, Ortho Diagnostics, Raritan, New Jersey) and confirmed by RIBA (Chiron Corporation, Emeryville, California). None of the patients in the study had chronic liver disease other than that caused by HCV infection and none of the patients were infected with HIV-1 or HIV-2. Immediately prior to starting interferon α therapy a serum sample was obtained from each patient for routine biochemical and haematological tests and for analysis of HCV genotype and titre. All the patients were followed prospectively for 12 months following cessation of therapy.

Definition of Response

Initial response was defined as complete normalisation of liver enzyme levels within three months of starting therapy and persistence of normal enzyme levels until therapy was stopped. Transient response was defined as an initial response followed by a relapse. A sustained response was defined as an initial response that was maintained for the 12 months following therapy. Patients with all other patterns of liver enzyme fluctuation were considered to be non-responders.

Quantification of HCV RNA

RNA was extracted using the RNAzol A method (Section 2.2.1) and reverse transcribed using MMLV-RT (as described in Section 2.3.1). PCR amplification and quantification of the products were performed as described previously (Section 2.4.1) using *Pfu* polymerase and the enzyme linked oligonucleotide assay (ELONA; protocol 6, Section 2.4.2).

Genotyping

Genotyping was carried out using the principles described by Stuyver *et al.* (1993), using commercially available reagents (Innogenetics, Ghent Belgium) and by following the manufacturer's instructions. Briefly, a biotin labelled fragment amplified from the 5'NCR is hybridised to genotype specific oligonucleotide probes which have been immobilised on a nitrocellulose strip. The bound 5'NCR sequences are detected using streptavidin alkaline phosphatase conjugate and a chromogenic substrate. The genotype can be easily inferred from the pattern of bands produced (Stuyver *et al.*, 1993).

Results

Pre-treatment samples from all the patients had detectable HCV RNA ($7x10^2$-$2x10^7$ HCV genomes/ml, geometric mean $3x10^5$ HCV genomes/ml). HCV genotyping revealed infection with HCV-1b in 16 (53%) patients, HCV-2a in 9 (30%), HCV-3 in 3 (10%) and HCV-4 in 2 (7%). None of the patients had evidence of infection with more than one genotype. Clinical and demographic characteristics according to HCV genotype are shown in Table 8 and Figure 17. The mean age of patients infected with HCV-3 was significantly lower than that of patients infected with other genotypes and the mean serum gamma-glutamyl transpeptidase (GGTP) level was significantly higher in patients infected with genotype 1b than in those infected with non-1b. Other clinical and demographic characteristics, including the geometric mean viral titres, did not vary significantly between the different genotypes.

Interferon α therapy induced an initial response in 16 patients (53%), which was sustained in 5 cases (17%). Fourteen patients (47%) were non-responders and 11 (36%) were transient responders. Comparison of pre-treatment clinical characteristics between patients who had an initial response to therapy and those who did not (Table 9) revealed that responders were significantly younger (45.6 ± 3.1 compared with 55.4 ± 2.7 years) and less frequently cirrhotic on liver biopsy than nonresponders (2/16 compared with 7/14). A similar comparison between patients with and without a sustained response to interferon α therapy revealed no clinical characteristic that was significantly associated.

Geometric mean pre-treatment HCV-RNA titres were similar in patients who had an initial response to interferon α and in non-responders (5.3 ± 0.3 compared with 5.6 ± 0.2 $\log_{10}$ HCV genomes/ml). However, patients who had a sustained response to therapy had a geometric mean HCV RNA titre approximately ten fold lower than those who did not have a sustained response (4.5 ± 0.75 compared with 5.6 ± 0.2 $\log_{10}$ HCV genomes/ml), but the difference did not reach statistical significance at the 5% level (p=0.1; Figure 18). In contrast, statistically significant differences were found between the rate of response (initial and sustained) of patients infected with different genotypes. Patients infected with HCV-2a were significantly more likely to respond (89%) than those with HCV-1b (37%) and they were also more likely to sustain that response (33% compared with 6%). The number of patients infected with HCV genotypes 3 and 4 was too small to permit meaningful evaluation of their interferon α response rate.

Table 8: Patient Characteristics and HCV Titre by HCV Genotype

Characteristic	HCV-1b (n=16)	HCV-2a (n=9)	HCV-3 (n=3)	HCV-4 (n=2)	P value*
Sex (M/F)	6/10	3/6	3/0	0/2	NS[a]
Age (yr)	54.2 (±2.7)	49.2 (±3.7)	30.3 (±2.8)	52.5 (±8.5)	< 0.05[b]
Duration of disease (yr)	8.6 (±1.0)	9.2 (±2.0)	4.0 (±0.0)	5.0 (±3.0)	NS
Source of infection					
Transfusion -related	3	2	0	0	NS
Sporadic	13	7	3	2	NS
ALT (IU/L)	170.0 (±31.5)	158.8 (±36.0)	155.3 (±45.7)	158.0 (±118)	NS
AST (IU/L)	109.9 (±18.9)	74.7 (±18.4)	56.0 (±20.8)	106 (±74.0)	NS
GGT (IU/L)	64.5 (±9.5)	35.3 (±8.5)	42.3 (±6.9)	31.0 (±4.0)	< 0.05[c]
γ-Globulin (g/dl)	1.7 (±0.1)	1.6 (±0.1)	1.3 (±0.1)	1.54 (±0.3)	NS
Platelet count ($x10^3/\mu l$)	159.9 (±15.1)	175.0 (±10.3)	194.3 (±2.9)	213 (±57)	NS
Liver histology					
CAH	12	6	3	0	NS
Cirrhosis	4	3	0	2	NS
Log_{10} HCV genomes/ml[d]	5.49 (±0.24)	5.13 (±0.51)	5.9 (±0.56)	6.15 (±0.65)	NS

* Tested by multigroup one-way ANOVA or Chi-squared unless otherwise stated
[a] NS = Not significant
[b] $P < 0.05$ by unpaired *t* test for HCV type 3 versus non-type 3
[c] $P < 0.05$ by unpaired *t* test for HCV type 1b versus non-type 1b
[d] Geometric mean titres (± S.E.)

Figure 17: Pre-Treatment HCV Viraemia Level by Genotype (Italian Patients)

(Geometric mean titres are represented by the horizontal line)

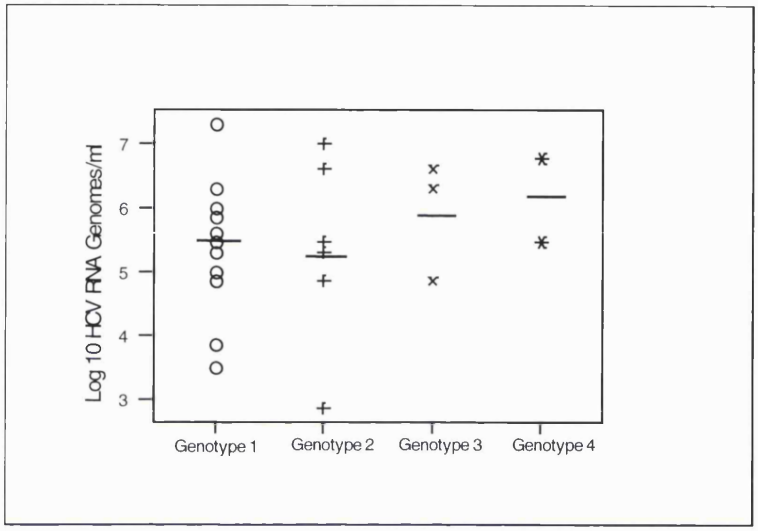


Figure 18: Geometric Mean Pre-Treatment Viraemia Levels and Interferon α Response

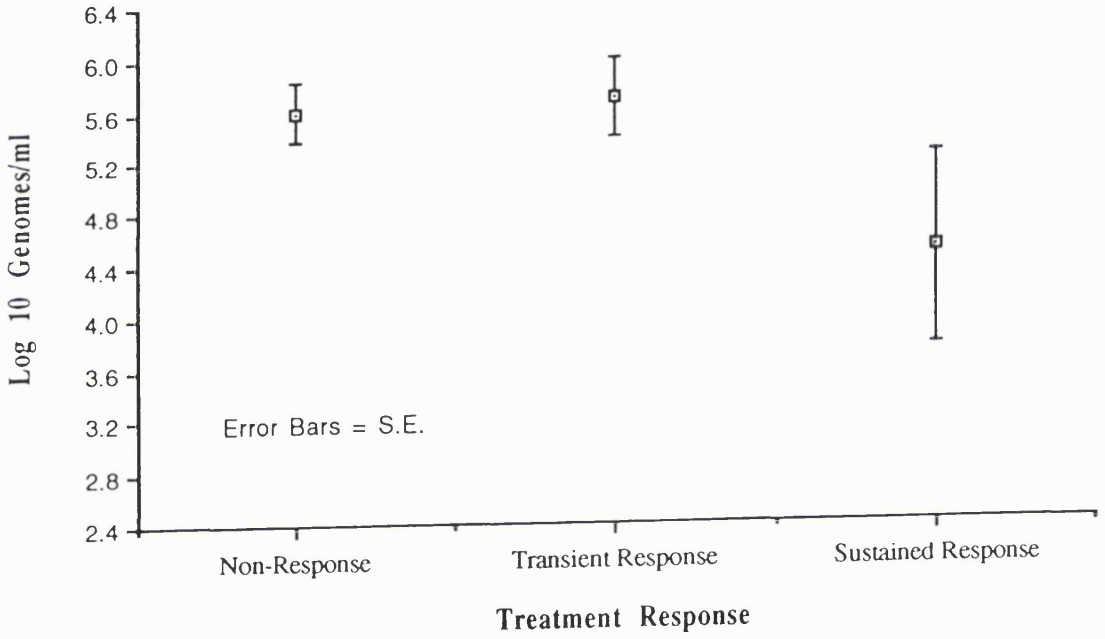


Table 9: Clinical and Virological Characteristics in Relation to Interferon α Response

Characteristic	Non-responders (n=14)	Transient-responders (n=11)	Sustained-responders (n=5)	P value*
Sex (M/F)	4/10	6/5	2/3	NS[a]
Age (yr)	55.4 ± 2.7	46.8 ± 4.0	43.0 ± 5.5	< 0.05[b]
Duration of disease (yr)	7.4 ± 0.7	9.6 ± 2.0	6.6 ± 1.4	NS
Source of infection				NS
Transfusion -related	1	3	1	
Sporadic	13	8	4	
ALT (IU/L)	133.4 ± 26.1	214.5 ± 42.1	140.8 ± 23.3	NS
AST (IU/L)	101.0 ± 22.0	97.9 ± 18.3	63.8 ± 15.2	NS
GGT (IU/L)	61.1 ± 10.5	38.5 ± 6.8	51.4 ± 16.2	NS
γ-Globulin (g/dl)	1.65 ± 0.13	1.51 ± 0.11	1.58 ± 0.14	NS
Platelet count ($x10^3/\mu l$)	157.6 ± 14.5	184.6 ± 16.4	181.0 ± 12.0	NS
Liver histology				<0.05[c]
CAH	7	10	4	
Cirrhosis	7	1	1	
Log_{10} HCV genomes/ml	5.6 ± 0.24	5.7 ± 0.31	4.5 ± 0.75	NS
HCV genotype				
HCV-1b	10	5	1	<0.05[d]
HCV-2a	1	5	3	<0.05[d,e]
HCV-3	1	1	1	NS
HCV-4	2	0	0	NS

* Tested by multigroup one-way ANOVA or Chi-squared unless otherwise stated

[a] NS = Not significant

[b] $P < 0.05$ by unpaired *t* test for non-responders versus initial responders (initial = transient + sustained)

[c] $P < 0.05$ by Fisher's exact test for non-responders versus initial responders

[d] $P < 0.05$ by Fisher's exact test comparing HCV-1b with HCV-2a for non-responders versus initial responders and for non-responders versus sustained responders

[e] $P < 0.05$ by Fisher's exact test comparing HCV-2a with non-2a for non-responders versus initial responders and for non-responders versus sustained responders

Discussion

Numerous therapeutic trails have demonstrated that interferon α is an effective therapy in a proportion of patients with chronic HCV infection, however, the majority of patients gain no long term benefit from this therapy. Interferon α therapy is associated with unpleasant side effects and is expensive. In order to better target therapy to those most likely to benefit, there is an urgent need to identify factors predictive of a successful outcome to therapy. This study investigated the effect of interferon α on 30 patients with chronic HCV infection in an attempt to identify any clinical, demographic, or virological factors which may be associated with therapeutic outcome.

The proportions of initial, transient and sustained responders observed in this cohort were similar to the proportions reported by others (Tine *et al.*, 1991). The only host factors significantly associated with initial response to interferon α were patient age and liver histology, responders being younger and less frequently cirrhotic than nonresponders.

The influence of pre-treatment viraemia level on interferon α responsiveness is unclear. Several groups have reported a connection between low pre-treatment HCV RNA titre and response to interferon α (Lau *et al.*, 1993; Hagiwara *et al.*, 1993), while others have not (Magrin *et al.*, 1992). In this study the mean pre-treatment was lower in those with a sustained response than in those without, but the difference failed to reach statistical significance, perhaps due to the relatively low number of patients studied.

Previous studies have suggested that infection with genotype 1b may be associated with more severe hepatic damage (Pozzato *et al.*, 1994) and a poorer response to interferon α therapy (Yoshioka *et al.*, 1992; Hino *et al.*, 1994). Our findings appear to lend support to both these notions; first, HCV 1b infected patients had higher mean ALT, AST and GGTP levels than those infected with other genotypes (although only the difference in GGTP level reached statistical significance) and

second, HCV 1b infected patients were less likely to respond to interferon α therapy (37% for HCV 1b verses 89% for HCV 2a).

The mechanism of genotype dependent interferon α responsiveness is unknown. The poor response rate associated with type 1b infections may reflect some form of intrinsic resistance of this genotype to interferon α. Alternatively, it has been proposed that the poor response rate of type 1b may be due to a higher replicative capacity. This has be inferred from the observation that the mean pre-treatment HCV RNA titre of HCV 1b cases is significantly higher than that of other genotypes (Yoshioka *et al.*, 1992; Hino *et al.*, 1994). However our findings do not support this theory, as no significant difference in HCV RNA was observed between the genotypes. This discrepancy may be due partly to methodological differences between the studies; Yoshioka *et al.* (1992) acknowledged that their results may have been due in part to genotype dependent differences in quantitative PCR sensitivity. The PCR primers PT3, PT4 and probe INT1DNP were selected from a highly conserved segment of the 5'NCR (Table 19) in order to eliminate such genotype dependant differences in amplification and detection efficiency. Thus we conclude that the apparent resistance of genotype 1b to interferon α therapy may not simply be an indirect consequence of a higher level of viraemia.

Although this and other studies have identified a number of host and viral factors which are clearly associated with therapeutic outcome, no single factor is able to predict with certainty whether or not a given patient will respond.

Using methodology such as the limit dilution/Poisson distribution technique (Simmonds *et al.,* 1990b) to quantify the HCV RNA levels would typically have involved preparing several ten fold dilutions for each of the 30 patient samples. This would provide an approximate measure of RNA titre. The repeat testing, at least 10 times, of one or more of the dilutions would have been required to achieve the same levels of accuracy as reported in the study described above. As each PCR test for the limit dilution technique requires double or 'nested' PCR reactions, the total number of individual reactions would have been over 1000. The fact that the same data was generated in only 30 PCR reactions represents a time and cost saving of over 30 fold.

3.2 Quantitative Analysis of HCV RNA in Patients Treated with a Combination of Interferon α and Ribavirin

Background

Showing that QRT-PCR could provide quantitative data without losing diagnostic sensitivity encouraged the application of the same technique to a cohort of 20 Italian patients involved in a pilot trial of IFN-α and Ribavirin combination therapy. The QRT-PCR technique was, however, applied not only to pre-treatment samples, but was also used to monitor viraemia levels during therapy and the post therapeutic follow up period. This involved the quantification of HCV RNA in 120 serum samples. The results of the trial were published in *Gastroenterology* (Brillanti *et al.*, 1994) and are described below.

Introduction

Treatment with interferon α monotherapy produces a sustained response in less than a quarter of patients and retreatment rarely results in viral clearance (Davis *et al.*, 1989), as has been discussed earlier (Section 3.1). Drug trials, in which combination therapy has been used to treat HIV-1 infection proved more effective than monotherapy in reducing viraemia. *In vitro* studies (Hayden *et al.*, 1984) have suggested that combination therapy with interferon α and Ribavirin, a nucleoside analogue, may have an additive or synergistic effect on RNA virus replication. Ribavirin has a broad spectrum of activity against both DNA and RNA viruses. It has been shown to be well tolerated as a monotherapy against HCV infection and causes a transient reduction in serum aminotransferase levels (Dusheiko *et al.*, 1996b).

This pilot study was the first designed to determine whether the combination of IFN-α and Ribavirin is an effective therapy in chronic HCV patients who fail to respond to interferon α monotherapy.

Methods

Patients

20 patients with chronic community-acquired hepatitis C were enrolled in this study. All had previously been treated in a trial using 3MU of natural human leukocyte IFN-α (Alfaferone, Alpha-Wassermann), given three times per week for six months (Brillanti *et al.*, 1991). The group comprised 10 consecutive patients who had had a transient response to the previous course of IFN-α followed by relapse and 10 consecutive patients who had not responded at all. None of the 20 patients had received IFN-α or other antiviral treatment within the previous 12 months. Entry criteria for this study were as follows: persistent elevations of serum alanine (ALT) and aspartate (AST) aminotransferase levels for at least the previous 6 months; a liver biopsy, obtained within the last month, with histological findings compatible with the diagnosis of chronic hepatitis of viral origin; the presence of antibodies to HCV by second generation ELISA test (Ortho Diagnostic Systems, Raritan, New Jersey), confirmed by second generation RIBA (Chiron Corporation, Emeryville, California); the absence of circulating anti-IFN-α antibodies detected by ELISA (BioNative AB, Umeå, Sweden).

Patients with decompensated liver disease, other serious illnesses, or infection with HIV-1 or HIV 2 were not included in the study. None of the patients had a history of exposure to blood or blood products or of intravenous drug abuse. Active hepatitis B virus infection, autoimmune hepatitis and other causes of chronic liver disease were excluded by conventional clinical and laboratory tests.

Protocol

The 20 patients were randomly assigned into 2 groups of 10 to receive a 6 month course of either a combination of natural human leukocyte IFN-α (3 MIU three times per week; Alfaferone, Alfa-Wassermann) and Ribavirin (800mg by mouth daily; Alfa-Wassermann, Alanno, Italy), or retreatment with IFN-α monotherapy (3MIU thrice weekly).

The patients in the two treatment arms were matched as follows:

Table 10: Patient Characteristics of the Two Treatment Groups at the Start of Therapy

Characteristics	IFN-α+Ribavirin (n=10)	IFN-α alone (n=10)
Male/Female	4/6	4/6
Age (yr.)[a]	43.3 ± 6.1	48.3 ± 4.4
ALT (IU/L)[a]	168.3 ± 47.8	174.5 ± 43.1
AST (IU/L)[a]	102.9 ± 19.7	101.9 ± 17.4
CAH/Cirrhosis	5/5	6/4
HCV RNA positive	10	10
NR/RR to previous IFN therapy	5/5	5/5

[a]Data expressed as mean ± SE; NR, non-responder; RR, responder with relapse.

Blood Samples and Liver Histology

Blood was taken before therapy began, at three monthly intervals during therapy and for nine months following therapy. Full blood counts and routine biochemical tests were performed 'blind' without knowledge of treatment or HCV RNA data. Serum was separated and stored at -20°C for QRT-PCR. Two liver biopsies were taken, one preceding therapy and one 6-9 months following cessation of therapy.

Quantification of HCV RNA

QRT-PCR was performed 'blind' without knowledge of treatment or ALT data, using MMLV-RT and *Pfu* DNA polymerase as described previously (QRT-PCR, Section 2.4.1; protocol 5). As well as quantitative PCR each sample was assayed for HCV RNA using a previously described non-quantitative 'nested' PCR (Garson *et al.,* 1991a). Briefly, HCV RNA extracted using the RNAzol A method (Section 2.2.1) was reverse transcribed using MMLV-RT and random hexamers (Section 2.3.1) and amplified using primers PT1 and PT2 and *Taq* DNA polymerase. A portion of the products of amplification were re-amplified using the internal or "nested" primers PT3 and PT4. Reaction products can be visualised as bands of uniform intensity on an ethidium bromide stained agarose gel.

Statistical Methods

Differences between paired observations were analysed by the Wilcoxon signed rank test. Fisher's exact test was used to analyse differences between proportions. Results are expressed as mean ±SE. To compare circulating HCV RNA levels, the geometric means of HCV RNA titres were calculated.

Results

Effects on Serum ALT and Liver Histology

During treatment, normalisation of ALT levels was observed in 7 patients treated with Ribavirin plus IFN-α and in 4 patients treated with IFN-α alone (Table 11 and Figure 19). In the combination therapy group, ALT normalisation occurred in 5/5 patients who had had a temporary response to the previous IFN-α treatment and in 2/5 of those patients who had not responded previously. In patients who received IFN-α monotherapy, normalisation of ALT levels occurred in 4/5 who had had a temporary response, but none of those who had not previously responded. Nine months after cessation of therapy 4 patients treated with combination therapy had sustained normalisation of ALT levels, compared with only one patient treated with IFN-α monotherapy. The response to therapy was not influenced by the appearance

of antibodies reactive against IFN-α, as all the patient sera from both treatment groups tested negative for these antibodies at the start and at the end of therapy.

Pre-treatment liver biopsy revealed chronic active hepatitis (CAH) in all 20 patients, associated with cirrhosis in 9 cases (Table 10). Between 6 to 9 months after stopping treatment a repeat biopsy was taken in each of the 5 patients with a sustained biochemical response. A reduction in the necroinflammatory features was observed in all cases.

Table 11: A Comparison of Combination Therapy with Monotherapy

	Ribavirin+IFN-α (n=10)		**IFN-α alone (n=10)**	
	ALT Normal	HCV RNA Negative	ALT Normal	HCV RNA Negative
Before treatment	0	0	0	0
After 3 mo. of treatment	6	5	2	3
After 6 mo. of treatment	7	5	4	3
3 mo. after treatment end	6	5	1	0
6 mo. after treatment end	5	5	1	0
9 mo. after treatment end	4	4	1	0

Effects on Serum HCV RNA

HCV RNA was detected in serum from all 20 patients before treatment. After 6 months of therapy, serum HCV RNA was undetectable in 5/10 patients treated with Ribavirin plus IFN-α and in 3/10 treated with IFN-α monotherapy (Table 11 and Figure 19). During the 9 month post treatment follow up period, recurrence of hepatitis C viraemia was observed in only 1/5 of the initial responders treated with combination therapy. In contrast, viraemia reappeared after therapy in 3/3 initial responders treated with IFN-α alone. The proportion of patients whose sera remained negative for HCV RNA after treatment was significantly higher in the Ribavirin plus IFN-α group than the IFN-α monotherapy group ($p<0.05$). In patients receiving combination therapy, sustained loss of viraemia was less common in those with

cirrhosis (1/5) than in those without (3/5). Although the mean age of those patients who experienced a sustained loss of viraemia was lower than those who failed to attain a sustained response (45.2 ± 5.5 vs. 54.1 ± 4.7 years), this difference did not reach statistical significance.

Disappearance of serum HCV RNA was always accompanied by normalisation of ALT values and reappearance of serum HCV RNA was accompanied by ALT elevation in all but one patient. This patient had been treated with IFN-α monotherapy and experienced sustained normalisation of ALT levels despite recurrence of HCV replication after cessation of therapy.

Amongst patients assigned to receive combination therapy the geometric mean pre-treatment viraemia level was 7.9×10^5 HCV genomes/ml (range 4.4×10^4 to 3×10^7 HCV genomes/ml). Similarly, pre-treatment titres of HCV RNA varied from 7×10^4 to 2×10^6 HCV genomes/ml with a geometric mean titre of 2.3×10^5 HCV genomes/ml in the patient group assigned to receive IFN-α alone. The pre-treatment geometric mean titre in patients who achieved a sustained disappearance of viraemia (3.9×10^5 HCV genomes/ml) was lower than that in patients who did not (1.2×10^6HCV genomes/ml). The serial viraemia and ALT levels in patients who received combination therapy are shown in Figure 19.

Side Effects

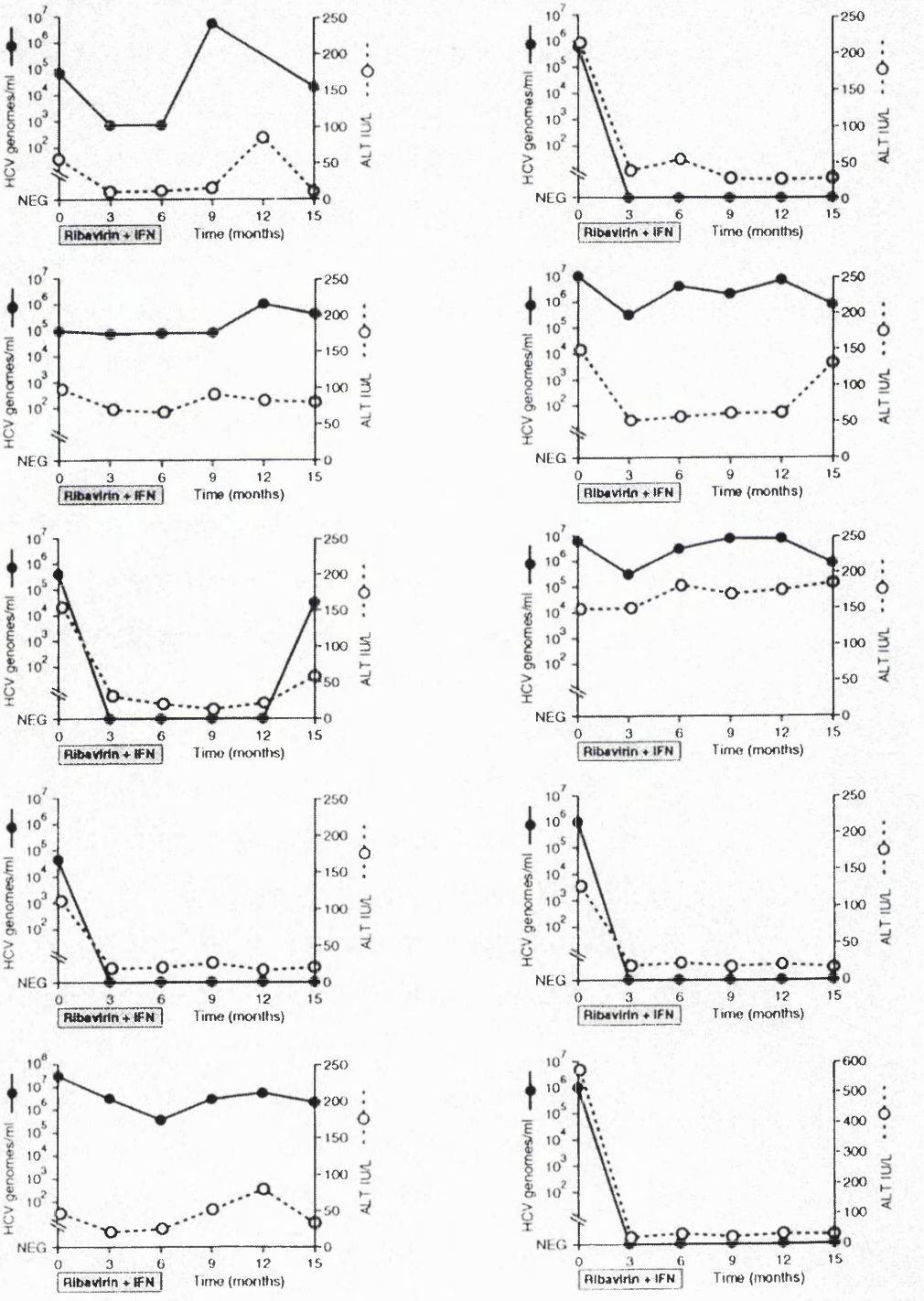
Both combination therapy and monotherapy were well tolerated. Monitoring side effects by questioning and examination revealed mild flu-like symptoms during the first week of therapy in patients from both treatment groups. During treatment, mild to moderate fatigue was more frequently reported by patients in the group receiving combination therapy.

The most common side effect of combination therapy was mild anaemia. The mean haemoglobin level decreased from 13.7 ± 0.7 g/dl before therapy to 11.4 ± 0.4 g/dl at the end of therapy ($p = 0.001$) and red blood cell counts decreased from 4.3 ± 0.1 x

10^6/µl to 3.6 ± 0.1 x 10^6/µl (p = 0.002). Serum bilirubin levels increased slightly from 0.7 ± 0.1 mg/dl to 0.8 ± 0.1 mg/dl and serum uric acid levels increased from 4.7 ± 0.6 mg/dl to 5.1 ± 0.6 mg/dl. Increases in bilirubin and uric acid levels were not statistically significant. The mild anaemia did not require reduction of Ribavirin dose in any patients and all side effects were reversible following cessation of therapy.

Figure 19: Serial ALT and HCV RNA Titres in 10 Patients Receiving Ribavirin + IFN-α Combination Therapy

Ten patients were treated with a combination of IFN-α (3 MIU thrice weekly) and Ribavirin (800mg daily) for 6 months. Patients were monitored for a further 9 months post therapy.



Discussion

Antiviral monotherapy of RNA viruses such as HIV-1 is associated with the rapid development of resistance. This may in part explain the relatively poor response rate to IFN-α monotherapy (approximately 15-20%). *In vitro* studies have suggested that Ribavirin combined with IFN-α may have an additive or synergistic antiviral effect on RNA virus replication (Hayden *et al.,* 1984) and studies of combination therapy of HIV-1 infection have shown significant benefit (Delta Coordinating Committee, 1996). These studies combined with the relatively low toxicity of both drugs suggests that clear benefits may be achieved using this therapeutic strategy for the treatment of chronic HCV infection. In this study the effect of combination therapy was compared with IFN-α monotherapy in patients who had failed to achieve a sustained response to previous IFN-α monotherapy.

The findings confirm that, as reported by others (Hoofnagle, 1993; Gerken *et al.,* 1995), retreatment with a standard dose of IFN-α alone does not give rise to a sustained clearance of HCV viraemia, although transient suppression of viraemia and ALT levels may occur. In contrast, treatment with a combination of Ribavirin plus a standard dose of IFN-α appears to be able to produce a sustained clearance of viraemia and sustained normalisation of ALT levels in a significant proportion (40%) of patients resistant to IFN-α alone. These findings have subsequently been confirmed in a series of small pilot studies re-evaluated by meta-analysis (Schalm *et al.,* 1996). More recently a much larger study showed that similar response rates can be achieved in previously untreated patients (Reichard *et al.,* 1998).

In all but one patient in this study persistent normalisation of ALT level was associated with sustained loss of detectable HCV RNA in serum. In the remaining case (a patient in the IFN-α monotherapy group), normal ALT levels were maintained despite the recurrence of HCV replication indicated by the reappearance of serum HCV RNA. This apparently paradoxical situation has been observed previously (Garson *et al.*, 1992; Kakumu *et al.*, 1993) and it has been suggested that this may be due to extra-hepatic replication, possibly in peripheral blood

mononuclear cells. Longer term follow up will be required to clarify the outcome in such patients.

Combination therapy with Ribavirin and IFN-α was well tolerated. Side effects included fatigue and anaemia, but these symptoms were mild and reversible following cessation of therapy. The use of Ribavirin has been associated with haemolysis (Di Bisceglie *et al.*, 1992b; Reichard *et al.*, 1991), but in this study haemolysis although observed did not require the dose of Ribavirin to be reduced.

Although complete clearance of chronic HCV infection is an achievable goal using IFN-α as a single agent, the sustained response rate to this expensive treatment is low (15-20%). These results suggest that a significant improvement in the sustained response rate of chronically infected patients can be achieved by using combination therapy. The fact that these findings have been supported by more recent work (Davis *et al.,* 1998; Poynard *et al.,* 1998), justifies serious consideration of the use of IFN-α and Ribavirin, not only for the treatment of those patients who fail to sustain a response to interferon α monotherapy, but also as the front line therapy for the treatment of chronic carriers of HCV.

The QRT-PCR used in this study provided informative quantitative data and was able to detect HCV RNA in all the samples found positive by the qualitative 'nested' PCR. This version of the technique although more cumbersome than later adaptations was still able to follow the decline in viraemia seen in patients who respond to therapy (Figure 19) and this decline was mirrored invariably by a similar decline in serum levels of liver enzymes.

3.3 Virological, Biochemical and Histological Effects of Human Lymphoblastoid Interferon in Swedish Patients with Chronic Hepatitis C

Background

The simplification of the ELONA technique achieved by the introduction of a directly conjugated alkaline phosphatase labelled probe (INT1AP; Table 5) is described in protocol 7, Section 2.4.2. The removal of the two, one hour antibody incubation steps prior to the addition of the chemiluminescent substrate by this modification reduced the time taken to perform the ELONA from 5 hours to less than 3 hours. The modified protocol was applied to 190 samples from 38 Swedish patients enrolled in a trial of lymphoblastoid IFN. The results of this study were published in 1997 in the *Journal of Viral Hepatitis* (Garson *et al.*,1997) and are described below.

Introduction

Hepatitis C virus infection often progresses to chronic liver disease. Interferon α is currently the only licensed therapy for chronic HCV infection and has been shown to be effective in normalising liver function tests, reducing the level of hepatic inflammation, lowering the risk of progression to hepatocellular carcinoma and the viral titre. The majority of trials assessing the efficacy of IFN-α have used interferon generated by recombinant DNA technology. The advantage of this strategy is that the drug has a high level of homogeneity and therefore predictable pharmacological activity. However, patients treated with recombinant IFN-α may produce antibodies which neutralise or reduce the drugs activity.

A diverse range of factors including viral titre, genotype, patient age, liver histology, interferon α dosage regimen and the induction of anti-interferon α antibodies are thought to influence the therapeutic outcome of HCV infection. However, the relative importance and interactions between these factors are as yet unclear (Hino *et al.*, 1994; Poynard *et al.*, 1995; Section 3.1). This study was designed to investigate

these factors and to assess the therapeutic efficacy of human lymphoblastoid interferon α in a cohort of Swedish patients with chronic hepatitis C.

Methods

Patients

Thirty eight Swedish patients were investigated as part of a randomised, double-blind, international multicentre trial of human lymphoblastoid interferon. These patients all had elevated serum alanine transaminase (ALT) levels for at least six months prior to therapy, histological evidence of hepatitis, antibodies to HCV (anti-HCV) detectable by a second generation ELISA, HCV viraemia and were attending participating centres in Malmö, Uppsala, Lund, Örebro and Umeå. Other causes of liver disease including hepatitis B, autoimmune hepatitis, Wilson's disease and α_1 anti-trypsin deficiency were excluded by appropriate laboratory investigations. Patients with HIV-1 or HIV 2 infection or decompensated cirrhosis were excluded from the study as were those that had received antiviral or immunomodulatory therapy within the preceding 6 months.

Interferon α Dosage Regimens

Human lymphoblastoid interferon-α-n1 (Wellferon, Glaxo Wellcome Research Laboratories, Beckenham, Kent) was given by subcutaneous injection three times per week. Patients were randomly assigned to one of the following four treatment groups:

1. 3 MIU for 6 months (n=12),
2. 3 MIU for 12 months (n=10),
3. 5 MIU for 6 months (n=8),
4. 5 MIU for 12 months (n=8).

Following cessation of therapy, all patients were followed up for a further 12 months.

Biochemical, Serological and Histological Monitoring

Liver function tests and full blood counts were performed weekly for the first month of therapy and then at bi-monthly intervals until the end of the follow up period. A blind assessment of liver biopsies was performed by one histopathologist on biopsies taken within 6 months of the start of therapy and at the end of the 12 month follow up. Evaluation of the histological activity index (HAI) was based on the Knodell numerical scoring system (Knodell *et al.*, 1981) but with the exclusion of the fibrosis score. Neutralising anti-interferon α antibody formation was assessed by the assay described by Brand *et al.* (1994).

HCV Genotyping, Viral Genome Detection and Quantification

The HCV genotype was determined on baseline samples by using a reverse hybridisation line probe assay (Stuyver *et al.*, 1993; Innogenetics, Ghent Belgium). Qualitative analysis of serum HCV RNA was performed using a nested PCR, as previously described (Garson *et al.*, 1991a) on serial samples taken prior to and during interferon α therapy and at 6 and 12 months following cessation of therapy.

PCR positive samples were analysed using the QRT-PCR method described in Section 2.4.2 (protocol 7). All tests were performed 'blind' without knowledge of the patients' clinical, biochemical or histological data.

Definition of Response Types

In the context of the present study, response to interferon α was defined virologically. The term sustained response (SR) was used to signify loss of detectable serum HCV RNA during therapy without evidence of recurrence of viraemia throughout the 12 month follow up period. Transient response (TR) signified loss of detectable viraemia during therapy with subsequent reoccurrence of viraemia either during therapy or during the follow up period. Patients who remained viraemic throughout treatment and follow up were defined as non-responders (NR).

Statistical Methods

Pearson's χ^2-statistics and Fisher's exact tests were used to examine differences between proportions. Means (geometric means for HCV RNA titres) were expressed $\pm$ standard error (SE) and comparisons between means were performed using the two sample *t* test. The null hypothesis was rejected at a level of $p < 0.05$.

Results

Demographic and Baseline Characteristics of the Cohort

The mean age of the 38 Swedish patients (male to female ratio 26:12) was 39.2 ± 1.6 years. Prior to therapy the mean serum ALT concentration was 2.3 ± 0.18 $\mu katl^{-1}$ (upper limit of normal 0.7 $\mu katl^{-1}$) and the geometric mean titre log_{10} 5.94 HCV genomes/ml. Pre-treatment liver biopsies revealed cirrhosis in four of the 38 patients. The mean pre-treatment HAI score was 8.8 ± 0.58. HCV genotyping revealed that 50% of the patients were infected with genotype 1 (9 type 1a and 10 type 1b), 21% were infected with genotype 2 (8 type 2b) and HCV genotype 3a was found in 29% (n=11). The source of infection was parenteral in 32 of the patients (either transfusion or intravenous drug use related) and of unknown origin in the remaining six. Baseline characteristics of patients in the four treatment groups were not significantly different (Table 12).

Table 12: Baseline Characteristics of the Entire Cohort and of Patients in Each Treatment Group

	All Patients treated with IFN	Patients Rx with 3MU for 6 months	Patients Rx with 3MU for 12 months	Patients Rx with 5MU for 6 months	Patients Rx with 5MU for 12 months
Number of Patients	38	12	10	8	8
Mean age (years)	39.2 ± 1.6	38.7 ± 2.12	39.3 ± 3.98	38.4 ± 3.98	40.9 ± 4.18
Sex Ratio (M:F)	26:12	9:3	8:2	5:3	4:4
Mean ALT*[1]	2.3 ± 0.18	2.8 ± 1.5	1.8 ± 0.8	2.5 ± 0.6	2.0 ± 0.8
Mean HCV RNA titre*[2]	5.94	6.08	5.99	6.04	5.55
HCV type 1a*[3]	9	3	3	0	3
HCV type 1b	10	5	2	3	0
HCV type 2b	8	3	2	0	3
HCV type 3a	11	1	3	5	2
Cirrhosis	4	3	1	0	0
Mean Knodell HAI*[4]	6.1 ± 0.36	6.9 ± 0.55	5.6 ± 0.88	5.1 ± 0.58	6.4 ± 0.8
Parenteral source*[5]	32	11	9	5	7

*[1] Serum ALT concentration expressed as μkat/l
*[2] Geometric mean baseline HCV RNA titres expressed as $\log_{10}$ genomes/ml
*[3] One patient had evidence of dual infection with types 1a and 3a but was classified as HCV type 1a for the purposes of statistical analysis
*[4] Knodell HAI excluding fibrosis score
*[5] Parenteral source = HCV infection associated with blood transfusion or intravenous drug use

Response Rates and Response 'Predictors'

Overall, 22 (58%) of the patients lost detectable HCV viraemia during interferon α therapy but eight of these patients relapsed virologically leaving 14 (37%) sustained responders. Seven of the eight who relapsed did so within 6 months of cessation of therapy. Sixteen of the patients (42%) remained viraemic throughout the treatment and follow up period (NR). The relationship between response type, baseline characteristics and dosage regimen are summarised in Table 13.

The factor most clearly associated with a sustained response to interferon α was HCV genotype. Only two of 19 (10.5%) patients infected with HCV genotype 1 achieved a sustained response compared with 12 of 19 (63%) patients infected with other genotypes (p=0.001). No statistically significant difference was observed between the sustained response rates of HCV type 1a and type 1b or between type 2b and type 3a, although both type 1 sustained responders were type 1a. Although a higher proportion of patients treated with 5MU produced a sustained response compared with 3MU (50% vs. 25%) the difference did not reach statistical significance. The proportion of patients producing a sustained response following 12 months therapy was similar to that following 6 months therapy (39% vs. 35%). Patients infected with genotype 1 had similar baseline characteristics to those patients infected with other genotypes. This is in contrast to certain other studies which suggest that HCV type 1 infection is characterised by a higher viral load. As can be seen in Figure 20 the pre-treatment viraemia levels in patients involved in this study that are infected with genotype 1 are similar to those in patients infected with genotypes 2 and 3.

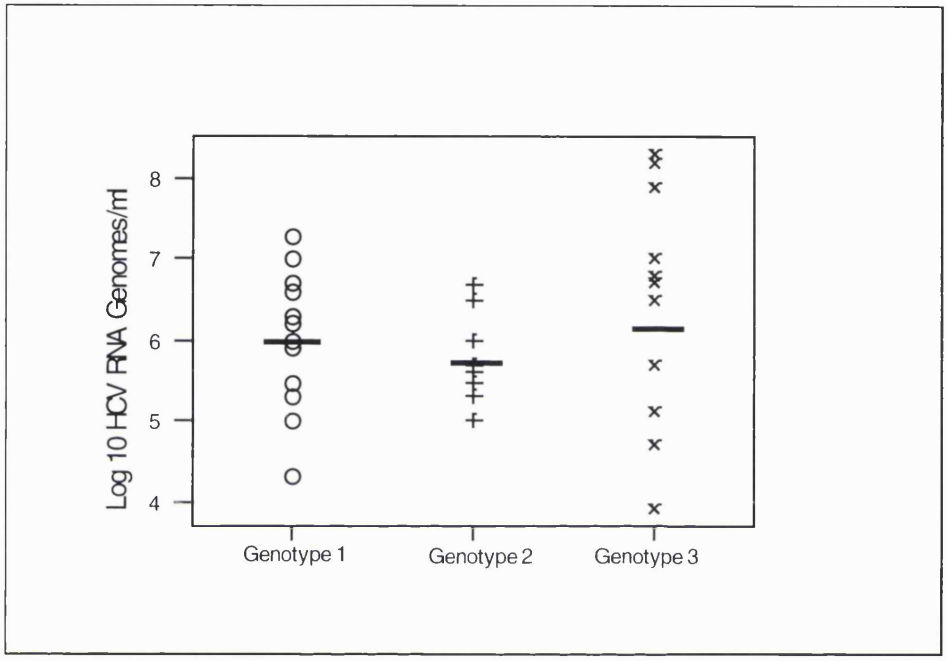
Table 13: Clinical and Laboratory Observations in Relation to the Type of Virological Response

	Non-responders (n=6)	Transient responders (n=8)	Sustained responders (n=14)	p value
Mean age (years)	39.5 ± 3.01	41.1 ± 2.52	37.8 ± 2.71	NS
Sex ratio (M:F)	12:4	7:1	7:7	NS
Mean baseline ALT*[1]	2.4 ± 0.33	2.5 ± 0.26	2.1 ± 0.28	NS
Mean baseline HCV RNA titre*[2]	6.08	5.78	5.86	NS
HCV type 1*[3]	14	3	2	<0.05
HCV non-type 1*[4]	2	5	12	
Cirrhosis on pre-Rx biopsy	3	1	0	NS
Parenteral source of infection*[5]	11	8	13	NS
3MU dose of IFN (n=22)	12	4	6	NS
5MU dose of IFN (n=16)	4	4	8	NS
6 months IFN Rx (n=20)	9	4	7	NS
12 months IFN Rx (n=18)	7	4	7	NS
Sustained ALT normalisation*[6]	0	1	14	<0.05
Mean pre-Rx Knodell HAI	6.3 ±0.45	6.0 ± 1.00	5.9 ± 0.65	NS
Mean post-FU Knodell HAI*[7]	6.6 ± 0.63	6.8 ± 0.83	3.3 ± 0.54	<0.05*[8]

*[1] Serum ALT concentration expressed as μkat l^{-1}
*[2] Geometric mean baseline HCV RNA titre expressed as $\log_{10}$ genomes/ml
*[3] HCV type 1 includes 9 type 1a and 10 type 1b
*[4] HCV non-type 1 includes 8 type 2b and 11 type 3a
*[5] Parenteral source = HCV infection associated with either transfusion or intravenous drug use
*[6] Sustained normalisation of serum ALT (<0.7 μkat/l) concentration throughout the 12 month follow up period
*[7] Mean Knodell HAI (excluding fibrosis score) of liver biopsies taken at the end of the 12 month follow up period
*[8] Tested by the 2-sample Wilcoxon rank sum test (sustained responders, pre-Rx vs. post-Rx)

Figure 20: Pre-Treatment HCV Viraemia Levels by Genotype (Swedish Patients)

(Geometric mean values are represented by the horizontal line)



Although the mean pre-treatment viraemia level in patients who achieved a sustained response (7.2 x 10^5 HCV genomes/ml) was approximately half of that seen in non-responders (1.2 x 10^6 HCV genomes/ml) the difference between the means was not statistically significant. Sustained virological responses were associated with lower patient age, lower pre-treatment Knodell HAI score and absence of cirrhosis, but these associations fail to reach statistical significance at the $p < 0.05$ level. None of the patients developed neutralising anti-interferon α antibodies detectable by the method of Brand *et al.* (1994).

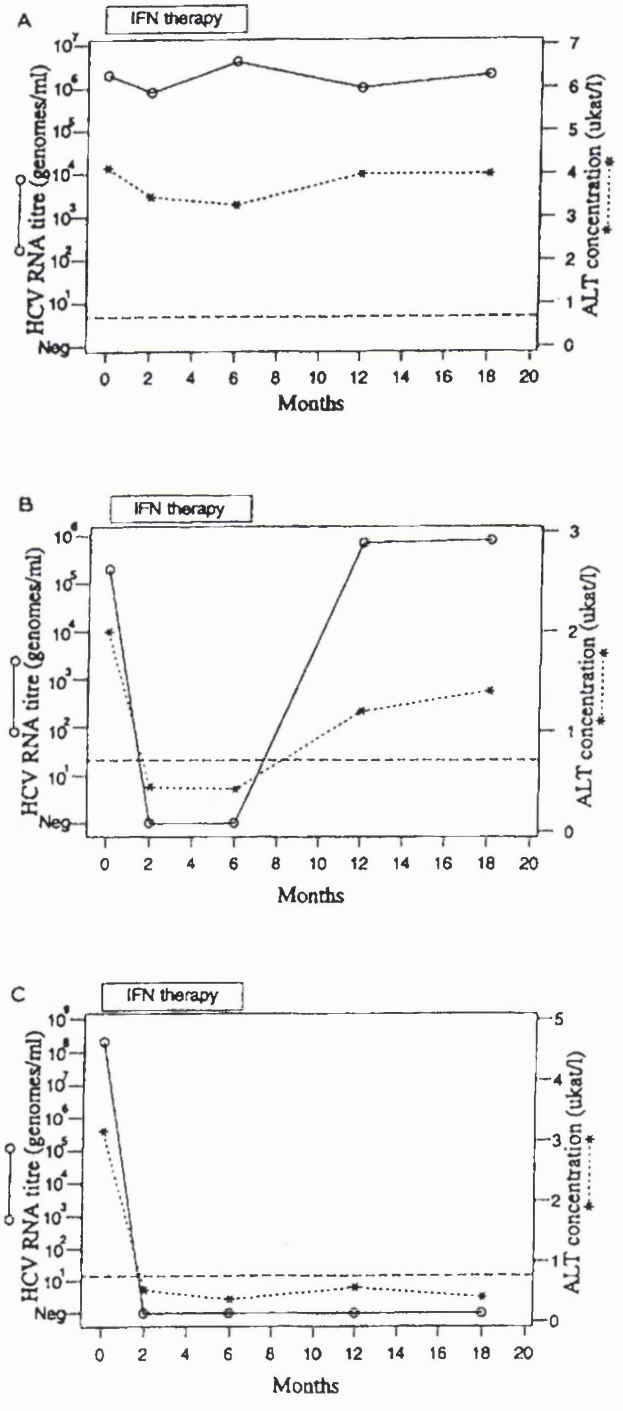
Viral, Biochemical and Histological Concordance

In 37 of 38 patients (97%) there was concordance between the response defined virologically (i.e. absence of detectable circulating HCV RNA) and serum ALT levels monitored during the 12 month follow up period (Figure 21). The one discordant case had a virological relapse at 12 months following cessation of therapy, which was not accompanied by a biochemical (ALT) relapse.

In patients who had a sustained virological response, the mean Knodell HAI score decreased significantly from 5.9 $\pm$ 0.65 to 3.3 $\pm$ 0.54 at 12 months post therapy ($p= 0.005$). By contrast, the mean Knodell HAI score increased over the same period in those who failed to achieve a sustained virological response.

Figure 21: Three Patterns of Viraemia and Transaminase Response Observed During Interferon α Monotherapy of Swedish Patients with Chronic HCV Infection

Patient A: Non-Responder, Patient B: Transient Responder, Patient C: Sustained Responder
(Note the striking correlation between the virological and biochemical response)



Adverse Effects

All four treatments regimens were generally well tolerated although there was a tendency for an increased incidence of adverse effects (mainly flu-like symptoms) with increasing dosage. Most adverse effects were mild, self-limiting and reversible on cessation of therapy. A more detailed description of interferon α related symptoms has been described elsewhere (Renault and Hoofnagle, 1989; Okanoue *et al.,* 1996).

Discussion

It is now well established that treatment with IFN-α can produce sustained biochemical and virological responses in a proportion of patients with chronic HCV infection. In the present cohort, the proportion achieving a sustained response (37%) was somewhat higher than the 15-20% reported in a number of earlier studies (Davis, 1990; Tine *et al.*, 1991). This finding may represent an improvement in response related to the use of lymphoblastoid IFN (Wellferon) rather than recombinant IFN used in many previous studies. Several (but not all) studies have now been published demonstrating higher response rates using lymphoblastoid IFN (Bacon *et al.*, 1995, Bardelli *et al.*, 1995; Malaguarnera *et al.*, 1995). This may be due to the heterogeneity of protein sequences found in lymphoblastoid IFN-α compared with the monotypic sequence of recombinant IFN-α. Alternatively, IFN purified from *in vitro* cultures of lymphoblastoid cells may have greater similarity, in terms of its post-translational modifications (e.g. glycosylation), to interferon α produced *in vivo* than does its recombinant counterpart, thereby allowing greater potency and lower immunogenicity. It is therefore interesting to note that, as reported in other studies using lymphoblastoid IFN (Antonelli *et al.*, 1991), no anti-IFN neutralising antibodies were detected in this cohort.

It is unlikely that the high rate of sustained response was due to an unusually favourable HCV genotype distribution or to particularly low viraemia levels because 50% of the patients were infected with genotype 1 virus and the viraemia levels reported in this study are very similar to those previously reported (Section 3.1).

However, the relatively low number of patients with cirrhosis (4 of 38 patients) and the relatively low mean age (39 years) in this cohort may have influenced the outcome favourably. Reichard and colleagues (Reichard *et al.*, 1994) also reported a high response rate following interferon α therapy in Swedish patients which was attributed to an extended duration of treatment.

The overall HCV genotype distribution (50% type 1, 21% type 2b and 29% type 3) observed in this study was the same as that reported by Shev *et al.* (1995) in HCV infected Swedish blood donors. The results here reinforce the view that HCV genotype is a critical factor in determining the efficacy of interferon α therapy (Hino *et al.*, 1994). Only 10.5% of patients infected with HCV type 1 achieved a sustained response as opposed to 63% of those infected with genotypes 2 or 3. The disappointing results repeatedly obtained with interferon α monotherapy for HCV type 1 infections should serve to stimulate the search for alternative treatment strategies for patients infected with this genotype.

There are conflicting reports regarding the optimal dose and duration of interferon α therapy in chronic HCV infection. Some authors report increased rates of sustained response with higher doses and longer treatment duration (Poynard *et al.*, 1995) while others find no benefit in increasing the dose beyond 3MU thrice weekly. This study finds that a greater proportion of sustained responses is associated with both longer duration of therapy and greater dose, but neither finding is of sufficient magnitude to reach statistical significance, perhaps because the number of patients in each subgroup is too small.

The results of the blind assessment of the pre and post-therapy liver biopsies are very encouraging and provide further evidence that the virological remissions induced by interferon α are accompanied by histological improvement. As inflammatory activity on liver biopsy is a marker of progression to cirrhosis, the striking reduction in this and other features of liver pathology 12 months after the end of therapy may herald long-term clinical benefit in this group of patients.

This study of Swedish patients was the last to be performed with the 'safety net' of parallel testing by qualitative 'nested' PCR. The fact that no samples were found to be positive by 'nested' PCR and negative by QRT-PCR provided strong evidence that the quantitative method was at least as sensitive as the qualitative method. The direct conjugation of alkaline phosphatase to the oligonucleotide probe used in the ELONA (Protocol 7) simplified the technique considerably and made the method more rapid.

The last of the quantitative data from this trial was generated towards the end of 1994 and this version of the protocol was then introduced to the routine diagnostic laboratory at UCLMS. Since the beginning of 1995 1,200 samples have been tested as part of the routine diagnostic service. The technique has been performed without problems by several operators and applied in numerous clinical contexts including:

- Anti-D immunoglobulin preparations implicated in the transmission of HCV to pregnant women in the Republic of Ireland were quantified by QRT-PCR and this data has been submitted for publication (Pers. Comm. Dr. J. Garson, UCLMS).
- The QRT-PCR has been used in this instance because of the sensitivity advantage that the method has over commercial detection methods.
- This sensitivity advantage is also exploited by the use of QRT-PCR for the confirmation of unexpected results generated using the Roche Amplicor HCV detection test.
- The QRT-PCR has also been used to monitor the progress of antiviral therapy in a similar manner to that described in this thesis.
- The diagnostic laboratory at UCLMS currently chooses to employ the QRT-PCR for the routine quantification of HCV RNA, the comparative evaluation of commercially available alternatives including Chiron Quantiplex assay and Roche Monitor assay.

3.4 Combination Therapy with Interferon α and Ribavirin for Chronic Hepatitis C Virus Infection in Thalassaemic Patients

Background

The confirmation of the findings of our pilot study of combination therapy by several other groups (Braconier *et al.*, 1995; Chemello *et al.*, 1995; Schvarcz *et al.*, 1995) led to a trial of this therapy in patients with thalassaemia. These patients are frequently resistant to INF-α monotherapy and it was hoped that some might respond to combination therapy. The genotyping of HCV infections in this study was performed by the detection of genotype specific antibodies. This had the advantage that cDNA could be synthesised exclusively for QRT-PCR. The use of a specific primer for cDNA synthesis had been shown in optimisation experiments to provide a slight sensitivity advantage (Section 2.3.1). The results of this trial have been published in the *British Journal of Haematology* (Telfer *et al.*, 1997) and are described below.

Introduction

Patients with thalassaemia receive regular blood transfusions in order to maintain haemoglobin levels. This leads to a much increased risk of infection with blood transmissible agents such as HCV (Wonke *et al.*,1990). HCV infection can be treated using interferon α, however the rate of sustained response is particularly low in this group of patients. A high incidence of cirrhosis and hepatic iron overload may reduce the effectiveness of this therapy. As we have shown previously, a combination of Ribavirin and interferon α may reduce the rate of relapse in HCV infected patients undergoing therapy (See Section 3.2).

Methods

Eleven patients with transfusion-dependent thalassaemia major were selected for this open label pilot study. Evidence of chronic HCV infection was demonstrated in all the patients using second generation serological testing for anti-HCV and confirmed using PCR for HCV RNA. All had been previously treated with interferon α (Intron A, Schering Plough) at three mega units thrice weekly for 6 months to 1 year and either failed to attain a response or relapsed following cessation of therapy. The interval between previous therapy and entry into the trial was at least 4 months. Exclusion criteria included clinical evidence of hepatic decompensation, symptomatic cardiac impairment requiring diuretics, angiotensin converting enzyme inhibitors or anti-arrhythmic medication, psychiatric illness, autoimmune disease, hyperuricalcaemia, renal impairment, neutropenia $<1x10^9/l$, thrombocytopenia $<100x10^9/l$, and evidence of infection with hepatitis B or HIV. All patients were informed of known side effects of both drugs and signed consent to participate. Approval for the study was given by the Whittington Hospital Ethical Committee.

Recombinant interferon α (Intron A, Schering Plough Ltd.) three mega units thrice weekly, was self administered subcutaneously together with Ribavirin (Viratek, California) 1g/day orally. Haemoglobin levels were monitored during therapy and transfusion frequency was altered to maintain a level of 9-10g/dl. If the transfusion frequency was increased, iron chelation therapy with desferrioxamine was also increased.

Baseline assessment included quantification of units of red cells transfused during the six months prior to therapy, liver biopsy for histological examination and assessment of liver iron content, HCV serotyping, HCV RNA titre, serum ferritin, a screen for autoantibodies, full blood count, serum urate, aspartate and alanine aminotransferase levels (AST and ALT).

Patients were seen at weeks 1, 3, 5, 8, 12, 16, 20 and 24 of therapy and 6-monthly thereafter for assessment of compliance, adverse effects, transfusion requirements, full blood count and serum biochemistry (AST and ALT). Treatment with both drugs

was stopped following evidence of neutropenia or thrombocytopenia. When the counts had regenerated treatment was restarted at a lower dose regime (800mg/day of Ribavirin and 2MIU thrice weekly of IFN-α).

Serum samples were separated after standing for 2 hours and stored at temperatures -20°C. HCV viraemia was monitored following extraction with RNAzol A, reverse transcription with MMLV-RT and amplification with *Pfu* polymerase (Section 2.2, 2.3.1, 2.4.1 and 2.4.2 (protocol 7)). Baseline serum samples were assayed for HCV genotype specific antibodies (Murex serotyping 1-6 assay; Van Doorn *et al.*, 1996). Samples which could not be genotyped by this method were typed by means of a reverse hybridisation line-probe assay (Stuyver *et al.*, 1993).

Transient response to therapy was defined as loss of detectable serum HCV RNA by the end of treatment, with reappearance within 6 months. Sustained response was defined as a loss of detectable serum HCV RNA by the end of therapy, maintained for at least 6 months post cessation of therapy.

Table 14: Baseline Characteristics of Trial Patients

Case	Age at start of therapy (yr.)	Chelation	Response to previous IFN monotherapy	Histology	HCV RNA Titre (gen./ml)	HCV genotype	ALT (IU/ml)	Liver Iron (µg/100mg)	Ferritin (µg/l)
1	24.5	DFO	NR	Cirrhosis	$1x10^7$	3	110	270	6955
2	42.9	DFO	TR	Chr. Hep.	$2x10^6$	1	90	431	5341
3	28.6	DFO	TR	Chr. Hep	$1x10^6$	3	20	148	928
4	16.0	DFO	NR	Cirrhosis	$3x10^6$	1	219	393	5933
5	35.5	DFO	TR	Cirrhosis	$1x10^8$	3	114	190	2217
6	23.7	DFO	NR	Cirrhosis	$8x10^6$	1	156	2770	3522
7	36.4	L1	NR	Cirrhosis	$5x10^6$	5	144	2270	3126
8	24.9	DFO	NR	Cirrhosis	$7x10^5$	3	50	3850	474
9	28.1	L1	NR	Cirrhosis	$3x10^6$	3	187	387	1727
10	21.2	L1	NR	Cirrhosis	$2x10^7$	1	129	197	1563
11	11.2	DFO	NR	ND	$5x10^7$	1	56	590	855

Abbreviations: DFO: desferrioxamine; L1: deferiprone; ND: not done; NR: no response; TR: transient response; Chr. Hep.: Chronic Hepatitis.

Results

A sustained response was seen in five patients (45.5%; cases 1-5, Table 14). A transient response was seen in two patients (18.1%; cases 7 and 8). There was no response to therapy in the remaining four cases (36.4%). Loss of HCV RNA was rapid in the sustained responders (Figures 22 and 23). Two had cleared serum HCV RNA by the end of the first week of therapy and all had cleared HCV RNA by the twelfth week. Conversely, none of the patients who were still HCV RNA positive by week 12 attained a sustained response. Three of eight cirrhotic patients and two of the five patients infected with genotype 1 attained a sustained response to combination therapy. There was no significant difference in age, baseline HCV RNA levels, HCV genotype, liver iron concentration, serum ferritin, or baseline serum ALT levels between sustained responders and non-sustained responders.

In general, IFN-α related 'flu-like' symptoms were milder than had been experienced on prior IFN-α monotherapy and compliance was excellent in all but one patient. Mild neutropenia developed in three patients but was reversible following dosage reduction. During the 6 months of treatment, transfusion requirements were significantly increased compared with the previous period (median increase 41%, range 25-94%, p=0.004). These changes are illustrated in Figure 22.

Figure 22: Change in HCV RNA Titre During the First Week of Therapy

(Geometric means are represented by the horizontal line)

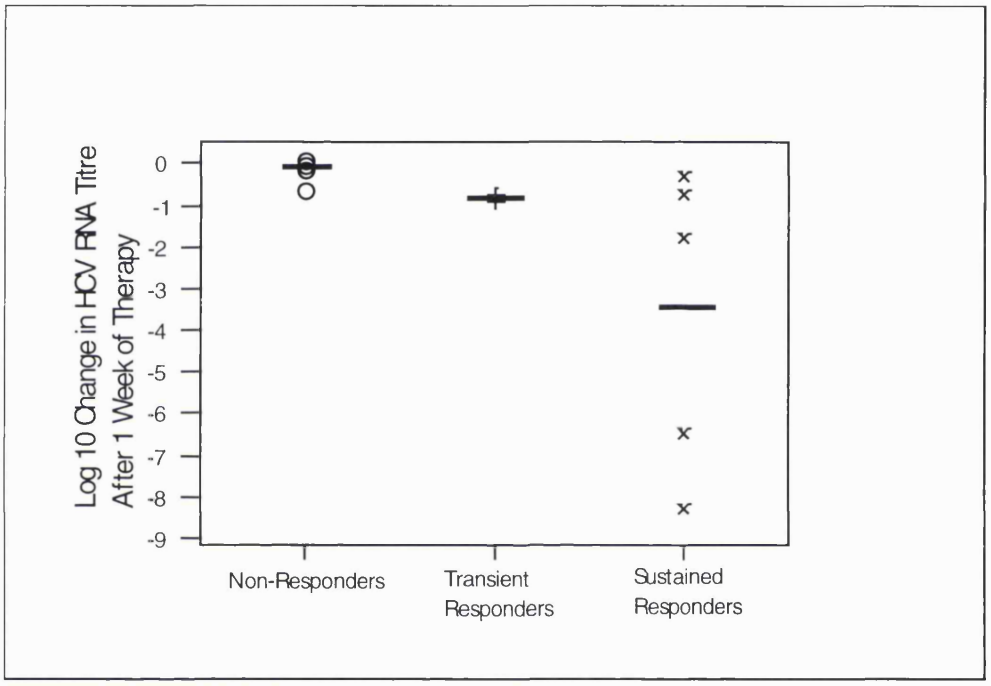


Figure 23: Serial HCV RNA Titres in Thalassaemic Patients Receiving Ribavirin + INF-α Combination Therapy

(a) Patients achieving a sustained response to therapy.

(b) Patients who failed to achieve a sustained response to therapy (the response in patients 7 and 8 was transient).

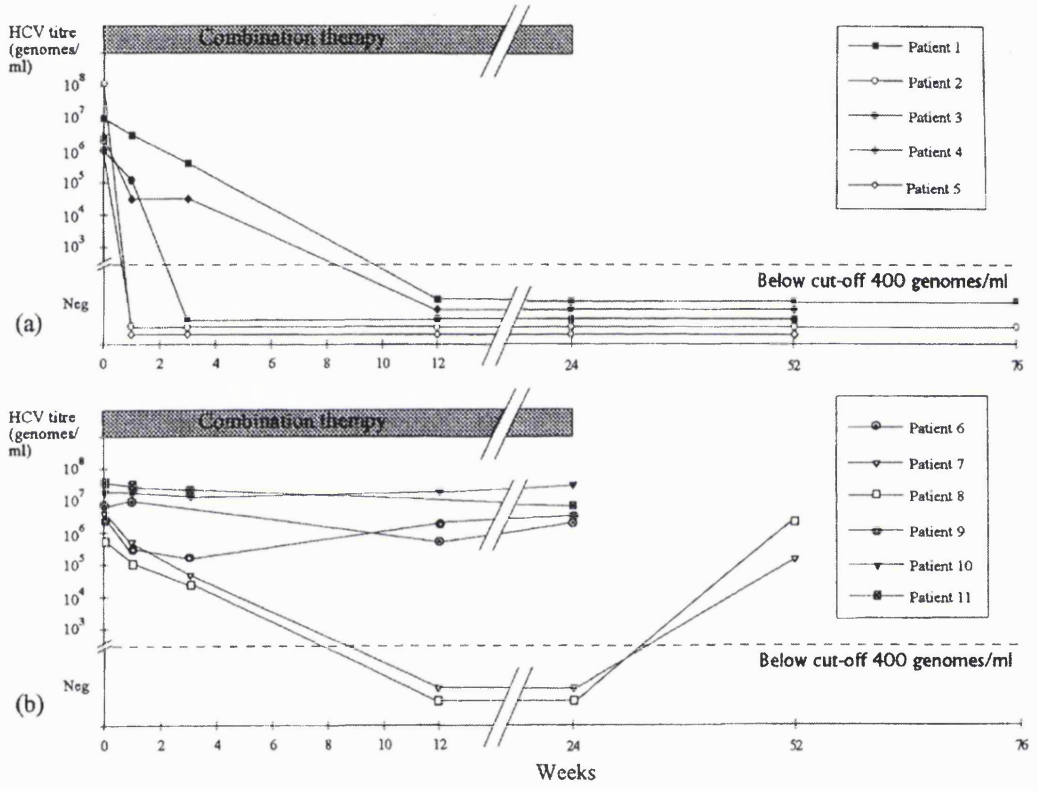
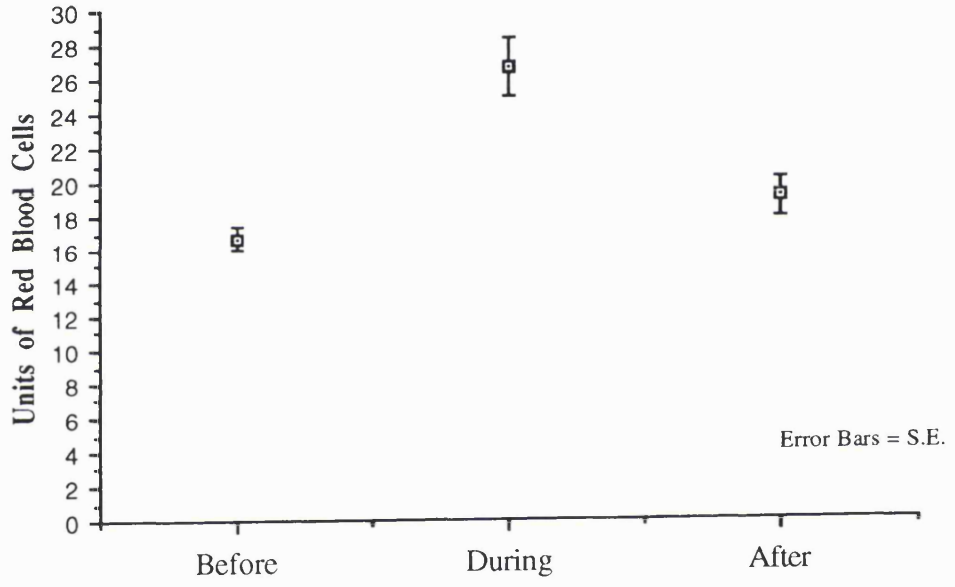


Figure 24: Transfusion Requirements (Red Cells) During the 6 Months Before During and After Therapy

Median values are represented by the horizontal line, the interquartile ranges are represented by the boxes and the full range is indicated by the vertical line



Conclusions

This pilot study has shown that sustained clearance of serum HCV RNA can be obtained in nearly half of thalassaemic patients with chronic HCV infection who have failed therapy with interferon α monotherapy. The major side effect was a significant increase in transfusion requirements during the treatment phase.

Significant mortality due to liver disease has been reported in the long term follow up of multiply-transfused thalassaemic patients (Zurlo *et al.,* 1989). Multiple transfusions present a significant risk of liver damage for two reasons. The haemolysis of transfused red blood cells causes parenchymal iron accumulation leading to cirrhosis, however with regular chelation therapy with desferrioxamine, progression of fibrotic changes on serial biopsy is predominantly due to viral hepatitis (Aldouri *et al.,* 1987). There is therefore an urgent requirement for effective therapy to eradicate HCV in the 75-90% of patients who do not obtain a long-term response with IFN-α monotherapy.

Previously in this thesis we reported that 4/10 patients who had failed to attain a sustained response to IFN-α monotherapy cleared detectable HCV RNA from their serum following combination therapy with IFN-α and Ribavirin (Section 3.2). These findings have now been confirmed by two further pilot studies (Schvarcz *et al.,* 1995; Braconier *et al.,* 1995). In addition Schalm and co-workers performed a meta analysis of both open label and randomised controlled trials the outcome of which supports the theory that this combination represents an effective therapy as a second line therapy for HCV infection following the failure of IFN-α monotherapy. The similar response rate in thalassaemics and non-thalassaemics is perhaps surprising given that many of the multiply transfused thalassaemic patients have cirrhosis and that cirrhotic patients have a poor response rate to IFN-α monotherapy. This may in part be due to the thalassaemic patients having a significantly lower mean age than the non-thalassaemic patients described in the previous section (Section 3.2; 26.6 [±2.8] years vs. 43.3 [± 6.1] years; p=<0.05).

We opted to use clearance of serum HCV RNA as the criterion for treatment response and have elected not to include normalisation of serum transaminase levels, since these may be elevated in multiply transfused patients due to the concomitant toxic effect of iron in the liver (Section 3.4). This choice has been justified by the demonstration of persistent and intermittent transaminase abnormalities in two of the patients who had a sustained loss of HCV RNA.

Patients who have no detectable HCV RNA in their serum 6 months following cessation of IFN-α monotherapy are unlikely to have a later relapse of HCV infection with reappearance of HCV RNA (Shindo *et al.,* 1991). Nevertheless, the theoretical possibility that combination therapy might simply delay the onset of a relapse needs to be kept in mind. However, persistent PCR negativity has been demonstrated in two of the sustained responders at 1 year post therapy.

In contrast to previous trials of antivirals active against HCV, the viral titre was assessed frequently during the early part of therapy and a rapid early drop in HCV RNA level appears to be associated with a sustained response (See Figure 22 and 23). All of those negative for HCV RNA after 3 weeks of therapy were sustained responders and 5 of the 7 who were negative by week 12. The presence of detectable HCV RNA at week 12 appears to predict a lack of sustained response and therapy could probably be discontinued at this stage for these patients.

In conclusion, combination therapy appears to bring about viral eradication in a significant proportion of thalassaemic patients with chronic HCV infection. For those who respond favourably the risk of hepatic failure or hepatocellular carcinoma in the future is likely to be much diminished. It seems likely that the risks associated with increased transfusion requirements for the duration of treatment are outweighed by the potential benefits. This therapeutic strategy is likely to become the treatment of choice for those patients who fail to attain a sustained response to IFN-α monotherapy, if not the front line therapy for the treatment of HCV infection.

The application of quantitative PCR at an early time-point (1 week) during therapy allowed the comparison of rates of decline in patients who achieved a sustained response to therapy with those who did not. Such an approach had previously been used to estimate the replication rate of HIV-1 (Ho *et al.,* 1995). If the fact that any of these patients achieved a sustained response to therapy was a little surprising, given that this group had a reputation for being resistant to therapy, the rate at which viraemia declined in 2/5 of the patients achieving a sustained response was quite dramatic. Clearing 1.2×10^8 and 2×10^6 HCV genomes/ml of HCV RNA to undetectable levels in the space of one week seemed remarkable (Figure 23). These results hinted that HCV might have a rapid replication rate and it has now been reported that the replication rate of HCV is estimated as 3.7×10^{11} genomes/day (Lam *et al.*,1997).

3.5 A Pilot Study of Lamivudine (3TC) and Zidovudine (AZT) Combination Therapy in HCV / HIV Co-Infected Patients

Background

In all of the studies described in this thesis so far the antiviral agents have produced large changes in HCV viraemia level in response to therapy. The next two studies describe trials of the nucleoside analogue 2',3'-dideoxy-3'-thiacytidine (3TC or Lamivudine, Glaxo Wellcome) which has been shown to be effective against both HIV-1 and HBV.

Introduction

3TC/Lamivudine has been shown to possess antiviral activity against HIV-1 (Sharp, 1996). Patients co-infected with hepatitis B virus (HBV) and HIV-1 that were treated with Lamivudine showed a marked reduction in the titre of both viruses (Benhamou *et al.,* 1996). Lamivudine has since been shown to be an effective treatment for HBV infection (Bain *et al.,* 1996). This study was designed to assess whether similar results might be obtained with HCV co-infected patients.

Methods

Protocol

Five patients, enrolled in a trial of the nucleoside analogue 3TC in combination with Zidovudine for the treatment of HIV-1 infection, were found to be co-infected with HCV. Trial participants came from 3 treatment arms: AZT monotherapy (200mg three times per day); AZT (same dose) plus 3TC (300mg twice daily); and AZT (same dose) plus 3TC (150mg twice daily). In addition, these patients may have received anti-microbial therapy to treat or prevent concurrent opportunistic infections.

Serum HCV RNA titres were measured by QRT-PCR (Sections 2.3.1, 2.4.1 and 2.4.2 (protocol 7)), immediately before treatment, after 12 weeks of treatment and after 24 weeks of treatment.

Statistical Analysis

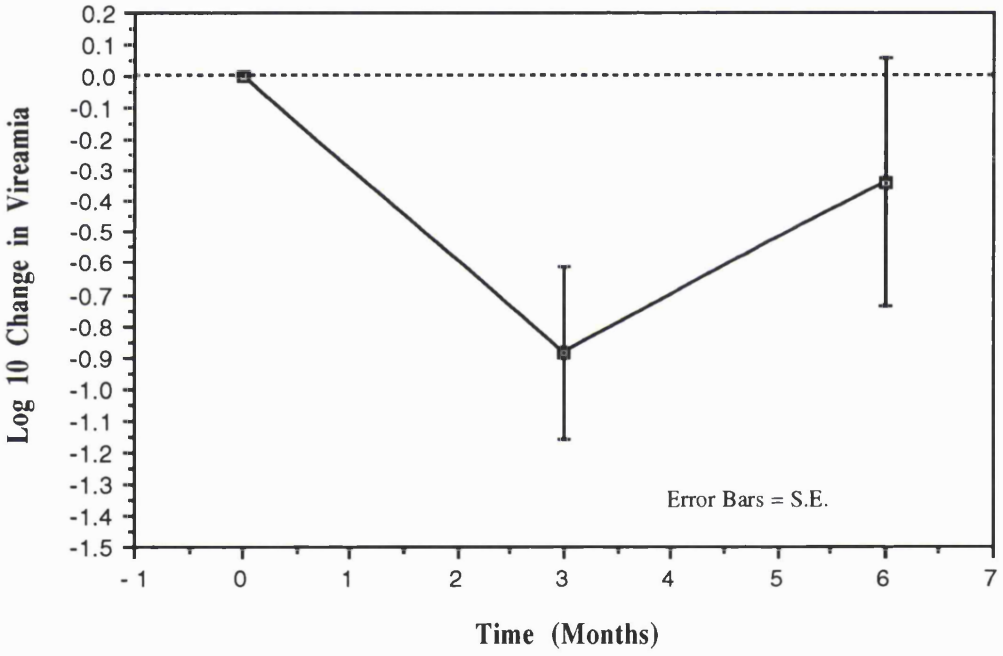
The statistical significance of any differences was measured using the paired *t* test.

Results

Viraemia levels prior to treatment ranged from 3×10^6 to 2×10^7 HCV genomes/ml with a geometric mean titre of 5.7×10^6. Four of the five patients treated with 3TC had a reduced level of viraemia following 12 weeks of therapy but this drop was not sufficiently great to have reached statistical significance (95% C.I. -1.43 to 0.41; p=0.1), but by week 24 when therapy was stopped all patients had viraemia levels below that of their respective baseline sample and this difference was statistically significant (95% C.I. -0.96 to -0.22, p=0.006; Figure 25).

Figure 25: Viraemia Levels During Therapy with 3TC in HCV and HIV Co-Infected Patients

Trial participants came from 3 treatment arms: AZT monotherapy (200mg three times per day); AZT (same dose) plus 3TC (300mg twice daily); and AZT (same dose) plus 3TC (150mg twice daily). HCV RNA titres were monitored for 6 months of therapy.



Conclusions

Experience from trials of nucleoside analogues in the treatment of HIV-1 infection demonstrate the rapid development of resistance to these agents and its detrimental effect on the outcome of therapy. Resistance also develops when antivirals are used in combination, although this tends to occur more slowly. This may be due to a slowing of the viruses replication rate and therefore its capacity to generate resistant mutants.

Unfortunately, sustained viral clearance is only seen in 15-20% of chronically HCV infected patients treated with IFN-α as a monotherapy. Improvements in sustained response rates have been observed using lymphoblastoid IFN (Bacon *et al.*, 1995; Section 3.3) and by using IFN-α in combination with the nucleoside analogue Ribavirin (Schalm *et al.*, 1996; Section 3.2 and 3.4). However, even with these important improvements in treatment less than half of the patients treated have a sustained clearance of viraemia. It is therefore important that new antiviral compounds are identified to complement or replace existing therapies.

The results of this small pilot study show that levels of HCV viraemia are reduced following the treatment of HIV-1/HCV co-infected patients with the nucleoside analogue 3TC in combination with AZT. 3TC has been used to treat HIV-1 infection and has been shown to produce a transient reduction in viraemia level with a concomitant improvement in immune function as measured by an increase in the level of circulating CD4 positive T lymphocytes (Phillips *et al.*, 1996). Combined use of the anti-retroviral agents 3TC and AZT increases the longevity of this response (Eron *et al.*, 1995). High levels of HCV viraemia have been reported in immunocomprimised patients either as a result of HIV-1 infection, immunosuppresive drugs, neoplasia, or genetic factors (Ghany *et al.*, 1996), presumably as a result of the immune system failing to clear the virus as quickly as it is being produced. The improvement of immune function in this cohort may have been sufficient to explain the reduction in HCV viraemia observed in this study. It is difficult to determine from these results if 3TC is also acting directly to suppress HCV replication. The results of this study were however regarded as sufficient to

warrant a second trial of this nucleoside analogue in the treatment in patients infected with HCV alone.

3.6 A Trial of Treatment of HCV Infection with Lamivudine (3TC)

Introduction

2',3'-dideoxy-3'-thiacytidine (3TC or Lamivudine, Glaxo Wellcome) has been shown to act as a nucleoside analogue capable of inhibiting the reverse transcriptase of HIV-1 and the polymerase of HBV. Standard therapeutic doses are of relatively low toxicity compared with other nucleoside analogues. The serum titre of HCV RNA in patients co-infected with HIV appears to decline during Lamivudine/AZT combination therapy (Section 3.5). This second pilot study was designed to determine if the effect is directly attributable to an antiviral action of Lamivudine against HCV.

Methods

Protocol

A total of nine patients shown to be infected with HCV, but not infected with HIV-1 or HBV were enrolled into this open label, single centre trial. Patients received 300mg of 3TC twice daily for 12 weeks. The study population comprised 8 males and 2 females. Immediately before therapy the serotype of HCV was determined (HCV serotyping kit 1-6, Murex) and HCV RNA titres were measured using QRT-PCR (Section 2.4). The HCV RNA titre was monitored at monthly intervals during the three months of therapy and also at one and three months post therapy. At each time point liver function was monitored by measuring serum ALT levels.

Results

The geometric mean pre-treatment HCV RNA titre of these 9 patients was shown to be 1 x 10^7 HCV genomes/ml (S.D. $\pm 10^{0.8}$ HCV genomes/ml). As can be seen in Figure 26 the mean HCV RNA titre in the serum of these patients declined during therapy. That decline reached statistical significance by the end of therapy (3 months; 95% C.I. -0.25 to -1.52 $\log_{10}$ HCV genomes/ml, p= 0.012). On cessation of therapy the mean HCV RNA titre and serum transaminase levels returned to pre-treatment levels and remained at this level throughout the follow-up period.

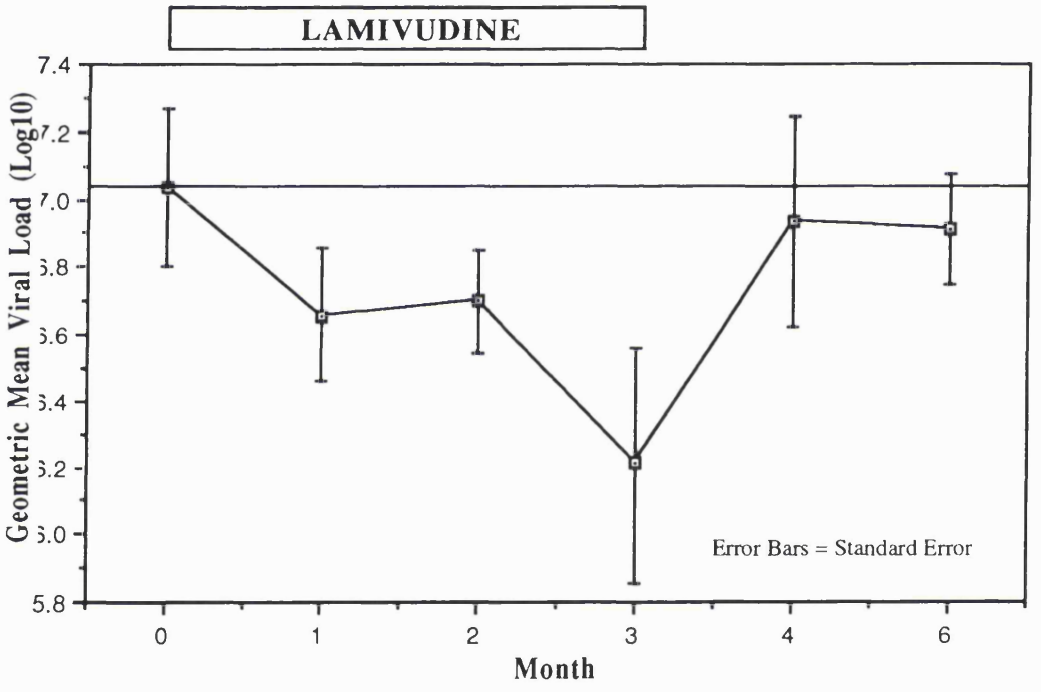
Table 15: HCV Viraemia Level During Therapy with Lamivudine (3TC)

Patient Code	Serotype **	Baseline	Month 1	Month 2	Month 3	Month 4	Month 6
1	✞	7.8	7.3 (-0.5)	7.0 (-0.8)	7.2 (-0.6)	6.9 (-0.9)	7.7 (-0.1)
2	2	6.2*	6.9 (0.7)	6.7 (0.5)	4.1 (-2.1)	nt[1]	6.9 (0.7)
3	2	8.0	5.8 (-2.2)	6.1 (-1.9)	6.5 (-1.5)	5.6 (-2.4)	6.5 (-1.5)
4	3	7.6	7.0 (-0.6)	6.9 (-0.7)	5.4 (-2.2)	nt[1]	6.6 (-1.0)
5	3	6.0	5.9 (-0.1)	6.2 (0.2)	5.9 (-0.1)	nt[2]	6.5 (0.5)
6	3	6.3	6.0 (-0.3)	7.0 (0.7)	5.7 (-0.6)	7.7 (1.4)	7.5 (1.2)
7	3	7.4	7.3 (-0.1)	nt[1]	7.0 (-0.4)	7.3 (1.0)	6.7 (-0.7)
8	1	6.7	7.0 (0.3)	6.4 (-0.3)	6.6 (-0.1)	6.6 (-0.1)	6.9 (0.2)
9	1	6.6	6.7 (0.1)	7.3 (0.7)	6.2 (-0.4)	7.5 (0.9)	nt[1]

HCV RNA Titres expressed as Log_{10} genomes/ml (figures in brackets indicate change in viral load with respect to baseline)
* Sample haemolysed, pre-screen sample 6.9.
** Simmonds' Nomenclature.
✞ Not Typable by Murex (1-6) Serotyping Kit.
nt[1] Unable to locate sample.
nt[2] Did not attend clinic following treatment, due to illness.

Figure 26: Geometric Mean HCV RNA Titres During an After Lamivudine Therapy

Nine HCV infected patients were treated with 3TC (200mg thrice daily) for three months. HCV RNA titres were measured using QRT-PCR before therapy, during therapy and during a 3 month follow up period.



Conclusions

A decline in viraemia level was observed in a previous study (Section 3.5) when HIV-1/HCV co-infected patients were treated with 3TC and AZT. This study demonstrates that a similar decline can also be observed in patients not infected with HIV-1, treated with 3TC alone. This provides evidence that the antiviral effect observed in the earlier study was not simply a result of the recovery of immune function, but probably a specific anti-HCV effect. 3TC is phosphorylated *in vivo* and has been shown to inhibit the function of HIV-1 reverse transcriptase by competing with deoxyribonucleotides for the enzyme's active site. One might speculate that the antiviral effect observed here occurs as a result of phosphorylated 3TC binding to the active site of the RNA dependant RNA polymerase encoded by HCV and blocking the incorporation of ribonucleotides.

IFN-α treatment of patients chronically infected with HCV results in a rapid decline in viraemia levels in approximately half of those treated. Unfortunately, sustained clearance of viraemia is only seen in 15-20 % of those treated. The rate of sustained clearance can be increased by treating with a combination of IFN-α and Ribavirin (Schalm *et al.*,1996; Section 3.2; Section 3.4). 3TC like Ribavirin is also a nucleoside analogue and both share relatively low toxicity compared with other nucleoside analogues. Ribavirin when used as a monotherapy for the treatment of HCV infection causes a transient normalisation of serum transaminases in a proportion of those treated (Hoofnagle *et al.*, 1996), but does not induce viral clearance or a significant reduction in viraemia. The development of novel candidate antivirals for the treatment of HCV infection has been hampered by a lack of an efficient culture system or convenient animal model for screening purposes. Trials to date have therefore been restricted mainly to pre-existing antivirals already shown to be effective against other RNA viruses. The end points chosen during these trials have usually been the normalisation of serum transaminase levels or clearance of viraemia. The trials described in this thesis demonstrate that virological monitoring is usually more reliable than biochemical monitoring presumably because ALT levels can be raised as a result of factors other than hepatic viral replication (Sections 3.4 and 3.10). The use of a sensitive, accurate QRT-PCR for the measurement of

viraemia allows the identification of more subtle antiviral effects. In this study monitoring viraemia has allowed the detection of a significant reduction in viraemia in the absence of viral clearance, thereby identifying 3TC as a candidate antiviral for potential use either as dual therapy with IFN-α, or as triple therapy in combination with both IFN-α and Ribavirin.

The application of QRT-PCR to the monitoring of HCV RNA levels in patients treated with 3TC has allowed a subtle antiviral effect to be detected which could not have been observed using qualitative techniques. Although this agent (like Ribavirin) is unlikely to have any significant contribution to make as a monotherapy for HCV infection, it would appear to be a sensible candidate for trial in combination therapy.

The two versions of the QRT-PCR used to monitor the trials of antivirals described in this thesis were both able to quantify HCV RNA over the full range of titres typically seen in patients chronically infected with HCV. This technology represented a significant improvement over the use of limit dilution/ Poisson distribution techniques in terms of ease of use, allowing the generation of the data reported here. Although alternative commercial assays became available, these QRT-PCR techniques have remained the method of choice within both the research and diagnostic areas of the UCLMS laboratory. This has been justified because these methods provide accurate quantitative data with greater sensitivity (10 to 100 fold) than commercial assays and at a fraction of the cost.

3.7 Acute Hepatitis C Viral Infection During Pregnancy: Failure of Mother to Infant Transmission

Background

In addition to quantitative virological monitoring of therapeutic trials QRT-PCR was also applied to other aspects of HCV research. The following study has been published in the *Journal of Medical Virology* (Zuckerman *et al.*, 1997) and describes the application of the QRT-PCR technique to the study of an acute HCV infection during pregnancy.

Introduction

Estimates regarding the extent of mother to infant transmission of HCV vary (Reesink *et al.*, 1990; Inoue *et al.*, 1991; Thaler *et al.*, 1991; Lam *et al.*, 1993). The rate of transmission appears to increase in mothers co-infected with HIV, possibly due to an increase in viral load secondary to immunosuppression (Zanetti *et al.*, 1995). This suggestion is supported by the findings of a prospective study of pregnant chronic HCV carriers in whom the risk of transmission was correlated with the maternal serum HCV RNA titre (Ohto *et al.*, 1994).

These and other reports of vertical transmission have focused on chronic carriers of HCV. However, the risk of transmission related to acute HCV infection is unknown. The clinical, serological and virological findings in a woman who developed an acute HCV infection during pregnancy and whose child was not infected, are reported here.

Case Report

A 23 year old woman visited Pakistan with her family, during her second trimester, between April and July 1991. Her pregnancy progressed uneventfully until October, when, at 29 weeks, she was icteric, with no other findings of note on examination. Ultrasound of the liver and common bile duct was normal, haemoglobin was 10.5g/dl, urinalysis revealed bilirubinuria and liver function tests were abnormal with serum levels of ALT 123 IU, alkaline phosphatase 186 IU and bilirubin 97 μmol/l. Hepatitis A IgM, HBsAg and HCV antibodies (Ortho, HCV ELISA Test System, second generation, Raritan, New Jersey, USA) were not detected. There was evidence of past HCMV and EBV infection and hepatitis E was considered in the differential diagnosis.

The mother had a vaginal delivery on November 14, 1991. A further maternal serum specimen was tested for HCV antibody and was reactive with a signal to cut-off ratio of 1.9, this had risen to 2.6 within 4 days. Supplemental testing produced indeterminate results, with only antibodies against the core antigen C22 detected (RIBA-2, Chiron RIBA HCV Test System, Raritan, New Jersey, USA). The presence of HCV specific antibodies was confirmed by January 1992. Despite a careful history being taken, no risk factors for acquiring hepatitis C were ascertained.

HCV antibody was not detected in serum collected from the baby at birth. The lack of passively acquired maternal antibody was likely to be due to seroconversion occuring during the perinatal period. HCV RNA was detected retrospectively in two maternal samples preceding birth (July 30th and October 10th) and in two samples taken immediately following birth (November 18th and November 22nd), using a nested PCR protocol previously described (Garson *et al.,* 1990b). The presence of hepatitis C viraemia in the absence of maternal antibody may have resulted in the mother potentially being highly infectious. The genotype [3a] was determined by a reverse hybridisation method (Stuyver *et al.,* 1993). The baby was breast fed, but aliquots of breast milk were shown not to contain detectable amounts of HCV RNA. A liver biopsy from the mother in April 1993 was consistent with the diagnosis of

chronic viral hepatitis. Therapy with IFN-α (3 MIU 3 times a week) was initiated in January 1995 but was discontinued after 3 months due to a lack of response.

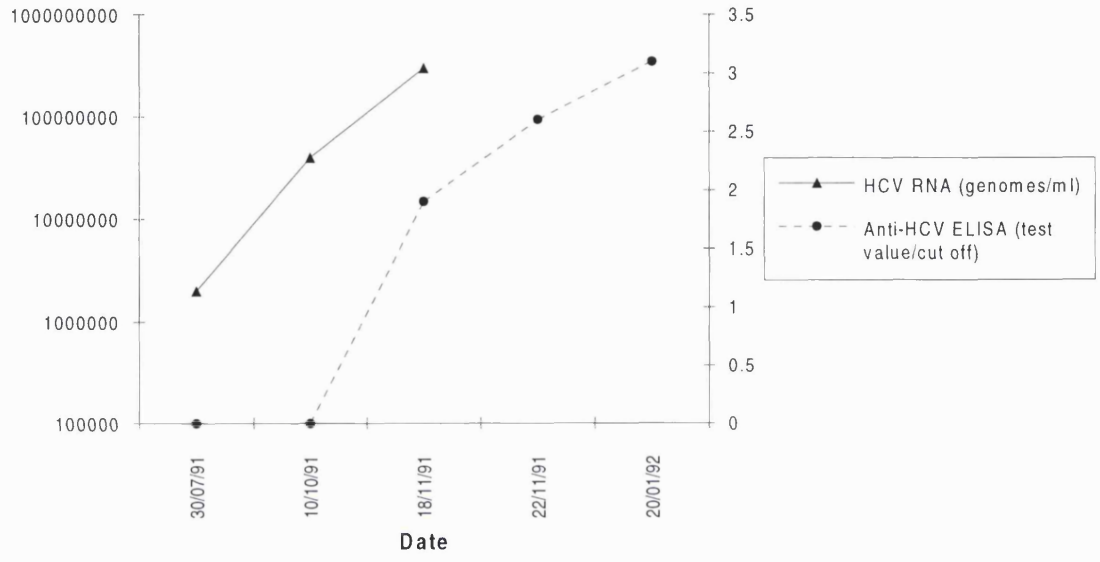
HCV RNA was not detected in serum samples collected from the baby over the first 10 months and neither HCV antibody nor HCV RNA was detected when the child was 4 years of age. HCV antibody was not detected in serum samples collected from the father and from two older children in 1992 and 1995.

Quantitative Analysis.

Aliquots of maternal serum stored at -20°C were tested retrospectively using the QRT-PCR method described in Section 2.4.2 (Protocol 7). By the time of delivery the viral titre had risen by over 2 $logs_{10}$ (Figure 27).

Figure 27: Maternal Serum Levels of HCV RNA and HCV Reactive Antibodies Following Acute HCV Infection During Pregnancy

The baby was born on November 14th 1991.



Conclusions

The large Japanese study described by Ohto *et al.* (1994) reported that mothers with a serum HCV RNA titre in excess of 10^6 HCV genomes/ml usually transmit the infection to their offspring. It is therefore surprising that a mother with a viraemia level of $>10^8$ HCV genomes/ml around the time of delivery did not give birth to an infected child. However, direct comparisons of viraemia level with those in other reports may lack validity, as without reference to a quantification standard different methods may yield widely discrepant estimates of viral load. The high viraemia level noted in this case may be due to viral replication prior to the development of a specific immune response. A rising viraemia level following infection reaching a high titre is the pattern typically seen in acute HCV infection; this is usually followed by a sharp decline as a result of partial immune clearance. However, HCV viraemia levels are known to be influenced by the level of immune competence of the infected individual and pregnancy itself itself is somewhat immunosuppressive (Davies and Browne, 1985).

One might speculate that in addition to the level of viraemia, genotype may influence the risk of transmission. Although materno-foetal transmission did not occur after this type 3a infection, a prospective study reported 6 children with HCV infection born to mothers with HCV reactive antibody, some of whom had HIV co-infection. Four of these children had genotype 3a infections (Zuccotti *et al.,* 1995).

Based on the data generated by Ohto *et al* (1994) it is reasonable to suggest that quantitative techniques for measuring HCV RNA will become important in the management of HCV infected mothers. Clearly the value of the information reported here and by Ohto *et al* could have been greatly increased had an internationally recognised quantification standard been available.

3.8 Monitoring of Viral Levels During Attempts to Culture HCV *In Vitro*

Background

The *in vitro* propagation of viruses has often proved to be a vital step in the development of vaccines and antivirals for the treatment of viral infections. The development of efficient tissue culture systems for the propagation of HCV has been the goal of many laboratories including our own.. There follows a description of one experiment which serves to illustrate the use of QRT-PCR in this context.

Introduction

The development of antivirals active against HCV has been hampered by the lack of a efficient tissue culture model of HCV replication. The tissue culture systems developed to date have included the infection of lymphocyte cell lines (Shimizu and Yoshikura, 1995), or the use of *ex vivo* infected human liver (Ito *et al.,* 1996). The lymphocyte lines tend to produce very low levels of virus intermittently. Although higher levels of virus are produced from *ex vivo* infected human liver, biopsy material is not usually readily available and adequate storage techniques have not yet been developed. In 1992 Moshage and Yap reported the maintenance of differentiated human hepatocytes in cell culture by using cocktails of differentiating agents. Currently a tissue culture system is being developed in our laboratory using such differentiating agents. By optimising the differentiation state of cultured hepatocytes it is hoped that HCV will be able to replicate in this system.

This section of the thesis describes the application of QRT-PCR to this optimisation process. The culture conditions described here were developed by JA Garson and D Lubach, UCLMS Department of Virology and do not represent the conditions currently in use (Garson *et al.,* 1999).

Methods

Culture Conditions

Hepatoma cell line HepG2 was grown as a monolayer in RPMI 1640 medium (Gibco BRL) containing 10% heat-inactivated foetal calf serum (FCS). All media contained 100 units/ml penicillin G, 100 µg/ml streptomycin and 2.5µg/ml fungizone to inhibit bacterial and fungal growth. Cultures were maintained at 37°C in a 95% air/5% CO_2 atmosphere in 25 cm^2 tissue culture flasks coated with collagen type 1 and fibronectin (Sigma). This cell line has previously been tested for HCV RNA by PCR and found to be negative (data not shown).

HepG2 had shown encouraging results during a preliminary screening experiment, designed to determine whether any of a number of hepatoma cell lines might be susceptible to HCV infection.

Once the cell numbers had increased sufficiently the growth medium was replaced with serum free medium (SFM) which was RPMI 1640 with synthetic FCS replacement (2% LPSR-1; Sigma), Na_2SeO_3 3 x 10^{-8} M, tri-iodo-L-thyronine 10^{-9} M (Sigma), 17-beta estradiol 10^{-8} M (Sigma) and apolipoprotein 5 x 10^{-8} M (Sigma).

Inoculation Technique

RPMI 1640 medium containing 10% FCS was replaced, 16 hours prior to inoculation, with serum free medium supplemented with a cocktail of differentiating agents (G medium). Sub-confluent monolayers were inoculated with three sera from three hepatitis C infected patients ("L", "W", and "P"), at a multiplicity of infection of approximately one HCV RNA molecule per cell and with normal human serum. All sera were diluted to 2% in G medium and incubated for 18-24 hours at 37°C. The inoculum was removed and the cells exposed for 12 or 24 hours to high concentrations (Bchini *et al.*,1990) of insulin (2.7 x 10^{-5} M) and dexamethasone (1.3 x 10^{-4} M) diluted in G medium. The cells were then washed five times in PBS (dilution factor approximately 10^8) and then passaged, using cell dissociation solution (Sigma), into new flasks coated with collagen and fibronectin. The cells in

these flasks were maintained in G medium supplemented with low concentrations of insulin and dexamethasone (2.7×10^{-6} M and 1.3×10^{-6} M respectively; Bchini *et al.*, 1990). Medium was changed weekly and a 200µl sample of supernatant was tested by PCR for HCV RNA by QRT-PCR as described in Sections 2.3.1, 2.4.1 and 2.4.2 (protocol 7). After 31 days the cells were harvested by removing the culture medium, washing the cells in PBS, incubating in dissociation solution, spinning the cells down in G medium (1200 rpm for 5 minutes) and resuspending in PBS. Half of the harvested cells were extracted using RNAzol A and HCV RNA levels were determined using the QRT-PCR method described in Sections 2.3.1, 2.4.1 and 2.4.2 (protocol 6).

Results

HCV RNA levels were monitored in tissue culture supernatant after 11, 19 and 31 days post-inoculation. RNA was only detectable in the supernatant of cells infected with 'Serum L' 11 days post-inoculation (Table 16). Five weeks post inoculation all culture supernatants were HCV RNA negative and the number of viable cell was dropping rapidly in all four of the cultures. In order to maximise the potential data generated from this experiment, RNA was extracted from half of each culture and the other half was replated using the same culture conditions. The HCV RNA levels were expressed as the estimated number of genomes per cell extracted. The cultures inoculated with 'Serum L', 'Serum W' and 'Serum P' contained 26, 1 and 12 HCV RNA molecules per cell respectively. Cells infected with normal human serum were, in contrast, negative for HCV RNA.

Table 16: HCV RNA Titre in Tissue Culture Supernatants

	11 Days	19 Days	31 Days
Serum L	5 x 10^4 HCV genomes/ml	Negative	Negative
Serum W	Negative	Negative	Negative
Serum P	Negative	Negative	Negative
Normal Human Serum	Negative	Negative	Negative

Table 17: HCV RNA Titre in Cultured Hepatocytes

	31 Days
Serum L	26 RNA molecules per cell
Serum W	1 RNA molecule per cell
Serum P	12 RNA molecules per cell
Normal Human Serum	Negative

Conclusions

Detection of HCV RNA at the first time point tested (day 11) may have been the result of residual inoculum. The cells were infected at a multiplicity of infection of one RNA genome per hepatocyte; the fact that the ratio of RNA copies per cell rose in two of the cultures (L and P) may indicate active replication of the genome. It is unfortunate that this experiment was designed to detect cell-free virus as these results would suggest that the virus is cell associated. Harvesting of the cells effectively terminated the experiment and cell pellet data could therefore only be generated at one timepoint. Repeating this experiment with multiple replicates of flasks would allow any variation of the RNA titre with time to be observed. In addition, further controls will be required to differentiate replicating virus from virus merely adhering to cells or the culture vessels. Long term culture of hepatocytes under these conditions results in many of the cells dying, indeed a second time point could not be measured using the replated cells as these died rapidly. It is therefore possible that the apparent rise in RNA copies per cell is merely a reflection of this cell death rather than of viral replication.

Unfortunately no cell culture system has been developed to date that produces high levels of replicating HCV. In order for the existing systems to be useful in screening antiviral compounds the amount of virus produced must be sufficiently high that HCV RNA is reproducibly detected. This may be achieved either by increasing the amount of virus produced, or by increasing the sensitivity of the detection system. The QRT-PCR system described proved to be able to detect RNA in the infected cultures, but not in the negative controls. The improved sensitivity of detection demonstrated with the RT-'Hot Start'-PCR using AMV RT and *Tfl* DNA polymerase (Section 2.5) has proved useful in monitoring more recent cultures (data not shown).

At the start of the work leading up to this thesis viral culture experiments were limited by the rate at which qualitative PCR and subsequent Poisson limit dilution PCR could be applied to quantify the samples produced. The application of QRT-PCR and more recently RT-HS-PCR to this area of research has increased the number of culture conditions and the number of timepoints that can be analysed. This should result in an increase in the speed at which optimisation of culture conditions can be achieved.

3.9 Lack of Susceptibility of the Cottontop Tamarin to Hepatitis C Infection

Background

The QRT-PCR was also applied in attempts to identify a practical small primate model of HCV infection. Accurate quantification of the sera used in these experiments allowed confirmation that inoculation had been successful. Special care needed to be taken to ensure that sera taken from tamarins did not behave differently from human samples. Serological assays for anti-HCV were modified by using monoclonal antibodies known to recognise human and tamarin IgG antibodies. In addition, tamarin serum samples were spiked with human serum containing known quantities of HCV in order to demonstrate that it was not inhibitory.

Introduction

Progress in HCV research has been hampered by the lack of a practical animal model. The availability of such a model would provide a system for the study of pathological mechanisms and greatly facilitate the development and evaluation of vaccines and antiviral drugs for the prevention and treatment of HCV infection. At present the only non-human model of HCV infection is the chimpanzee (Farci *et al.*, 1993). Although the chimpanzee has proven itself a useful model, it is virtually unobtainable for research purposes. Studies with two other tamarin species, *Saguinus mystax* and *Saguinus labiatus*, suggested that these species were susceptible to infection with agents of non-A, non-B hepatitis (Tabor, 1989). However, these studies were undertaken before the non-A, non-B hepatitis group had been subdivided into identifiable agents and before specific diagnostic tests for HCV were available.

We therefore decided to investigate the possibility that the cottontop tamarin, *Saguinus oedipus oedipus*, might be a suitable model for HCV infection. The cottontop tamarin was selected because it has been used extensively for many years

in EBV research and because it has been shown to be a practical animal model for viral pathogenesis studies and vaccine trials (Morgan, 1996; Finerty *et al.,* 1992).

Methods

Animals and Veterinary Procedures

One male and three female cottontop tamarins, bred in the colony at the University of Bristol, were taken from the main colony and housed under Category 2 containment conditions in a negative pressure isolator. The animals were healthy adults, aged between 3 and 5 years, each approximately 500g in weight. None had been inoculated previously with any hepatitis virus, blood, or plasma derived material. All regulated procedures were carried out under sedation (ketamine, Parke Davis, Pontypool, Gwent, UK; medetomidine, Pfizer, Sandwich, Kent, UK) or general anaesthesia (alphlaxone, Mallinckroot, Harefield, Middlesex, UK).

Inocula

Three high titre serum samples from HCV-infected patients were selected for inoculation. All had been separated within a few hours of venesection, snap frozen in liquid nitrogen and stored at -70°C. One was genotype 4, another genotype 1 and the genotype of the third was unknown. The titre of the genotype 4 serum was 3×10^6 HCV HCV genomes/ml and the two other sera had titres of 7×10^7. A serum from a healthy non-infected individual was used as a control inoculum. All the sera employed had no demonstrable markers of HIV (1&2), human T-cell leukaemia virus (HTLV1&2), or HBV infection.

Virological, Biochemical and Histological Monitoring

HCV RNA concentrations in the inocula were determined by QRT-PCR (Sections 2.3.1, 2.4.1 and 2.4.2 (protocol 7). Spiking experiments with known amounts of HCV RNA were performed to confirm that serum from the cottontop tamarin is a suitable analyte and does not inhibit either reverse transcription or PCR. Attempts to detect HCV reactive antibodies were performed using a commercial ELISA (Ortho Diagnostic Systems, Raritan, New Jersey, USA) modified by using an alternative peroxidase conjugate (Sigma, Pool, Dorset, UK; #A-8667 at a dilution of 1:2000) to be able to detect both human and tamarin IgG. The inocula and serum samples for PCR and antibody analysis were transported between London and Bristol frozen on dry ice and on arrival were stored at -20°C.

Biochemical assessment of liver function was undertaken by monitoring levels of bilirubin, GGT, AST, ALT, alkaline phosphatase, albumin, globulin and total protein. These assays were carried out on tamarin sera at the Department of Chemical Pathology, Bristol Royal Infirmary using standard automated methods. Histological examination of liver tissue was performed blind following the use of standard histological staining techniques.

Four animals were inoculated intravenously, one received normal human serum and each of the others received a different HCV-containing serum. The volume of serum administered was 500µl in each case. Serum samples were taken immediately prior to inoculation, 10 minutes following inoculation (from the contralateral limb), 1 week, 2 weeks, 3 weeks, 6 weeks, 8 weeks, 11 weeks, 13 weeks and monthly thereafter until 6 months post-inoculation. HCV-RNA was assayed in 100µl aliquots of serum at each time point.

Results

Neither HCV RNA nor HCV specific antibodies were detectable in any of the serum samples taken prior to inoculation. In each of the three HCV-inoculated animals HCV RNA was detected in the samples taken at 10 minutes post-inoculation, but not in any of the later samples. The titre of HCV RNA detected at 10 minutes was approximately fifty-fold lower than that of the respective inocula, a result consistent with the dilution effect resulting from mixing of the inoculum with the blood volume. HCV antibodies were detected in the 10 minute and 1 week samples from one of the tamarin inoculated with HCV. At 10 minutes the ELISA reading was 2090 mA_{450} units and at 1 week 690 mA_{450} units. The cut off for this assay was 640 mA units.

None of the enzyme, bilirubin, or protein assays on sera from any of the animals post-inoculation showed any significant deviation from the values obtained pre-inoculation and there was no evidence of weight loss throughout the experiment. Liver samples from each animal showed normal histology and none had detectable HCV RNA.

One of the HCV inoculated animals died at 4 weeks post inoculation, but the death was not apparently HCV related. Serum and liver samples taken at the time of death were negative by PCR for HCV RNA and the liver histology was normal. Post-mortem examination including histological assessment of the major organs did not reveal the cause of death.

Discussion

The presence of HCV RNA in the circulation of all three animals at 10 minutes post-inoculation confirms that the intravenous inoculation was technically successful. No serological evidence of an antibody response to the inoculated HCV was obtained. The presence of HCV reactive antibodies in the sample taken 10 minutes post-inoculation and at a much lower level in the sample taken after 1 week from one of

the HCV inoculated animals is consistent with detection of passively transferred anti-HCV rather than of an active immune response.

Since the volume of high titre inoculum injected into the tamarins was very much in excess of that required to infect humans (infection following needlestick accidents is well documented; Okamoto *et al*, 1992b), it is reasonable to conclude that the cottontop tamarin is not susceptible to HCV infection. This conclusion is in accordance with the findings of Abe *et al.* (1993) who demonstrated the inability of HCV to infect a number of non-human primates including Cynomologous monkeys, Rhesus monkeys, African Green monkeys, Japanese monkeys and Doguera baboons. In addition to the observation that we could not infect cottontop tamarins with HCV, we also demonstrated that a tamarin derived B-cell line could not be infected (data not shown). It appears therefore that the host range of HCV is extremely narrow, possibly being limited exclusively to chimpanzees and man.

The tamarin samples were often more heavily haemolysed than human samples and invariably arrived as volumes less than 250µl, presumably as a consequence of the tamarins being more than 100 times smaller than adult humans. However, the use of tamarin samples did not provide any technical barriers to generating HCV RNA quantification data, as demonstrated by the spiking experiments.

3.10 GBV-C Infection Amongst Blood Donors, Transfusion Recipients and Haemodialysis Patients

Background

The ELONA technique proved to be sufficiently simple and robust to be used in the quantification of PCR products amplified from several other viruses. The discovery of a new member of the *Flaviviridae* called GB virus C provided an opportunity to test further the flexibility of the techniques developed for the quantification of HCV. Random hexamers were used to prime reverse transcription thereby allowing PCR amplification of multiple regions within the genome. The conditions for the PCR and ELONA stages of the quantification of GBV-C were almost identical to those described for QRT-PCR for HCV. The only exceptions being the substitution of appropriate primers and a slight reduction in the anneal temperature to allow for the difference in their nucleotide composition.

Introduction

Three new members of the *Flaviviridae* have been identified (Simons *et al.*, 1995a). Two of these viruses were identified in tamarins inoculated with serum from a surgeon (initials GB) with acute hepatitis. These viruses were named GB virus A (GBV-A) and GB virus B (GBV-B) and their molecular characterisation allowed the identification of another virus, GBV-C, from a human blood specimen. Independently, an RNA virus designated hepatitis G virus (HGV), was identified from the plasma of a patient with chronic hepatitis (Linnen *et al.*, 1996). The viruses GBV-C and HGV are closely related to each other and distantly related to hepatitis C virus (HCV), GBV-A and GBV-B. Alignment of the nucleotide sequence of GBV-C and HGV has revealed a homology of 85% (95% at the amino acid level). GBV-C and HGV would appear to be two isolates of the same virus (Zuckerman,1996), referred to here as GBV-C.

The viral genome is a single stranded positive sense RNA of approximately 9.4kb that encodes a single polyprotein of 2873 to 2910aa in which, the structural proteins (core, occasionally absent, and two envelope glycoproteins E1 and E2) are encoded by the 5' end of the genome and the non-structural proteins (NS2, NS3, NS4a, NS4b, NS5a and NS5b) by the 3' end. The coding sequence of the genome is flanked by a 5' non-coding region (5'NCR) and a 3' non-coding region (3' NCR).

There is strong evidence that this virus can be transmitted parenterally (Linnen *et al.*, 1996; Schmidt *et al.*, 1996) and the virus has frequently been found in multiply transfused patients such as haemophiliacs (Linnen *et al.,* 1996) and patients undergoing maintenance haemodialysis (De Lamballerie *et al.*, 1996; Tsuda *et al.*, 1996). The virus was initially identified in patients with hepatitis and a causative role has been suggested. The virus has also been tentatively implicated in fulminant hepatitis, aplastic anaemia and abnormalities of the bile duct epithelium (Mushahwar and Zuckerman, 1998). However, insufficient data are yet available to draw firm conclusions about the possible pathogenicity of the virus (Alter *et al.,* 1997a).

This study was divided into three phases, the first of which was designed to assess the prevalence of GBV-C RNA in blood donors in South East England. The second phase of the study was designed to determine the prevalence of genomic (GBV-C RNA) markers of infection amongst transfusion recipients and to determine the strength of any association with biochemical markers of liver damage. In addition, the prevalence of serological markers prior to transfusion was also studied. Only transfusion recipients with known transfusion histories, for whom the implicated donor samples were available, were used in the second phase of the study. The third phase of this study involved measuring the prevalence of genomic and serological markers in patients undergoing maintenance haemodialysis and following the natural history of infection with GBV-C in these patients.

Methods

Blood Donor Prevalence

286 unselected non-commercial blood donor samples from the National Blood Authority-London and the South East (NBA-LSE, Colindale, London) were tested for GBV-C RNA by 'nested' PCR (Section 3.10.1) as part of a study to determine blood donor prevalence. Products generated by the first round of PCR were assayed by ELONA (Section 2.4.2, protocol 7) modified to detect GBV-C. The sample yielding the highest signal in the ELONA assay was assumed to contain the highest titre of GBV-C RNA and was quantified by the limit dilution/Poisson distribution method (Simmonds *et al.,* 1990b) for use as an external quantification standard.

Blood Transfusion Study

In a separate study, 187 blood transfusion recipients, with a lifetime exposure, prior to this study, of less than 5 units, were selected and anonymised. Prior to the samples being anonymised age, sex and ALT levels were recorded. These patients were all part of an on-going donor/recipient transmission study (Transfusion Transmission Initiative, NBS-LSE). Sera taken 6-9 months after the most recent transfusion were stored at -20°C, transported on dry ice and tested for GBV-C RNA using QRT-PCR amplifying a 185 base pair (bp) fragment of the 5' non-coding region (5'NCR). To differentiate patients infected as a result of transfusion or hospitalisation from those already chronically infected, a sample taken immediately prior to their most recent transfusion was also tested. Matched donor samples were tested if transfusion recipients became GBV-C RNA positive following the recent blood transfusion. Transmission events were confirmed using restriction fragment length polymorphism (RFLP) fingerprinting as described below.

Haemodialysis Patients

A new renal unit was opened at UCL Hospitals in November 1993 and now provides maintenance haemodialysis for 80-100 patients. In order to reduce the possibility of nosocomial infection amongst the patients, serum samples are taken at three monthly intervals and tested for antibodies and nucleic acid indicative of infection with hepatitis C virus (HCV), hepatitis B virus (HBV) and human immunodeficiency viruses types 1 and 2 (HIV-1 and HIV-2). Patients infected with HCV or HBV are treated separately from uninfected patients. Patients infected with HIV-1 or 2 are treated elsewhere.

GBV-C RNA Extraction and cDNA Synthesis

RNA was extracted from 200µl of serum using the acid guanidinium thiocyanate method (RNAzol A, Section 2.2.1). The precipitated RNA was resuspended in 20µl of RNase free water. Reverse transcription was performed using random hexamers as previously described (Section 2.3.1).

Qualitative ('nested') PCR for the 5'NCR

GBV-C cDNA was amplified by 'nested' PCR. The reaction conditions and cycling parameters for the first round of amplification (35 cycles) were as used for QRT-PCR of HCV cDNA as described in Section 2.4.1. The exceptions being that primers BM1 (5'CGG CCA AAA GGT GGT GGA TG3'; 1µl [100µg/ml]; position 101-120) and BM2 (5'biotin-CGA CGA GCC TGA CGT CGG G3', 1µl [100µg/ml]; position 285-267) were used in place of primers PT3BIO and PT4 and the temperature at which these primers were annealed was 50°C.

The nucleotide sequences of both BM1 and BM2 were supplied by Boehringer Mannheim GMb (Mannheim, Germany), but primer BM2 was modified to include a

5' biotin moiety. The nucleotide positions described are based on the numbering scheme of Linnen *et al.* (1996).

Second round of amplification was performed using similar reaction conditions to the first round, the exceptions being that the internal primer set KW3 (5'GTA GGT CGT AAA TCC CGG TC3'; 1µl [100µg/ml]; position 137-156) and KW4 (5'biotin CAA CAC CTG TGG ACC GTG CG3'; 1µl [100µg/ml]; position 245-226) were used and the reactions were run for 30 cycles. These second round primers were designed following the alignment of all 45 sequences available from the EMBL database at the beginning of 1996 (HDG249 - HDG254, HDGU59518 - HDGU59522, HDGU59524 - HDGU59555, HDGU59557 and HDGU59558).

Quantitative PCR for the 5' NCR

The method used to quantify the GBV-C RNA was adapted from the QRT-PCR method used in the quantification of HCV RNA (Section 2.4). Briefly, random primed cDNA was amplified by PCR using primers BM1 and BM2 and the same reaction parameters as used in the first round of the qualitative PCR (Section 3.10.1). The biotinylated products were captured onto streptavidin coated black microtitre plates, denatured with 0.15M NaOH and hybridised with an alkaline phosphatase conjugated oligonucleotide probe (BM-PROBE, AP-5'GGT AGC CAC TAT AGG TGG G3'; position 162-180). Primer BM-PROBE was adapted for chemiluminescence from a sequence provided by Boehringer Mannheim by conjugation with alkaline phosphatase. Quantification was achieved by measuring the intensity of light emitted by a dioxetane-based chemiluminescent substrate and comparison with a standard curve generated from a dilution series of quantified GBV-C cDNA.

Detection of Antibodies Reactive Against the Envelope Glycoprotein E2

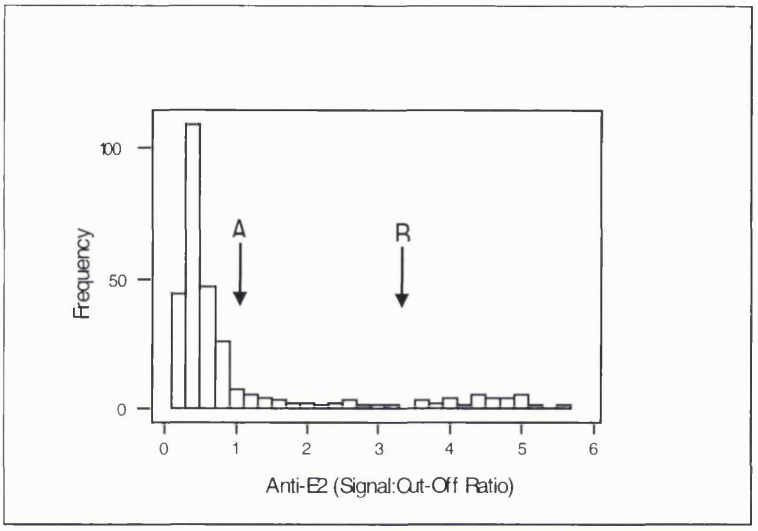
Antibodies were detected using the GBV-C anti-E2 Assay available from Boehringer-Mannheim (Mannheim, Germany). This assay is a 'sandwich' ELISA composed of a capture monoclonal, which is bound to the solid-phase via a biotin streptavidin linkage. This monoclonal antibody binds to a recombinant GBV-C E2 glycoprotein. Antibodies in the test sample that bind to the E2 glycoprotein are detected using a human IgG specific monoclonal antibody conjugated to horseradish peroxidase. In order to differentiate antibodies in the test sample binding specifically to the E2 glycoprotein from those binding non-specifically, samples yielding signals above the assay cut-off were tested again without adding the E2 glycoprotein. This system of confirmation is recommended by Boehringer Mannheim and remains the only commercially available method of 'confirming' serologically reactive results.

Selection of Cut-Off Values for the GBV-C anti-E2 Assay

The kit instructions were followed with the exception that in place of the horseradish peroxidase substrate ABTS, TMB (Murex Diagnostics) was used. This modification was shown to increase the signal to noise ratio without significantly changing the frequency of samples scored positive or negative, as defined by the cut-off formula [0.2 x (mean of positive controls + mean of negative controls)] recommended in the kit instructions. The performance characteristics of the modified assay are illustrated by the frequency distribution of absorbance values shown in Figure 28.

Figure 28: Frequency Distribution Histogram

Signals were generated using the modified assay expressed as a ratio of the cut-off value defined by the GBV-C anti-E2 Kit (data set includes all the results generated in our laboratory up to December 1998)



Positive and negative results generated by a well optimised assay should be easily resolvable. In view of this, it appears from the data illustrated in Figure 28 that the cut-off value generated using the recommended formula (arrow A) is too low. Although the absorbance values do appear to divide into two broad peaks the resolution between these peaks is poor. Based on the data shown, a second cut-off value (arrow B), 3.3 times that given by the recommended formula, could be proposed which might more accurately divide positives from negatives. In the absence of a serological test producing more clear cut results the data generated will be interpreted using both cut-off values.

Restriction Fragment Length Polymorphism (RFLP) Fingerprinting.

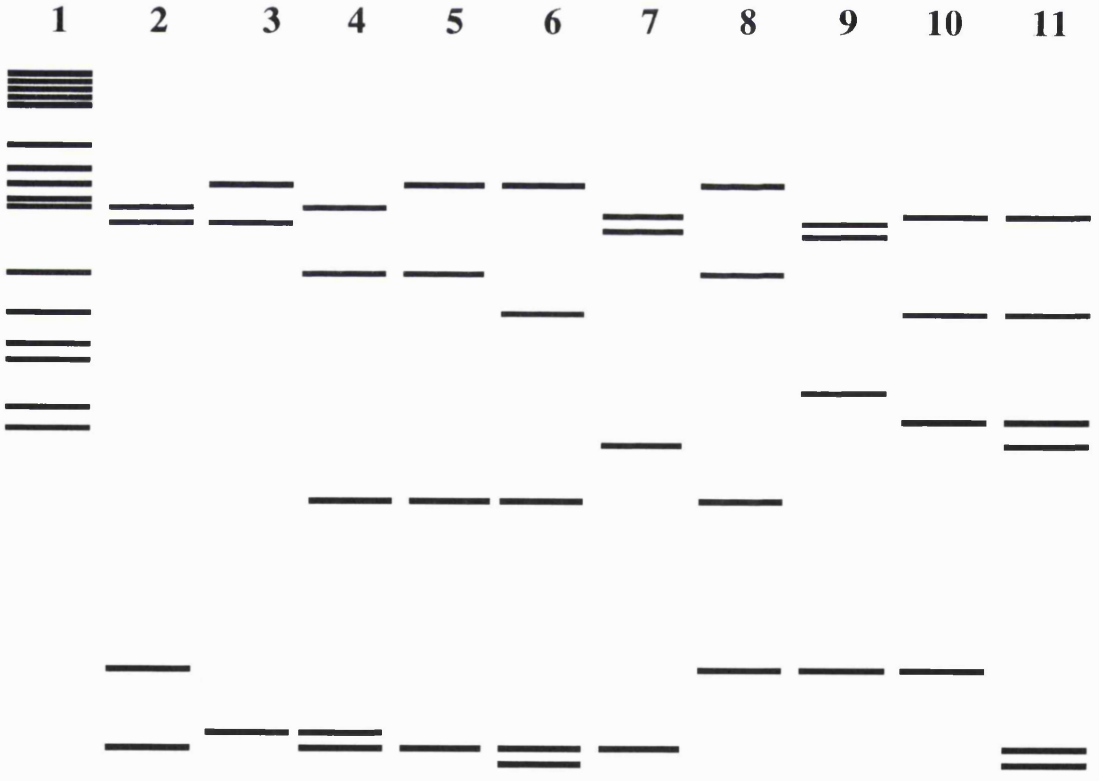
A 'nested' PCR was performed using the external primer pair YK 874 (5'CTG ATG TTG CTA GCC TGT GTG AGA3') and YK 877 (5'ACC GAC ACC TTA GAT CCC CAG CCC3') and the internal primer pair YK 1183 (5'CAG ACC CAT ACA GCC TAT TGT GAC3') and YK 876 (5'CCT TAC AGT CCT TAT TGC TTC CTC3') to produce a 402 base pair product from the NS5 gene (Lampe *et al.,* 1997). Following an initial denaturation of 95°C for 2 minutes, random primed cDNA was amplified for 30 cycles at 92°C for 45 seconds, 50°C for 45 seconds and 75°C for 45 seconds. Two microlitres of first round PCR product was amplified for a further 30 cycles with the same reaction conditions but using the nested primer pair. In order to minimise the number of misincorporated bases, amplification was performed using the proof-reading enzyme *Pfu* DNA polymerase. 10µl of PCR product was digested with 10 units of the restriction endonuclease *Hae* III for 1 hour at 37°C. To control for incomplete digestion 5µg of uncut plasmid pBR322 was diluted in PCR buffer and digested in parallel with the PCR product. The digested pBR322 also acted as a molecular weight marker when analysed by agarose gel electrophoresis (4% Metaphor agarose, NuSeive).

The NS5 primer set has previously been used to amplify GBV-C sequences from Brazilian haemodialysis patients (Lampe *et al.*, 1997). Predicted restriction patterns for sequences selected at random from the EMBL database (U86020 - U86024, U75356, U45966, U63715, D90601, U44402) were generated using the computer program Webcutter (Max Heiman, Massachusetts General Hospital, 1995). The sequence recognised by the restriction enzyme *Hae* III (GGCC) was found in 8 separate locations within the region amplified. None of the restriction sites were present in all the sequences analysed, thus providing over 40,000 potential RFLP patterns. Amongst the 10 sequences analysed using this computer simulation, no 2 sequences yielded the same pattern (Figure 29). Thus, in theory, each virus sequence amplified should have its own 'fingerprint' which could be used to follow transmission events.

Figure 29: Schematic Diagram Showing Computer Predicted RFLP Profiles for 10 GBV-C Sequences

Lane 1 (marker lane) shows the position of fragments produced by a restriction digest of pBR322 with *Hae* III (scale diagram from an ethidium bromide stained 4% agarose gel [Metaphor, NuSieve])

Lanes 2-11 show the computer predicted *Hae* III restriction fragment patterns of sequences (accession numbers: U86020 - U86024, U75356, U45966, U63715, D90601, U44402) from the EMBL database, relative to the marker lane.

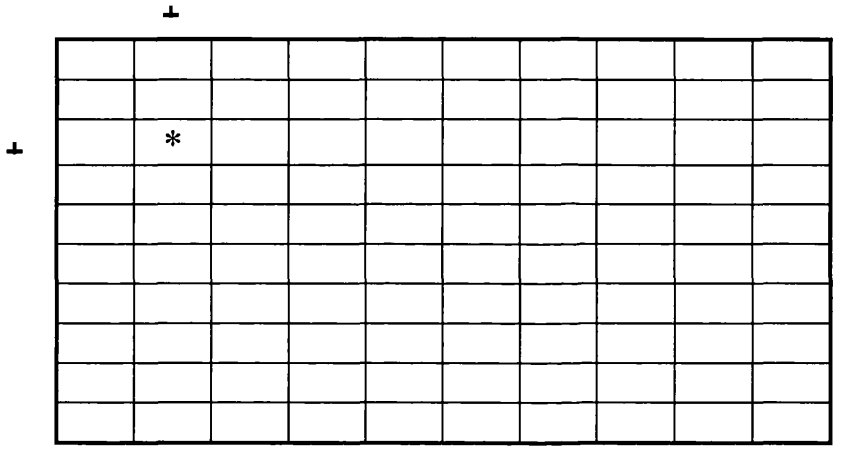


Results

Donor Prevalence Study

Of 286 unselected non-commercial blood donors 4 were found to have GBV-C RNA in their serum. Donor serum samples containing GBV-C RNA were initially identified as reactive pools from a two-dimensional matrix (10 x 10) and were confirmed by testing the individual donor sample. The donor prevalence in this cohort was therefore estimated to be 1.4%.

Figure 30: 10 x 10 Matrix



* Reactive well.

One of the four blood donor samples containing GBV-C RNA was positive with a very high signal to background ratio (2300:1) determined by ELONA. This sample was selected as a potential high titre positive control and quantified using the limit dilution Poisson distribution method (Simmonds *et al.*, 1990b). Random primed cDNA produced nested PCR products in 7 out of 10 reactions containing a 10^{-5} dilution and in 2 out of 10 reactions containing a 10^{-6} dilution, resulting in a predicted mean GBV-C RNA concentration of 6.9 x 10^{7} genomes/ml (Mean = -ln (frequency of negatives)). This high titre serum sample was used to generate standard curves in all subsequent quantitative PCR assays for GBV-C RNA.

Transfusion Transmission Study

Nineteen of the 187 transfusion recipients (10.2%) were found to have GBV-C RNA in their serum following their most recent transfusion, 8 of whom were also viraemic prior to that transfusion. The remaining 5.9% (11/187) of transfusion recipients therefore appear to have become viraemic during hospitalisation. These patients had each received between 1 and 5 units during hospitalisation (mean 2.4 units).

One hundred and seventy nine serum samples taken prior to the patients' most recent transfusion were analysed for the presence of anti-E2 antibodies. Insufficient serum was available from 8 patients for serological analysis. Twenty three (12.8%) of these sera contained E2-reactive antibodies as defined by cut-off A and eight (4.5%) defined by cut-off B. None of the samples taken from these patients prior to or following this transfusion contained both GBV-C RNA and anti-E2 antibodies (using either cut-off).

In order to determine if the presence of E2 reactive antibodies was indicative of a protective immune response, donor samples to which patients with pre-existing E2 reactive antibodies had been exposed were tested for the presence of GBV-C RNA. One patient had been exposed to viraemic blood with a viral titre in excess of 10^6 genomes/ml. Viraemia was not detectable in the recipient's sample taken 6-9 months following this transfusion. This patient was a male aged 62 years and the serum sample taken prior to his most recent transfusion produced a signal in the anti-E2 assay 4.7 times cut-off A and 1.4 times cut-off B.

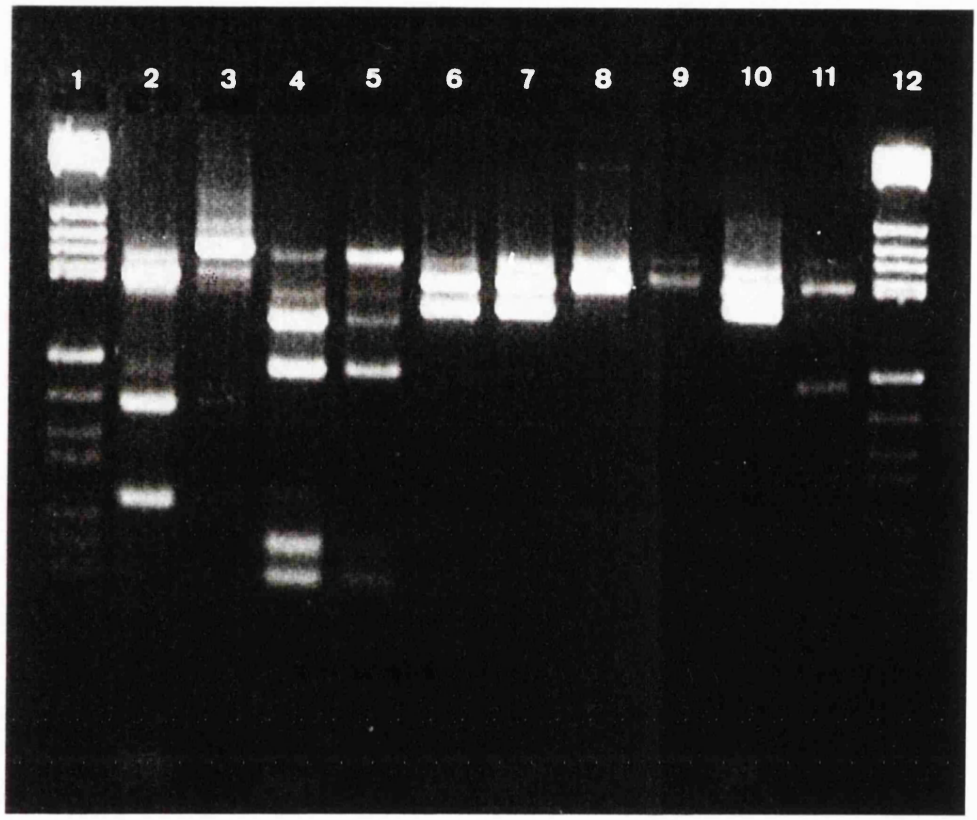
Of the 11 apparent transfusion transmission events, RT-PCR products from the NS5 gene were successfully amplified from both donor samples and their transfusion recipient pairs in 5 cases. These PCR products were digested with endonuclease *Hae* III and fragment patterns analysed by agarose gel electrophoresis (Figure 31). Pairs 1 to 4 had fragment patterns which shared common bands but pair 5 had clearly different fragment patterns.

All the donor samples that had no detectable RNA using NS5 primers also failed to produce a PCR product following re-extraction of the RNA from the serum sample, reverse transcription of the RNA and PCR amplification of the cDNA using primers specific for the 5'NCR region. In order to rule out the possibility of false positive results in the recipient samples in this study, the post transfusion samples from the patients who appeared to have become infected during hospitalisation, but whose donor samples did not contain detectable viral RNA, where re-analysed, by 'nested' PCR of the 5'NCR, in quadruplicate. All of these samples were confirmed to be reactive in at least 3/4 replicates.

In addition, one case could not be evaluated by RFLP because cDNA generated from the recipient sample failed to amplify using primers specific for the NS5 region. This was despite the successful amplification of cDNA generated from the relevant donor sample with these primers and a positive PCR signal from the recipient sample using primers specific for the 5'NCR region.

Figure 31: RFLP Profiles from 5 Donor (D) and Recipient (R) Pairs

Lanes 1 and 12 = pBR322 *Hae* III,
Lanes 2 and 3 contain donor and recipient samples from pair 1, lanes 4 and 5 contain donor and recipient samples from pair 2, lanes 6 and 7 contain donor and recipient samples from pair 3, lanes 8 and 9 contain donor and recipient samples from pair 4, and lanes 10 and 11 contain donor and recipient samples from pair 5.



Haemodialysis Study

Of 84 serum samples taken from haemodialysis patients between March and April 1996 GBV-C RNA was present in the serum of 17 (20.2%). The level of viraemia ranged from 4 x 10^2 to 1 x 10^7 genomes/ml, with a geometric mean titre of 5.1 Log_{10} genomes/ml (± 0.35 Log_{10} genomes/ml).

Antibodies reactive against E2 were found in 13 of 70 haemodialysis patient sera that were tested (18.6%) when cut-off A was used but in only one patient using cut-off B, none of these sera contained GBV-C RNA.

RFLP profiles from 5 haemodialysis patients are shown in Figure 32. Two of the 5 patient sera analysed contained sequences whose RFLP profiles shared common bands as can be seen in lanes 2 and 4. The other three RFLP profiles shown in lanes 3, 5 and 6 do not share common bands, suggesting that there may be as many as four separate sources of infection present in this cohort.

Sera from patients with markers of GBV-C infection in March and April 1996, were tested serially over a 15 to 18 month time period. A typical time course of viraemia is shown in Figure 33A. None of the patients whose sera contained antibodies against E2 (defined by either cut-off) had detectable viraemia in the samples following, or preceding March and April 1996 (e.g. Figure 33C). One of the patients shown to be viraemic in March 1996 had cleared the virus by June 1996. No detectable antibodies were present in March 1996 but the patient had seroconverted by January 1997 (Figure 33B).

Figure 32: RFLP Profiles of 5 Haemodialysis Patients

Lanes 1 and 7: molecular weight marker ϕX174 *Hae* III; lanes 2 and 4: a similar profile perhaps indicating a common source of infection; lanes 3, 5 and 6: unique "fingerprints" indicate a different sources of infection

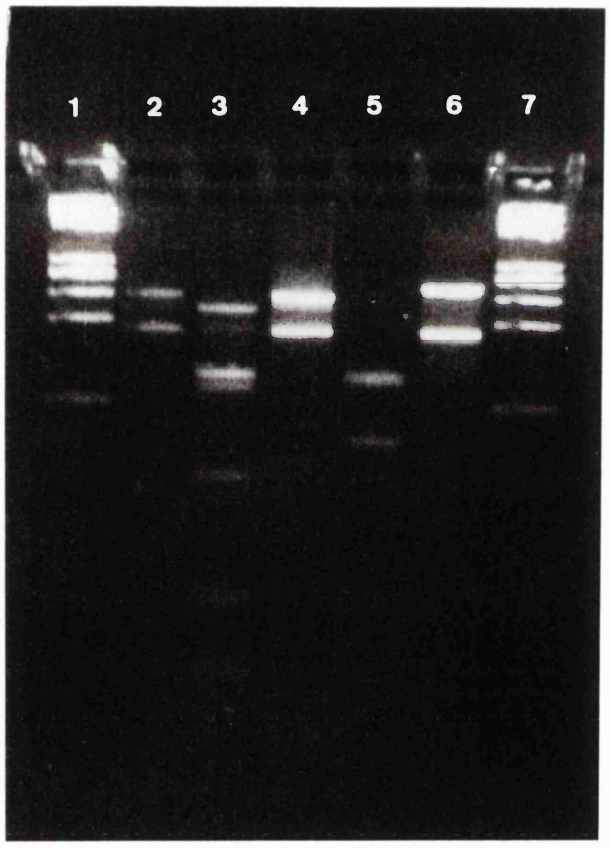
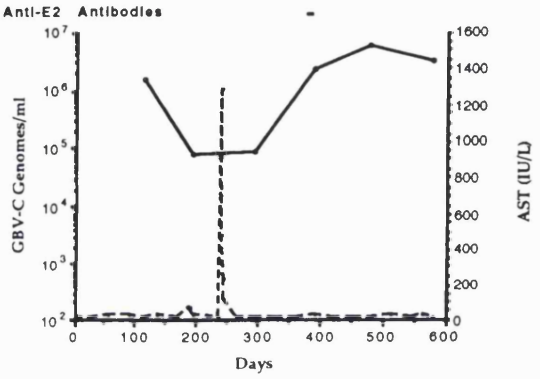


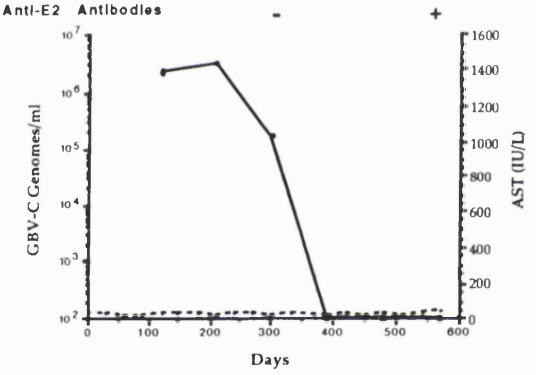
Figure 33: Three Patterns of GBV-C Viraemia Observed in Haemodialysis Patients

Solid Line = GBV-C Viraemia Dotted Line = Transaminase Level

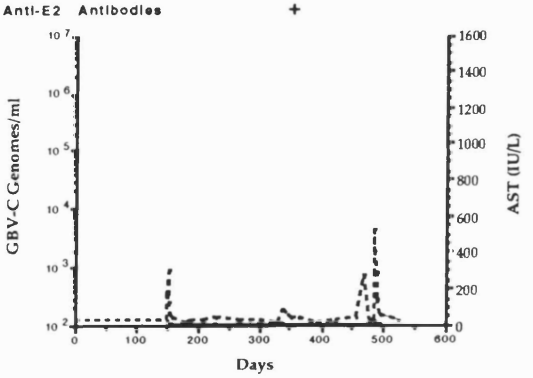
A



B



C



Discussion

The prevalence of GBV-C amongst transfusion recipients (10.2%) and amongst patients undergoing maintenance haemodialysis (20.2%) is higher than the prevalence amongst blood donors (1.4%). A high prevalence of GBV-C has been reported amongst haemodialysis patients, but varies widely from country to country. GBV-C RNA has been found in 3.1% of Japanese haemodialysis patients (Masuko *et al.,* 1996), 55% in Indonesia (Tsuda *et al.,* 1996) and 58% in France (De Lamballerie *et al.,* 1996). This variation may reflect differences in detection sensitivity either as a result of the assays used, or because of genotypic differences between virus found in each country. The rate reported here is similar to that found in the United States (20%; Alter *et al.,*1996). The prevalence amongst the non-commercial blood donors in this study was comparable with that in Japanese non-commercial blood donors (0.9%; Tameda *et al.,* 1996) and non-commercial blood donors in the United States (1.7% Linnen *et al.,* 1996; 1.4% Alter *et al.,* 1997a).

The 187 transfusion recipients described here had, prior to this study, received between 0 and 5 units of donor blood (prevalence of viraemia amongst donors = 1.4%) and therefore have a predicted transfusion related exposure of between 0% and 7% (5 units x 1.4% = 7%). This calculation assumes that the prevalence of GBV-C amongst blood donors has remained constant over time. Eight of these 187 patients (4.3%) had detectable viraemia prior to the transfusion studied here, possibly as a result of previous transfusion exposure.

An apparent mutual exclusivity of GBV-C viraemia and the presence of anti-E2 antibodies, has been reported previously (Hassoba *et al.,* 1997). From this observation it has been inferred that the presence of anti-E2 antibodies be indicative of GBV-C having been cleared. Therefore, in addition to the viraemic transfusion recipients a further 4.5% (cut-off B) to 12.8% (cut-off A) of the cohort may have been exposed to GBV-C. Thereby suggesting that the total number of patients with evidence of previous exposure is between 8.8% and 17.1% (viraemic recipients [4.3%] + recipients with anti-E2 antibodies [4.5-12.8%]). In the absence of any

certainty as to the accuracy of either cut-off it is difficult to judge the level of previous exposure to GBV-C in this cohort. However both values are higher than the transfusion related exposure, possibly as a result of infection from sources other than blood transfusion.

Following the transfusion of between 1 and 5 units of blood (mean 2.4 units) the prevalence of GBV-C viraemia rose from 4.3% to 10.2%, a rise of 5.9%. This rise may represent an underestimate, as a proportion of those exposed to the virus may have produced an immune response and cleared the virus. A prospective study of Taiwanese transfusion recipients exposed to GBV-C RNA positive plasma found that fewer than half became infected following transfusion (Wang *et al.*, 1998), although previous studies found that infection invariably resulted in persistent viraemia (Lefrere *et al.*, 1997; Alter *et al.*, 1997a). Unfortunately, insufficient serum was available to measure the antibody prevalence amongst the transfusion recipients post transfusion and therefore we were unable to properly assess the true exposure.

The sequence of a 402bp fragment of GBV-C NS5 gene from the sera of both donors and recipients was analysed by RFLP. In 5 of the 11 sets of donor samples none of the sera had detectable NS5 or 5'NCR RNA. This finding suggests that a source of infection other than blood transfusion may be responsible for the GBV-C infection of these transfusion recipients. However it also remains possible that the donor samples contained a level of viraemia below the detection limit of the RT-PCR assay. Alternatively false negative results may have occurred as a result of poor storage or inhibitory contaminants of the donor samples, although we have no evidence to support either of these theories. NS5 sequence from 5/6 transfusion recipients corresponding to positive donors were successfully amplified. The chances of two unlinked samples having the same RFLP profile would appear to be minimal as the potential number of restriction fragment patterns that can be generated by digesting the NS5 PCR product with *Hae* III is over 40,000. Comparison of RFLP profiles in four of the five apparent transmission events produced results consistent with a transmission event having occured. The fifth donor recipient pair displayed differing RFLP profiles. Several possibilities exist to

explain this result. One or both of the PCR products may have been generated from contaminating cDNA or PCR product. However, this seems extremely unlikely as none of 72 negative controls tested generated a positive result and re-analysis of both samples by RFLP showed that the patterns were reproducible. The sequence of the virus may have mutated to produce a different restriction pattern, or a sub-population may have expanded to become the predominant quasispecies. The RFLP profile in another patient tested repeatedly over an 18 month period was however remarkably stable (data not shown). The possibility of incomplete digestion is controlled for by digesting the plasmid pBR322 in parallel (Figure 31, lane 1), although it is possible that an inhibitor of restriction endonuclease digestion may have been present in one of the samples. Finally, the explanation may simply be that a source other than the blood donor may have caused the infection.

Patient to patient transmissions result in RFLP profiles which share common bands (e.g. Figure 31, pair 3). Therefore a nosocomial outbreak would be expected to result in several patients sharing RFLP profiles with common bands. As can be seen from Figure 32, three haemodialysis patients sera tested produced different RFLP profiles and two patients shared a profile with common bands. This suggests that this is not a single source outbreak and that there are at least four sources of infection, but the two patients with common profiles may provide evidence of transmission within the dialysis unit. However, patients whose sera contained markers of infection had undergone maintenance haemodialysis for a similar length of time to those without markers of infection, thus suggesting that the source(s) of infection may be outside the renal unit. A study of Brazilian haemodialysis patients concluded that blood transfusion was the most significant risk of infection as they found no evidence of common viral sequences (Lampe *et al.,* 1997).

As can be seen from Figure 33A, infection can produce persistent viraemia, but does not result in consistently raised aspartate transaminase levels (AST). Occasional peaks of abnormally raised AST levels are not unusual amongst haemodialysis patients and can be seen in patients without viraemia (Figure 33C). This data would suggest that GBV-C is not a cause of significant hepatitis amongst these patients.

Transfusion recipients with GBV-C RNA in their serum were of similar age and had a similar male to female ratio as recipients with no detectable viraemia (Table 18). None of the viraemic transfusion recipients had raised serum ALT levels.

Several studies have been described in the literature where an elevated prevalence of GBV-C viraemia has been reported amongst patients with unexplained hepatitis (Simons *et al.*, 1995; Linnen *et al.*, 1996; Tameda *et al.*, 1996). However, studies comparing the frequency of hepatitis in patients with GBV-C viraemia with the frequency in GBV-C RNA negative controls have failed to find significant differences between the two groups of patients (Alter *et al.*, 1997a; Alter *et al.*, 1997b; Bjorkman *et al.*, 1998). This study found no significant differences in ALT levels in the serum of transfusion recipients with detectable GBV-C RNA compared with GBV-C RNA negative transfusion recipients (Table 18). Several authors have now questioned the role of GBV-C in the aetiopathogenesis of non-A to E hepatitis, suggesting that the virus is an innocent bystander perhaps more appropriately named human orphan flavivirus (Alter 1996; Theodore and Lemon 1997).

Table 18: A Comparison of Transfusion Recipients with GBV-C RNA in their Serum and Recipients with no Detectable GBV-C RNA

	GBV-C RNA STATUS			
	POSITIVE n=19 Mean (±S.D.)	**NEGATIVE n=168 Mean (±S.D.)**	**STATISTICAL TEST**	**INTERPRETATION**
AGE (YEARS)	67.7 (±12.6)	68 (±13)	2 Sample t Test	p=0.94 (NS)
ALT (IU/ml)	18.4 (±6)	20 (±10.2)	2 Sample t Test	p=0.37 (NS)
SEX RATIO (M:F)	8:11	79:85	χ^2 Test	p=0.62 (NS)

Having spent considerable time determining optimal conditions for the quantification of HCV RNA, the simplicity with which a method for quantifying GBV-C RNA could be developed suggested that the QRT-PCR may be broadly applicable. It appears that GBV-C may not be a significant clinical problem in

humans, but may serve as a model for flaviviral infections, in which case quantitative PCR may prove a valuable tool. The primers used in this study were designed based upon the sequence data available at the start of 1997. As much more sequence data is available now it would seem wise to reassess the primer design in light of any sequence polymorphism (Section 4.2.2).

4. General Discussion

4.1 Introduction

PCR has proven itself an invaluable tool in the study of hepatitis C virus infection not only for use in the research setting but also as a practical method for the clinical investigation of HCV infected patients. During the period of study leading up to this thesis (1992-1997) PCR techniques were adapted and refined, moving PCR from being a low throughput tool for the highly trained molecular biologist, to being a relatively high throughput quantitative technique sufficiently robust and simple for use in a routine diagnostic service.

This thesis describes the development and application of quantitative PCR for the analysis of large numbers of clinical samples, and for samples produced during the development of a system for the *in vitro* propagation of HCV. The techniques developed have been used to define the course of viraemia during antiviral therapy of HCV infected patients; to measure hepatitis C viraemia during pregnancy; to study a potential animal model and a tissue culture system. In addition, quantitative PCR has been adapted for the ultra sensitive detection of HCV in pooled plasma, and for use in defining the natural history of infection with the closely related virus, GBV-C.

4.2 The Future of Quantitative PCR in HCV Research

4.2.1 PCR:- Technological Improvements

Although methods for the quantification of HCV RNA have advanced significantly over the last five years there are several areas in which the PCR technique can be further refined. These improvements include: a reduction in the lower limit of detection, increased speed and sample throughput and greater accuracy of quantification.

Improvements in Sensitivity

Well optimised PCR methods are capable of detecting single molecules of intact cDNA (Simmonds *et al.,* 1990a). Improvements in sensitivity must therefore be achieved by generating cDNA molecules from viral RNA more efficiently, extracting more RNA from the analyte, or increasing the volume of sample added to the reaction. Improvements in sensitivity achieved by increasing the efficiency of reverse transcription would appear to have only a limited mileage, as RT-PCR sensitivity is now approaching that of DNA PCR, with detection limits as low as 4-8 RNA molecules per reaction (Section 2.5).

The sensitivity of RNA extraction methods can be improved by increasing the volume of sample analysed. Methods employing RNA capture, using particles coated with oligonucleotides, antibody or silica, can be used to concentrate virus or viral RNA from large sample volumes. Clearly this technique is only applicable in circumstances where large sample volumes are routinely available, such as when testing for the presence of HCV RNA in plasma pools. It will also be important to ensure that inhibitors of reverse transcription or PCR are not also concentrated to the extent that they negate the gains in sensitivity achieved or produce variable quantitative results.

The proportion of the extracted RNA added to the reverse transcription reaction is limited by a number of factors. Extraction often ends with an elution step or a resuspension step. The volume of liquid required for efficient elution or dissolution may be too large for all the sample to be practically included in the RT reaction. In addition, extraction may lead to the co-purification of impurities such as proteins and salt which may have an inhibitory effect if included in the RT reaction at too high a concentration.

The volume of cDNA added to a PCR reaction following reverse transcription is often limited by the high concentrations of reaction components such as DTT. The development of single tube RT-PCR protocols such as that described in Section 2.5 was dependent on the resolution of this problem, and a four fold increase in detection sensitivity is presumably achieved purely by adding all the products of reverse transcription compared with one quarter.

Increased Speed and Sample Throughput

A dramatic increase in the number of samples that can reliably be quantified in a given space of time has occurred over the last five years, and is described in this thesis. This has largely been achieved by the simplification of the methodology. Firstly, by reducing the number of rounds of amplification from 2 to 1, and then by using protocols for reverse transcription and amplification in a single reaction tube. Both of these time saving modifications have been achieved without a loss in the sensitivity of the technique. In addition the methods described avoid performing multiple repeats for each sample. Further improvements will undoubtedly be achieved by the increasing use of automated techniques, and the early stages of automated RNA extraction are described in Section 2.2.2. It seems extremely likely that liquid handling robots such as those used for RNA extraction will be developed in parallel with methodological improvements to allow the automation of the entire process. Increased automation is extremely attractive for several reasons, not least the removal of the operator and therefore operator error from many of the stages of HCV RNA quantification.

The advantages of automation are however potentially negated if the handling of large numbers of samples in close proximity increases the risk of cross contamination. Data generated by international collaborative quality control (QC) studies have shown that a large number of laboratories performing PCR on a routine basis fail to achieve acceptable levels of sensitivity or contamination avoidance (Zaaijer *et al.,* 1993). Although there has undoubtedly been improvement as evidenced by more recent studies (Damen *et al.,* 1996), and it has been suggested (Dore *et al.*,1997) that improvements in PCR technology have made it a universally reliable technique, not all laboratories are able to perform PCR without contamination problems. The introduction of contamination prevention strategies such as the use of uracil-N-glycosylase (UNG; Longo *et al.,* 1990) may prevent the reamplification of contaminating PCR products, but cannot prevent false positive results caused by sample cross-contamination. It is therefore difficult to approve the use of any new protocol for performing PCR without assessing the ability of individual laboratories to reliably produce accurate results. This may best be achieved using test panels of coded standards.

In order for any diagnostic technique to be deemed useful it must fulfil the following criteria (placed in order of relative importance):

- *Sensitivity*:- it must identify as positive samples containing that which it is designed to detect.
- *Specificity*:- it must not incorrectly identify samples as positive if they do not contain that which it is designed to detect.
- *Reliability*:- it must produce the same result each time it is performed on a sample.
- *Practicability*:- it must be possible to perform the technique within time, skill and budget constraints.

Accuracy of Quantification

In many ways the fact that PCR has been used with such success to yield undoubtedly useful quantitative data, as described in this thesis, is an achievement in itself. As shown in Figure 3 small differences in the efficiency of amplification can have a large effect on the number of product molecules produced, as a result of the exponential nature of the process. Many factors can cause differences in the reaction conditions in individual reaction vessels, and techniques have been developed take these factors into account. Including alternative amplification substrates, known as internal calibrators, at predetermined levels in each reaction vessel, as described in Section 1.4, may provide an increased level of accuracy and greater security against false negative results. Internal calibrators, however, by definition must compete with the target molecules for reaction components. This may reduce the efficiency of amplification of the target molecules, and therefore diminish the sensitivity of the assay. Accurate measurement of this loss in sensitivity, and comparison with the gains in quantification accuracy and reliability will be required in order to make sensible decisions as to which methods are appropriate under what circumstances.

4.2.2 Importance of Quantitative PCR in the Study of HCV

Viraemia Level as a Pre-Treatment Predictor of Response to IFN-α Monotherapy

Interferon α therapy of HCV infection often fails to produce a sustained response, it is expensive, and more importantly it is associated with significant side effects. The ability to provide indicators of successful therapy prior to treatment should save patients unnecessary suffering, and hospital budgets unnecessary expense. This thesis describes two studies examining IFN-α monotherapy as a means of treating cases of chronic HCV infection (Section 3.1, and Section 3.3). In both studies the pre-treatment level of viraemia was measured, and assessed as a predictor of treatment response. The first study found that the pre-treatment viraemia level was approximately 10 fold lower in sustained responders from a cohort of 30 Italian patients with chronic HCV (Figure 17). However, the second study found that the

sustained responders amongst a cohort of 38 Swedish patients with chronic HCV had viraemia levels intermediate between those of transient responders, and those who did not respond. In both studies the differences in viraemia level did not reach statistical significance. Neither study provided firm support for the frequently reported finding that pre-treatment viraemia level is an important factor in determining the response to interferon α (Martinot-Peignoux *et al.*, 1995; Yamada *et al.*,1995). The fact that our results are at odds with many other reports may be a statistical quirk, but may also reflect important differences in the way HCV RNA was quantified. The several papers which report that low pre-treatment viraemia is a strong predictor of response to IFN-α therapy have used the branched DNA (bDNA) technology developed by the Chiron Corporation (Quantiplex) (Martinot-Peignoux *et al.*, 1995; Yamada *et al.*,1995). This technology differs significantly from nucleic acid amplification techniques such as PCR, LCR and NASBA in that the method depends upon signal amplification (Figure 6). Early versions of the bDNA kits for HCV RNA appear to have overestimated the viraemia levels of genotype 1 sequences (Suzuki *et al.*, 1995; Dusheiko *et al.*, 1996a; Hawkins *et al.*, 1997) and genotype 1 is associated with a poor response to IFN-α. This may have been a result of maximising the sensitivity of the assay using panels of sera from the United States where genotype 1 is predominant, however, more recent modifications have resolved this anomaly (Dusheiko *et al.*, 1996a).

Despite the somewhat limited sensitivity of the bDNA system it does allow direct detection of viraemia in laboratories unable to perform PCR. It is clear from the data presented in this thesis that direct detection of virus is an important part of monitoring the response to antiviral therapy. Surrogate markers of HCV infection such as transaminase levels can give rise to misleading information. Either as a result of extrahepatic viral replication, as evidenced by the persistent normalisation of ALT levels in a patients treated with IFN-α (Garson *et al.*, 1992; Kakumu *et al.*, 1993) who had not cleared HCV from their serum; or in circumstances where transaminases may be raised as a result of other causes of liver damage, as reported in thalassaemic patients infected with HCV (Section 3.4), and patients undergoing maintenance haemodialysis who are infected with GBV-C (Section 3.10).

The QRT-PCR and RT-HS-PCR described in this thesis amplify a subgenomic fragment from the 5'NCR. By aligning the sequences of HCV genotypes 1, 2 and 3 (the three most frequently occuring genotypes in the UK population) against those of primers PT2, PT3, PT4 and INT1AP a high level of sequence homology can be seen (Table 19). None of the sequences shown vary by more than one base from the primer sequences and the pleomorphic bases are towards the centre of the primers. Given the low level of sequence variation in this region of the 5'NCR the results generated would not be expected to vary from genotype to genotype. However, it is only by assaying equimolar concentrations of each HCV genotype that the lack of bias predicted can clearly be shown. When such experiments were performed by the Chiron Corporation using early versions of the bDNA assay, a clear over-prediction of the level of type 1 sequences was shown.

The single base mismatches in PT3 were observed in two locations both close to the middle of the primer. This polymorphic region in PT3 was noted by Smith and co-workers (1995) in a paper describing polymorphisms effecting oligonucleotide primers commonly used for detecting HCV RNA. This paper also describes two primers which do not contain bases which vary between genotypes. A higher level of quantification accuracy is potentially achievable by replacing PT3 with one or other of the primers described.

Viraemia Levels Prior to IFN-α Ribavirin Combination Therapy

A recent large scale (100 patients) trial of IFN-α and Ribavirin used in combination to treat chronically infected Swedish patients who had not previously received IFN-α monotherapy reported that 38% attained a sustained clearance of virus (Reichard *et al.,* 1998). Neither combination therapy trial described in this thesis contained sufficient numbers to be able to stratify responders and non-responders in terms of pre-treatment viraemia levels. The trial of IFN-α naive Swedish patients (Reichard *et al.,* 1998) found that the largest increase in response rates compared with IFN-α monotherapy was seen in patients with higher pre-treatment viraemia level. It is noticeable also in this larger trial as in the two studies reported in this thesis, that the initial response rates with IFN-α Ribavirin combination therapy are similar to those reported for IFN-α monotherapy, but relapse appears to be much less frequent when combination therapy is used, but the mechanism for this is unclear. This may reflect the development of less complete resistance to combination therapy than to monotherapy.

Early Kinetics of Viraemia During Antiviral Therapy

Given the findings reported in this thesis (Sections 3.1 and 3.3) it would seem unwise to take high pre-treatment viraemia level as a contraindication of IFN-α monotherapy. For example one patient with a pre-treatment viraemia level of 2×10^8 HCV genomes/ml attained a complete sustained response to interferon α monotherapy (Section 3.3, Figure 21). In addition, Nishiguchi *et al.* (1996) found that IFN-α may help to prevent hepatocellular carcinoma independently of viral clearance.

Reports have suggested that a failure to clear viraemia during the first few weeks of therapy is however predictive of a failure to attain a sustained response to IFN-α monotherapy (Orito *et al.,* 1995). A recent paper by Lam *et al.* (1997) demonstrated that much can be gained by following viraemia levels early during therapy. By following the rate of decline of viraemia level in patients infected with type 1 HCV

during treatment with differing doses of IFN-α, and the applying a mathematical model similar to that used to determine the replication rate of HIV-1 (Ho *et al.*, 1995), they were able to demonstrate a dose dependent response and predict a minimum replication rate of 3.7×10^{11} virions per day. From this they were able to suggest that it seems likely that IFN-α acts directly to inhibit HCV replication, and not by blocking the infection of susceptible cells as has previously been suggested (Zeuzem *et al.*,1996).

Identification of Novel Antivirals

Whereas, the improvement in response rates gained by using Ribavirin in combination with IFN-α, as demonstrated in Sections 3.2 and 3.4 and reinforced in the literature, provides great hope to those suffering with HCV infection, experience with other RNA virus infections such as HIV-1 suggests that the development of viral resistance to antiviral agents occurs rapidly. It is therefore important to identify compounds able to supplement or replace those antivirals already in use. The absence of efficient *in vitro* replication systems, or convenient animal models for HCV infection makes the development of new treatment strategies difficult. The safety of agents used in clinical trials for the treatment of HCV has to date largely depended upon data generated during trials of their use against other viruses (e.g. Lamivudine, etc.). Efficacy has often been judged by their ability to reduce serum levels of transaminases, but as discussed previously this is not always appropriate. Ribavirin was considered to be a candidate for use in combination therapy on the basis of its ability to induce a transient reduction in ALT levels. Patients treated with Ribavirin monotherapy do not normally attain viral clearance, sustained or otherwise. Monitoring of viraemia during Ribavirin trials has shown only slight changes in level (Hoofnagle *et al.*, 1996). It is therefore difficult to judge the potential effectiveness of antivirals during combination therapy strategies based upon data from monotherapy trials. The data presented in Sections 3.5 and 3.6 of this thesis demonstrate the usefulness of quantitative methodologies in providing evidence of subtle antiviral effects. Using qualitative PCR no change in the hepatitis C viraemia status of chronically infected patients treated with 3TC would have been

observed, but using the QRT-PCR evidence for a significant reduction in the geometric mean titre of HCV RNA was obtained.

Hepatitis C Viraemia Levels During Pregnancy

Measuring the level of circulating HCV RNA during pregnancy may represent a further clinical application of quantitative PCR. However, the lack of standardisation of quantitative techniques provides an obstacle to the development of clear guidelines for obstetricians. It seems unsurprising that a higher level of maternal viraemia provides a higher risk of neonatal infection, a fact that has already been established for HIV-1 infection. It remains to be determined with certainty what numerical relationship exists between maternal viraemia level and the rate of infection in neonates. Until this is established it will be difficult to provide accurate information on risks for infected mothers, or to assess whether antivirals should be used to reduce viraemia during pregnancy. The data generated by Ohto *et al.*, (1994) required the collaboration of a large number of obstetric departments to yield the reported data. It may be more practical to reassess the viraemia levels reported using international standards than to repeat such a study. However the possibility that different genotypes may amplify with different efficiencies may further cloud the data, and it may be necessary also to standardise the quantification methodology for this application.

Currently the use of INF-α and Ribavirin are contraindicated during pregnancy, but further information as to the risk of neonatal infection may change this advice. Clearly antivirals providing less risk of foetal abnormality would be of benefit, but more information on the natural history of HCV infection during pregnancy will be required before recommendations can be given as to optimal timing of any antiviral treatment. Lamivudine, is not contraindicated for use during the second or third trimesters, but it is yet to be determined whether its antiviral effect is sufficient to reduce the risk of materno-foetal transmission. The use of AZT therapy has been shown to be effective in reducing the rate of vertical transmission from HIV infected mothers (Wales and Smith, 1996).

It has also been reported that the rate of vertical transmision from HCV infected mothers is also dependant on the mode of delivery. A study conducted in Italy suggested a lower rate of transmission in babies born by Caesarean section compared with vaginal delivery (6% vs 32%; Paccagnini *et al.*, 1995). This was confirmed in a further study where 5.6% of infants born by Caesarean section became infected with HCV compared to 13.9% born vaginally (Thomas *et al.*, 1998). This effect is presumably a result of a reduced risk of blood contamination of the infant.

4.3 Development of Novel Inhibitors of Viral Replication

Following the encouraging introduction of proteinase inhibitors for HIV-1 therapy efforts to develop similar treatment strategies for HCV have been pursued. The serine proteinase encoded by HCV non-structural gene 3 (NS3) is responsible for the post-translational cleavage of the viral polyprotein at four sites, and it is considered essential for virus replication. Soluble proteinases can be generated as 20kDa N-terminal fragments of the 70kDa NS3 protein using standard methods for recombinant protein expression. These fragments form the basis of many of the high throughput screening assays used by pharmaceutical companies to identify potential inhibitors. Chemical modification of these compounds may result in improved potency, reduced toxicity and increased bioavailability.

Love *et al.* (1996) have solved the X-ray structure of the NS3 proteinase domain at 2.4Å resolution (Love *et al.*, 1996). NS3 appears to be a zinc containing metallo-proteinase with a catalytic triad of His-57, Asp-81 and Ser-139. Somewhat surprisingly Love *et al* reported that the Asp-81 side chain was orientated away from His-57, despite the fact that this would be expected to reduce the stability of the active site.

Simultaneous publication (Kim *et al.*, 1996) of the crystal structure of NS3 complexed with a synthetic NS4a peptide at a similar level of resolution, showed that NS4a forms an integral part of the proteinase structure. The structural anomalies reported by Love *et al.*, could be an artefact of crystalisation, but would be more conveniently explained by the absence of NS4A. Expressing the major proteinase as a non-covalent complex of two intricately associated viral proteins may allow a second site for antiviral action (Clarke, 1995).

These findings will accelerate efforts to develop better anti-HCV agents via 'rational drug design' strategies. However, progress in this area of HCV research is still hampered by the lack of an efficient *in-vitro* replication system.

4.4 Development of Tissue Culture Systems

The development of tissue culture systems supporting *in vitro* replication has frequently proven to be an essential step in the development of treatment and prevention strategies for viral infection in man. Vaccinations against poliovirus and the archetypal flavivirus yellow fever virus (YFV) resulted from the development of tissue culture propagation of these viruses; and the large scale screening of anti-retroviral agents remains largely dependant on HIV-1 susceptible lymphocyte lines such as H9 (Dezube *et al.,* 1997).

The development of efficient and reproducible *in vitro* culture systems for HCV has so far proven elusive. Several strategies have been tried including the use of *ex vivo* liver samples, transfection of apparently 'full length' cDNA, growth of the virus in cultured lymphocytes or lymphocyte lines and the infection of hepatocyte lines. Although high levels of HCV RNA are reported to be produced from *ex vivo* material and this approach may prove a useful for assessing antivirals, this system is limited by the lack of a ready supply of biopsy material, and attempts to preserve such material are not yet fully evaluated. The lack of success of transfection protocols has previously been attributed to the failure to synthesise truly full length cDNA. The recent extension of the HCV genome by a further 98 nucleotides has allowed the successful transfection of chimpanzee liver, resulting in the production of full length HCV RNA and viral particles (Yanagi *et al.,* 1997). The transfer of this transfection technology to the culture flask may also provide a useful system for screening antivirals, and removes the potential problem of neutralising antibodies preventing infection. The relative infectivity of *in vivo* samples could not be assessed using this system and unless subsequent rounds of replication occur it could not be used to determine the nature of the HCV receptor. Several groups have described the use of lymphocytes to propagate HCV (Shimizu and Yoshikura, 1995), and these experiments have included evidence of viral passaging and the presence of whole viral particles. However, recent reports have provided evidence that the efficient replication of HCV may not be supported by lymphocytes *in vivo* (Lanford *et al.,* 1995; Mellor *et al.,* 1998).

The approach described in Section 3.8 may have several advantages. Firstly, undifferentiated hepatocyte lines grow readily in culture. The differentiation procedure described is reproducible, thus providing a convenient supply of differentiated hepatocytes. As this approach depends upon infection rather than transfection, the infectivity of serum samples can be judged, and receptor binding studies can be performed. Although, reproducibly positive tissue cultures can be demonstrated by this approach (data not shown) irrefutable evidence of viral replication has not been produced here. The demonstration of negative stand HCV RNA has often been used elsewhere as evidence of viral replication, but convincing controls are often not reported. The use of HCV RNA extracted from serum as a negative control may give a false sense of security, because RNA extracted from cells contains considerably more nucleic acid, thereby increasing the risk of spurious priming.

4.5 Animal Models of HCV Infection

Animal models remain the most valuable method of assessing potential efficacy of drugs or vaccines prior to clinical trails in man. Many agents showing significant antiviral activity *in vitro* fail to reproduce this effect *in vivo*, often as a result of poor bioavailability or short pharmacological half life. In addition, unexpected side effects can also be identified through the use of appropriate animal models. Some of this information can be inferred from the chemical structure of the antiviral, or obtained by tests in uninfected animals. However, in order to accurately determine the dose response relationship between a virus and an antiviral agent accurate animal models are invaluable.

To date the chimpanzee remains the only animal susceptible to HCV infection other than man. Research is limited to the small population of captive bred chimpanzees and is often prohibitively expensive. The failure of the study described in Section 3.9, and of several other published studies to identify susceptible primates suggests that HCV has an extremely narrow host range.

The development of antiviral agents against HBV has also been hampered by the resistance of primates other than chimpanzees and man to infection. However, using infections with closely related hepadnaviruses infecting other mammals (woodchuck hepatitis virus) and birds (duck hepatitis virus) as models of infection has led to a significant improvement in the understanding of the biology of these viruses. The most closely related viruses to HCV are the three recently discovered flaviviruses GBV-A, GBV-B and GBV-C (HGV), all three of these viruses are able to infect tamarins and may in part explain the results of Tabor (1989) mentioned in Section 3.9. Although the GB viruses have been shown to cause hepatitis in tamarins there is little evidence that this is also true in man. Also, the assumption that a close genetic relatedness necessarily means that the viruses share similar susceptibility to antivirals is belied by the fact that GBV-C is even less susceptible to IFN-α monotherapy than HCV (Lau *et al.*, 1997). However, in the absence of alternatives these surrogate models may provide useful clues to the biology of HCV.

Table 19: A Comparison of Sequences from HCV Genotypes 1-3 Aligned with Oligonucleotides Used by the QRT-PCR and the RT-HS-PCR

				← INT1AP →			← PT2 →	
		← PT3 →				← PT4 →		
3	E-b1	*AGTGTCGT*	*GCAGCCTCCA*	*GG*-----**CCC**	**TCCCGGGAGA**	**GCCAT***AGTGG*	*TCTGCGGAAC*	*CGGTGAGTAC*	ACCG
3	E-b2	--------	----------	----------	----------	----------	----------	----------	----
3	E-b3	--------	----------	----------	----------	----------	----------	----------	----
3	E-b4	--------	----------	----------	----------	----------	----------	----------	----
3	E-b5	--------	----------	----------	----------	----------	----------	----------	----
3	E-b6	--------	----------	----------	----------	----------	----------	----------	----
3	E-b7	--------	----------	----------	----------	----------	----------	----------	----
3	E-b8	--------	----------	----------	----------	----------	----------	----------	----
2	K2a	------A-	----------	----------	----------	----------	----------	----------	----
2	HC-J6	--------	A---------	----------	----------	----------	----------	----------	----
2	E-b9	--------	A---------	----------	----------	----------	----------	----------	----
2	E-b10	--------	A---------	----------	----------	----------	----------	----------	----
2	E-b11	--------	A---------	----------	----------	----------	----------	----------	----
2	E-b12	--------	A---------	----------	----------	----------	----------	----------	----
2	K2b-1	------A-	----------	----------	----------	----------	----------	----------	----
1	E-b13	--------	----------	----------	----------	----------	----------	----------	----
1	E-b14	--------	----------	----------	----------	----------	----------	----------	----
1	E-b15	--------	----------	----------	----------	----------	----------	----------	----
1	E-b16	--------	----------	----------	----------	----------	----------	----------	----
1	E-b17	--------	----------	----------	----------	----------	----------	----------	----
1	E-b18	--------	----------	----------	----------	----------	----------	----------	----
1	HCV-1	--------	----------	----------	----------	----------	----------	----------	----
1	Pt-1	--------	----------	----------	----------	----------	----------	----------	----
1	H77	--------	----------	----------	----------	----------	----------	----------	----
1	H90	--------	A---------	----------	----------	----------	----------	----------	----
1	GM1	--------	----------	----------	----------	----------	----------	----------	----
1	GM2	--------	----------	----------	----------	----------	----------	----------	----
1	J1	--------	----------	----------	------A---	----------	----------	----------	----
1	A1	--------	----------	----------	------A---	----------	----------	----------	----
1	S1	--------	----------	----------	----------	----------	----------	----------	----
1	T1	--------	----------	----------	----------	----------	----------	----------	----
1	U18/I24	--------	----------	----------	----------	----------	----------	----------	----
1	HCV-J	------T-	----------	----------	----------	----------	----------	----------	----

N.B. Reverse Transcription Primer PT2 underlined; PCR Primers PT3 and PT4 *in italics*; ELONA hybridisation probe **in bold**.

References

Abe K., Kurata T., Teramoto Y., Shiga J., Shikata T. (1993). Lack of susceptability of various primates and woodchucks to hepatitis C virus. Journal of Medical Primatology 22: 433-434.

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

Abravaya K., Hu HY., Khalil O. (1997). Stratagies to avoid amplicon contamination. In: Nucleic Acid Amplification Technologies: application to Disease Diagnosis by Lee H., Morse S and Olsvik Ø. BioTechniques Books, Eaton Publishing, Natick, Massachusetts, USA.: 125-134.

Afzal MA., Minor P. (1994). Instant RNA isolation from virus-infected tissue culture fluid for the polymerase chain reaction. Vaccine 12 (11): 976-977.

Aldouri MA., Wonke B., Hoffbrand AV., Flynn DM., Laulicht M., Fenton LA., Scheuer PJ., Kibbler CC., Allwood CA., Brown D., *et al.* (1987). Iron state and hepatic disease in patients with thalassaemia major, treated with long term subcutaneous desferrioxamine. Journal of Clinical Pathology 40 (11): 1353-1359.

Alter HJ., Purcell RH., Shih JW., Melpolder JC., Houghton M., Choo QL., 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 (22): 1494-1500.

Alter HJ (1996). The cloning and clinical implications of HGV and HGBV-C. New England Journal of Medicine 334: 1536-1537.

Alter HJ., Nakatsuji Y., Melpolder J., Wages J., Wesley J., Shih JW., Kim JP. (1997a).The incidence of transfusion associated hepatitis G virus infection and its relation to liver disease. New England Journal of Medicine 336: 747-754.

Alter MJ, Gallagher M, Morris TT, Moyer LA, Meeks EL, Krawczynski K, Kim JP, Margolis HS (1997b). Acute non-A-E hepatitis in the United States and the role of hepatitis G virus infection. Sentinel Counties Viral Hepatitis Study Team. New England Journal of Medicine. 336 (11): 741-746.

Antonelli G., Currenti M., Turriziani O., Dianzani F. (1991). Neutralising antibodies to interferon α: relative frequency in patients in patients treated with different interferon preparations. Journal of Infectious Disease 163: 882-885.

Atrah HI., Ala FA., Ahmed MM., Hutchinson F., Gough D., and Baker K. (1996). Unexplained hepatitis C virus antibody seroconversion in established blood donors. Transfusion 36 (4): 339-343.

Bacon BR., Farrell G., Benhamou JP., Hopf U., Barcena R., Feinman V., Rizzetto M., Wright T., Warwick S., Horton J. (1995). Lymphoblastoid interferon improves the long term response to a 6-month course of treatment when compared with recombinant interferon alpha 2b - results of an international trial. Hepatology 22: 181.

Baier B., Telford D., Gulbins E., Yamada N., Kawakami T., Altman A. (1993). Improved specificity of RT-PCR amplifications using nested cDNA primers. Nucleic Acids Research 21 (5): 1329-1330.

Bain VG., Kneteman NM., Ma MM., Gutfreund K., Shapiro JA., Fischer K., Tipples G., Lee H., Jewell LD., Tyrrell DL. (1996). Efficacy of lamivudine in chronic hepatitis B patients with active viral replication and decompensated cirrhosis undergoing liver transplantation. Transplantation 62 (10): 1456-1462.

Balayan MS., Andjaparidze AG., Savinskaya SS., Ketiladze ES., Braginsky DM., Savinov AP., Poleschuk VF. (1983). Evidence for a virus in non-A, non-B hepatitis transmitted via the fecal-oral route. Intervirology 20 (1): 23-31.

Bardelli F., Messori A., Rampazzo R., Alberti A., Martini N. (1995). Effect of recombinant or lymphoblastoid interferon-alpha on alanine aminotransferase in patients with chronic hepatitis-C or chronic non-a, non-b-hepatitis - a meta-analysis. Clinical Drug Investigations 9: 239-254.

Bchini R., Capel F., Dauguet C., Dubanchet S, Petit M-A. (1990). *In vitro* infection of human hepatoma (HepG2) cells with hepatitis B virus. Journal of Virology 64: 3025-3032.

Beck S and Köster H. (1990) Application of dioxetane chemiluminescent probes to molecular biology. Analytical Chemistry. 62: 2258-2270.

Bell H., Hellum K., Harthug S., Maeland A., Ritland S., Myrvang B., von der Lippe B., Raknerud N., Skaug K., Gutigard BG., Skjaerven R., Prescott LE., Simmonds P. (1997) Genotype, viral load and age as independent predictors of treatment outcome of interferon-alpha 2a treatment in patients with chronic hepatitis C. Scandinavian Journal of Infectious Disease 29 (1): 17-22.

Benhamou Y., Katlama C., Lunel F., Coutellier A., Dohin E., Hamm N., Tubiana R., Herson S., Poynard T., Opolon P.(1996). Effects of lamivudine on replication of hepatitis B virus in HIV infected men. Annals of Internal Medicine 125 (9): 705-712.

Bjorkman P, Sundstrom G, Widell A. (1998). Hepatitis C virus and GB virus C/hepatitis G virus viremia in Swedish blood donors with different alanine aminotransferase levels. Transfusion. 38 (4): 378-384.

Bjoro K., Froland SS., Yun Z., Samdal HH., Haaland T. (1994). Hepatitis C infection in patients with primary hypogammaglobulinaemia after treatment with contaminated immune globulin. New England Journal of Medicine 331: 1607-1611.

Bloch KJ. (1992). Cryoglobulinaemia and hepatitis C virus. New England Journal of Medicine 327: 1521-1522.

Blumberg BS., Gerstly BJS., Hungerford DA., London WT., Sutnick AI. (1967). A serum antigen (Australian antigen) in Down's syndrome, leukaemia and hepatitis. Annals of Internal Medicine 66: 924-931.

Blumberg BS., Sutnick AT., London WT. (1968). Hepatitis and leukemia. Their relation to Australia antigen. Bullitin of the New York Academy of Medicine 44: 1566-1586.

Boom R., Sol CJ., Salimans MM., Jansen CL., Wertheim van Dillen PM., van der Noordaa J. (1990): Rapid and simple method for purification of nucleic acids. Journal of Clinical Microbiology. 28 (3): 495-503.

Braconier JH., Paulsen O., Engman K., Widell A. (1995). Combined alpha-interferon and Ribavirin treatment in chronic hepatitis C: a pilot study. Scandinavian Journal of Infectious Disease 27 (4): 325-329.

Bradley D., McCaustland K., Krawczynski K., Spelbring J., Humphrey C., Cook EH. (1991). Hepatitis C virus: buoyant density of the factor VIII-derived isolate in sucrose. Journal of Medical Virology 34 (3): 206-208.

Brand CM., Leadbeater L., Budiman R., Lechner K., Gisslinger H. (1994). Successful re-treatment of an anti-interferon resistant polycythaemia vera patient with lymphoblastoid interferon-alpha N1 and *in vitro* studies on the specificity of the antibodies. British Journal of Haematology 86 (1): 216-218.

Brechot C., Nalpas B., Feitelson MA. (1996). Interactions between alcohol and hepatitis viruses in the liver. Clinical Laboratory Medincine 16 (2): 273-287.

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

Brillanti S., Garson J., Foli M., Whitby K., Deaville R., Masci C., Miglioli M., Barbara L. (1994). A pilot study of combination therapy with ribavirin plus interferon alfa for interferon alfa - resistant chronic hepatitis C. Gastroenterology 107: 812-817.

British Medical Association and Royal Pharmaceutical Society of Great Britain. (1996). Hepatitis A Vaccine; British National Formulary 31:503.

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

Busch MP., Wilber JC., Johnson P., Tobler L., Evans CS. (1992). Impact of specimen handling and storage on detection of hepatitis C virus RNA. Transfusion 32 (5): 420-425.

Carrino JJ., Chan C., Canavaggio M., Lee H. (1997). Ligation-Based Nuceic Acid Probe Methods. In: Nucleic Acid Amplification Technologies: application to Disease Diagnosis by Lee H., Morse S and Olsvik Ø. BioTechniques Books, Eaton Publishing, Natick, Massachusetts, U.S.A.: 61-79.

Chemello L., Cavalletto L., Bernardinello E., Guido M., Pontisso P., Alberti A. (1995). The effect of interferon alfa and Ribavirin combination therapy in naive patients with chronic hepatitis C. Journal Hepatology 23 Suppl 2: 8-12.

Chen PJ., Lin MH., Tai KF., Lui PC., Lin CJ., Chen DS. (1992). The Taiwanese hepatitis C virus genome: Sequence determination and mapping of the 5' termini of viral genomic and antigenomic RNA. Virology 188: 102-113.

Chomczynski P, and Sacchi N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analytical Biochemistry 162 (1): 156-159.

Choo QL., 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 (4902): 359-362

Choo QL., 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 organisation and diversity of the hepatitis C virus. Proceedings of the National Academy of Sciences of the U.S.A. 88: 2451-2455.

Choo QL., Kuo G., Ralston R., Weiner A., Chein D., van Nest G., Han J., Berger K., Thudium K., Kuo C., Kansopon J., McFarland J., Tabrizi A., Ching K., Moss B., Cummins LB., Houghton M., and Muchmore E. (1994). Vaccination of chimpanzees against infection by the hepatitis C virus. Proceedings of the National Acadamy of Science of the U.S.A. 91: 1294-1298

Chou Q., Russell M., Birch DE., Raymond J., Bloch W. (1992). Prevention of pre-PCR mis-priming and primer dimerization improves low-copy-number amplifications. Nucleic Acids Research 20: 1717-1723.

Chuang WL., Chang WY., Lu SN., Lin ZY., Chen SC., Hsieh MY., Wang LY., You SL., Chen CJ. (1993). The role of hepatitis C virus in chronic hepatitis B virus infection. Gastroenterology Japan 28 (Suppl. 5): 23-27.

Clarke BE. (1995). Approaches to the development of novel inhibitors of hepatitis C virus replication. Journal of Viral Hepatitis 2: 1-8.

Cuypers HT., 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 (12): 3220-3224.

Damen M., Cuypers HT., Zaaijer HL., Reesink HW., Schaasberg WP., Gerlich WH., Niesters HG., Lelie PN. (1996). International collaborative study on the second EUROHEP HCV RNA reference panel. Journal of Virological Methods 58 (12): 175-185.

Dane D. S., Cameron C. H., Briggs M. (1970). Virus-like particles in serum of patients with Australia-antigen associated hepatitis. Lancet 1 (649): 695-698.

Darwin C. (1859). On the origin of species. Murray: London.

Davies M., Browne C. M. (1985). Pregnancy-associated non-specific immunosuppression: mechanism for the activation of the immunosuppressive factors. American Journal of Reproductive Immunology and Microbiology 9 (3): 84-90.

Davis GL., Balart LA., Schiff ER., Lindsay K., Bodenheimer HC., Perrillo RP., Carey W., Jacobson IM., Payne J., Dienstag JL., Van Theil DH., Tamburro C., Lefkowitch J., Albrecht J., Meschievitz C., Ortega T., Gibas A, Hepatitis Interventional Therapy Group. (1989). Treatment of chronic hepatitis C with recombinant interferon alfa. A multicenter randomized, controlled trial. Hepatitis Interventional Therapy Group. New England Journal of Medicine 321 (22): 1501-1506.

Davis GL. (1990). Recombinant alpha-interferon treatment of non-A, and non-B (type C) hepatitis: review of studies and recommendations for treatment. Journal of Hepatology 11 Suppl 1: S72-7.

Davis GL., Esteban-Mur R., Rustgi V., Hoefs J., Gordon SC., Trepo C., Shiffman ML., Zeuzem S., Craxi A., Ling MH., Albrecht J., Hepatitis Interventional Therapy Group. (1998). Interferon Alfa-2b Alone or in Combination with Ribavirin for the

Treatment of Relapse of Chronic Hepatitis C. New England Journal of Medicine 339(21): 1493-1499.

De Lamballerie X., Charrel RN., Dussol B. (1996) Hepatitis GB virus C in patients on hemodialysis . New England Journal of Medicine. 334 (23): 1549.

Deka N., Sharma MD., Mukerjee R. (1994) Isolation of the novel agent from human stool samples that is associated with sporadic non-A, non-B hepatitis. Journal of Virology 68 (12): 7810-7815.

Deinhardt F., Holmes AW., Capps RB., Popper H. (1967). Studies of the transmission of human viral hepatitis to marmoset monkeys: 1. transmission of disease, serial passages and description of liver lesions. Journal of Experimental Medicine 125: 673-688.

Deinstag JL. (1983). Non-A, Non-B Hepatitis. I. Recognition, Epidemiology, and Clinical Features. Gastroenterology 85: 439-462.

Delta Coordinating Committee (1996). Delta: a randomised double-blind controlled trial comparing combinations of zidovudine plus didanosine or zalcitabine with zidovudine alone in HIV-infected individuals. Lancet. 348 (9023): 283-291.

Dezube BJ., Ahlers CM., Lawrence JP., Teng EI., Silberman SL., Pardee AB., Finberg RW. (1997). Tenidap inhibits replication of the human immunodeficiency virus 1 in cultured cells. Journal of Acquired Immune Deficiency Syndrome and Human Retrovirology 14 (1): 13-17.

Di Bisceglie AM., Martin P., Kassianides C., Lisker-Melman M., Murray L., Waggoner J., Goodman Z., Banks SM., Hoofnagle JH. (1989). Recombinant interferon alfa therapy for chronic hepatitis C. A randomized, double-blind, placebo-controlled trial. New England Journal of Medicine. 321 (22): 1506-1510.

Di Bisceglie AM., Goodman ZD., Ishak KG., Hoofnagle J., Melpolder JJ., Alter HJ. (1991). Long-term clinical and histopathalogical follow-up of chronic post-transfusion hepatitis. Hepatology 14: 969-974.

Di Bisceglie AM., Axiotis CA., Hoofnagle JH., Bacon BR. (1992a). Measurements of iron status in patients with chronic hepatitis. Gastroenerology 102: 2108-2113.

Di Bisceglie AM., Shindo M., Fong TL., Fried MW., Swain MG., Bergasa NV., Axiotis CA., Waggoner JG., Park Y., Hoofnagle JH. (1992b). A pilot study of Ribavirin therapy for chronic hepatitis C. Hepatology 16 (3): 649-654.

Dixit V., Quan S., Martin P., Larson D., Brezina M., DiNello R., Sra K., Lau JY., Chien D., Kolberg J. (1995). Evaluation of a novel serotyping system for hepatitis C virus: strong correlation with standard genotyping methodologies. Journal of Clinical Microbiology 33 (11): 2978-83.

Donahue JG., Munoz A., Ness PM., Brown DE., Yawn DH., M^{c}Allister HA., Reitz BA., Nelson KE. (1992). The declining risk of post-transfusion hepatitis C virus infection. New England Journal of Medicine 327: 369-373.

Dore GJ., Kaldor JM., McCaughan GW. (1997). Systematic review of role of polymerase chain reaction in defining infectiousness among people infected with hepatitis C virus. British Medical Journal 315 (7104): 333-337.

Dusheiko GM., Khakoo S., Soni P., Greillier L. (1996). A rational approach to the management of hepatitis C infection. British Medical Journal 312: 357 364.

Dusheiko G., Main J., Thomas H., Reichard O., Lee C., Dhillon A., Rassam S., Fryden A., Reesink H., Bassendine M., Norkrans G., Cuypers T., Lelie N., Telfer P., Watson J., Weegink C., Sillikens P., Weiland O. (1996b). Ribavirin treatment for patients with chronic hepatitis C: results of a placebo-controlled study. Journal of Hepatology 25 (5): 591-598.

El Zayadi A., Simmonds P., Dabbous H., Prescott L., Selim O., Ahdy A. (1996). Response to interferon α of Egyptian patients infected with hepatitis C virus genotype 4. Journal of Viral Hepatitis 3: 261-264.

English JC., Peake MF., Becker LE. (1996). Hepatitis C and porphyria cutanea tarda. Cutis 57 (6): 404-408.

Eron JJ., Benoit SL., Jemsek J., MacArthur RD., Santana J., Quinn JB., Kuritzkes DR., Fallon MA., Rubin M. (1995). Treatment with Lamivudine, Zidovudine, or both in HIV-Positive Patients with 200 to 500 $CD4^+$ Cells per Cubic Millimeter. New England Journal of Medicine 333 (25): 1662-1669.

Farci P., Alter HJ., Govindarajan S., Wong DC., Engle R., Lesniewski RR., Mushahwar IK., Desai SM., Millar RH., Ogata N. (1992). Lack of protective immunity against reinfection with hepatitis C virus. Science 258: 135-140.

Feinstone KSM., Kapikian AZ., Purcell RH. (1973). Hepatitis A: detection by immune electron microscopy of a virus-like antigen associated with acute illness. Science 182: 1026-1028.

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

Fert V., and Baret A. (1990). Preparation and characterisation of xanthine oxidase-antibody and hapten conjugates for their use in sensitive chemiluminescent immunoassays. Journal of Immunological Methods 131: 237-247.

Ferré F., Marchese A., Pezzoli P., Griffin S., Buxton E., Boyer V. (1994). Quantitative PCR: An Overview. In:The Polymerase Chain Reaction by Mullis KB., Ferré F., Gibbs RA. Birkhauser, Boston, Massachusetts, USA.: 67-88.

Findlay GM., and MacCallum FO. (1938). Hepatitis and jaundice associated with immunisation against certain virus diseases. Proceedings of the Royal Society of Medicine 31: 799-805.

Finerty S., Tarlton J., Mackett M., Conway M., Arrand JR., Watkins PE., Morgan AJ. (1992). Protective immunisation against Epstein-Barr virus induced disease in cottontop tamarins using the virus envelope glycoprotein gp340 produced from a bovine papillomavirus expression vector. Journal of General Virology 73: 449-453.

Fong TL., Shindo M., Feinstone SM., Hoofnagle JH., Di BisceglieAM. (1991). Detection of replicative intermediates of hepatitis C viral RNA in liver and serum of patients with chronic hepatitis C. Journal of Clinical Investigation 88: 1058-1060.

Fredricks DN., Relman DA. (1996). Sequence Based Identification of Microbial Pathogens: a Reconsideration of Koch's Postulates. Clinical Microbiology Reviews 9 (1): 18-33.

Garson JA., Tedder RS., Briggs M., Tuke P., Glazebrook JA., Trute A., Parker D., Barbara JA., Contreras M., Aloysius S. (1990). Detection of hepatitis C viral sequences in blood donations by "nested" polymerase chain reaction and prediction of infectivity. Lancet; 335 (8703): 1019-1022.

Garson JA., Preston FE., Makris M., Tuke P., Ring C., Machin SJ., Tedder RS. (1990a). Detection by PCR of hepatitis C virus in factor VIII concentrates. Lancet 335 (8703): 1473.

Garson JA., Ring C., Tuke P., Tedder RS. (1990b) Enhanced detection by PCR of hepatitis C virus RNA. Lancet 336 (8719): 878-879.

Garson JA., Tuke PW., Makris M., Briggs M., Machin SJ., Preston FE., Tedder RS. (1990c). Demonstration of viraemia patterns in haemophiliacs treated with hepatitis-C-virus-contaminated factor VIII concentrates. Lancet 336 (8722): 1022-1025

Garson JA., Ring CJ., Tuke PW. (1991a). Improvement of HCV genome detection with "short" PCR products . Lancet 338 (8780): 1466-1467.

Garson JA., Lenzi M., Ring C., Cassani F., Ballardini G., Briggs M., Tedder RS., Bianchi FB. (1991b). Hepatitis C viraemia in adults with type 2 autoimmune hepatitis. Journal of Medical Virology 34: 223-226.

Garson JA., Clewley JP., Simmonds P., Zhang LQ., Mori J., Ring C., Follett EA., Dow BC., Martin S., Gunson H. (1992). Hepatitis C viraemia in United Kingdom blood donors. A multicentre study. Vox Sanguinis 62 (4): 218-223.

Garson JA., Brillanti S., Whitby K., Foli M., Deaville R., Masci C., Miglioli M., Barbara L. (1995). Analysis of clinical and virological factors associated with response to alpha interferon therapy in chronic hepatitis C. Journal of Medical Virology 45 (3): 348-353.

Garson JA., Uhnoo I., Whitby K., Braconier JH., Deaville R., Duberg A., Wallmark E., Wistrom J., Goldin RD., Spacey B. (1997). Virological, biochemical and histological effects of human lymphoblastoid interferon in Swedish patients with chronic hepatitis C. Journal of Viral Hepatitis 4 (5): 325-331.

Garson JA., Lubach D., Passas J., Whitby K., Grant PR. (1999). Suramin Blocks Hepatitis C Binding to Human Hepatoma Cells In Vitro. Journal of Medical Virology 57: 238-242.

Gerken G, Teuber G, Goergen B, and Meyer zum Büschenfelde K. (1995): Interferon-alpha retreatment in chronic hepatitis C. Journal of Hepatology 22 (Suppl. 1): 118-121.

Ghany MG., Chan TM., Sanchez Pescador R., Urdea M., Lok AS. (1996). Correlation between serum HCV RNA and aminotransferase levels in patients with chronic HCV infection. Digestive Disease Science 41 (11): 2213-2218.

Gingeras TR., Prodanovich P., Latimer T., Guatelli JC., Richman DD., Barringer KJ. (1991). Use of self-sustained sequence replication amplification reaction to analyze and detect mutations in zidovudine-resistant human immunodeficiency virus. Journal of Infectious Disease 164(6): 1066-74.

Han HJ., Shyamala V., Richman KH., Brauer MJ., Irivine B., Urdea MS., Tekamp Olsen P., Kuo G., Choo QL., Houghton M. (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 of the U.S.A. 88: 1711-1715.

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.

Hassoba HM., Terrault NA., El-Gohary AM., Scheffel C., Jou C., Brackett J., Hunt J., Lou SC., and Wright TL. (1997). Antibody to GBV-C Second Envelope Glycoprotein (Anti-GBV-C E2): Is it a marker for immunity? Journal of Medical Virology 53: 354-360.

Hawkins A., Davidson F., Simmonds P. (1997). Comparison of plasma virus loads among individuals infected with hepatitis C virus (HCV) genotypes 1, 2, and 3 by quantiplex HCV RNA assay versions 1 and 2, Roche Monitor assay, and an in house limiting dilution method. Journal of Clinical Microbiology 35 (1): 187-192.

Hayden FG., Schlepushkin AN., Pushkarskaya NL. (1984). Combined interferon-alpha 2, rimantadine hydrochloride, and Ribavirin inhibition of influenza virus replication *in vitro*. Antimicrobial Agents and Chemotherapy 25 (1): 53-57.

Higuchi R. (1989). Simple and rapid preparation of samples for PCR. In PCR Technology (ed. Erhlich HA) Stockton Press, New York.

Higuchi R., Fockler C., Dollinger G., Watson R. (1993) Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Biotechnology. 11(9): 1026-30

Hill AB. (1965). The enviroment and disease: association or causation? Proceedings of the Royal Society of Medicine 58: 295-300.

Hillingsø JG., Jensen IP., Tom-Petersen L. (1998). Parvovirus B19 and acute hepatitis in adults. Lancet 351: 955-956.

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

Ho DD., Neumann AU., Perelson AS., Chen W., Leonard JM., Markowitz M. (1995). Rapid turnover of plasma virions and CD_4 lymphcytes in HIV-1 infection. Nature 373: 123-126.

Holland PV., Barrera JM., Ercilla MG., Yoshida CF., Wang Y., de-Olim GA., Betlach B., Kuramoto K., Okamoto H. (1996). Genotyping hepatitis C virus isolates from Spain, Brazil, China, and Macau by a simplified PCR method. Journal of Clinical Microbiology 34 (10): 2372-2378.

Hoofnagle JH., Lau D., Conjeevaram H., Kleiner D., Di Bisceglie AM. (1996). Prolonged therapy of chronic hepatitis C with Ribavirin. Journal of Viral Hepatitis 3 (5): 247-252.

Inoue Y., Miyamura T., Unayama T., Takahashi K., Saito I. (1991). Maternal transfer of HCV. Nature 353: 609.

Ito T., Mukaigawa J., Zuo J., Hirabayashi Y., Mitamura K., Yasui K. (1996). Cultivation of hepatitis C virus in primary hepatocyte culture from patients with

chronic hepatitis C results in release of high titre infectious virus. Journal of General Virology 77 (Pt 5): 1043-1054.

Jouet P., Roudot-Thorval F., Dhumeaux D., Metreau JM. (1994). Comparative efficacy of interferon-alpha in cirrhotic and noncirrhotic patients with non-A, non-B, C hepatitis. Gastroenterology 106: 686-690.

Kaito M., Watanabe S., Tsukiyama-Kohara K., Yamaguchi K., Kobayashi Y., Konishi M., Yokoi M., Ishida S., Suzuki S., and Kohara M. (1994). Hepatitis C virus particle detected by immunoelectron microscopic study. Journal of General Virology 75: 1755-1760.

Kakumu S., Yoshika K., Tanaka K., Higashi Y., Kurokawa S., Hirofuji H., Kusakabe A. (1993). Long-term carriage of hepatitis C virus with normal aminotransferase after interferon treatment in patients with chronic hepatitis C. Journal of Medical Virology 41 (1): 65-70.

Kaneko S., Murakami S., Unoura M., Kobayashi K. (1992). Quantitation of hepatitis C virus RNA by competitive polymerase chain reaction. Journal of Medical Virology 37 (4): 278-282.

Kato N., Sekiya H., Ootsuyama Y., Nakazawa T., Hijikata M., Ohkoshi S., Shimotohno K. (1993) Humoral immune response to hypervariable region 1 of the putative envelope glycoprotein (gp70) of hepatitis C virus. Journal of Virology 67 (7): 3923-3930.

Kemp DJ., Smith DB., Foote SJ., Samaras N., and Peterson MG. (1989). Colorimetric detection of specific DNA segments amplified by polymerase chain reaction. Proceedings of the National Academy of Science of the U.S.A. 86: 2423-2427.

Kievits T., van Gemen B., van Strijp D., Shukkink R., Dircks M., Adriaanse H., Malek L., Sooknanan R., and Lens P. (1991). NASBA™ isothermal enzymatic *in*

vitro nucleic acid amplification optimized for the diagnosis of HIV-1 infection. Journal of Virological Methods 35: 273-286.

Kim JL., Morgenstern KA., Lin C., Fox T., Dwyer MD., Landro JA., Chambers SP., Markland W., Lepre CA., O'Malley ET., Harbeson SL., Rice CM., Murcko MA., Caron PR., Thomson JA. (1996). Crystal structure of the hepatitis C virus NS3 protease domain complexed with a synthetic NS4A cofactor peptide. Cell 87 (2): 343-355.

Knodell RG., Ishak KG., Black WC., Chen TS., Craig R., Kaplowitz N., Kiernan TW., Wollman J. (1981). Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. Hepatology 1 (5): 431-435.

Ko YC., Ho MS., Chiang TA., Chang SJ., Chang PY. (1992). Tattooing as a risk of hepatitis C virus infection. Journal of Medical Virology 38: 288-291.

Koonin EV. (1991). The phylogeny of RNA-dependant RNA polymerases of positive strand RNA viruses. Journal of General Virology 72: 2197-2206.

Kuo G., Choo QL., Alter HJ., Gitnick GL., Redeker AG., Purcell RH., Miyamura T., Dienstag JL., Alter MJ., Stevens CE., *et al.* (1989). An assay for circulating antibodies to a major etiological virus of human non-A, non-B hepatitis. Science 244: 362-364.

Krugman S., Giles JP., Hammond J. (1967). Infectious hepatitis. Evidence for two distinctive clinical epidemiological and immunological types of infection. Journal of the American Medical Association 200: 365-373.

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

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

Lam NP., Neuman AU., Gretch DR., Wiley TE., Perelson AS., Layden TJ. (1997). Dose dependant acute clearance of hepatitis C genotype 1 virus with interferon alpha. Hepatology 26 (1):226-231.

Lampe E., Saback FL., Yoshiba CFT., and Niel C. (1997). Infection with GB virus C/hepatitis G virus in Brazilian hemodialysis and hepatitis patients and asymptomatic individuals. Journal of Medical Virology 52: 61-67.

Lanford RE., Sureau C., Jacob JR., White R., Fuerst TR. (1994). Demonstration of *in vitro* infection of chimpanzee hepatocytes with hepatitis C virus using strand specific RT/PCR. Virology 202 (2): 606-614.

Lanford RE., Chavez D., Chisari FV., Sureau C. (1995). Lack of detection of negative-strand hepatitis C virus RNA in peripheral blood mononuclear cells and other extrahepatic tissues by the highly strand-specific rTth reverse transcriptase PCR. Journal of Virology 69 (12): 8079-8083.

Lau JYN., Davis GL., Kniffen J., Qian KP., 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.

Lau JY., Qian K., Detmer J., Collins ML., Orito E., Kolberg JA., Urdea MS., Mizokami M., Davis GL. (1997). Effect of interferon-alpha and ribavirin therapy on serum GB virus C/hepatitis G virus (GBV-C/HGV) RNA levels in patients chronically infected with hepatitis C virus and GBV-C/HGV. Journal of Infectious Disease 176 (2): 421-426.

Leary TP., Muerhoff AS., Simons JN., Pilot Matias TJ., Erker JC., Chalmers ML., Schlauder GG., Dawson GJ., Desai SM., Mushahwar IK. (1996). Consensus

oligonucleotide primers for the detection of GB virus C in human cryptogenic hepatitis. Journal of Virological Methods. 56 (1): 119-21

Lefrère JJ., Loiseau P., Maury J., Lasserre J., Mariotti M., Ravera N., Lerable J., Lefèvre G., Morand-Joubert L., Girot R. (1997). Natural History of GBV-C/Hepatitis G Virus Infection Through the Follow-Up of GBV-C/Hepatitis G Virus-Infected Blood Donors and Recipients Studied by RNA Polymerase Chain Reaction and Anti-E2 Serology. Blood 90 (9): 3776-3780.

Li X., Jeffers LJ., Shao L., Reddy KR., de Medina M., Scheffel J., Moore B., Schiff R. (1995). Identification of hepatitis C virus by immunoelectron microscopy. Journal of Viral Hepatitis 2: 227-234.

Linnen J., Wages J Jr., Zhang Keck ZY., Fry KE., Krawczynski KZ., Alter H., Koonin E., Gallagher M., Alter M., Hadziyannis S., Karayiannis P., Fung K., Nakatsuji Y., Shih JW., Young L., Piatak M Jr., Hoover C., Fernandez J., Chen S., Zou JC., Morris T., Hyams KC., Ismay S., Lifson JD., Kim JP., *et al.* (1996). Molecular cloning and disease association of hepatitis G virus: a transfusion-transmissible agent. Science 271 (5248): 505-508.

Lisitsyn N., Lisitsyn N., Wigler M. (1993). Cloning the differences between two complex genomes. Science 259: 946-951.

Longo MC., Berninger MS., Hartley JL. (1990). Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. Gene 93(1): 125-128.

Love RA., Parge HE., Wickersham JA., Hostomsky Z., Habuka N., Moomaw EW., Adachi T., Hostomska Z. (1996). The crystal structure of hepatitis C virus NS3 proteinase reveals a trypsin-like fold and a structural zinc binding site. Cell 87 (2): 331-342.

MacCallum FO., Bauer DJ. (1944). Homologous serum jaundice: transmission experiments with human volunteers. Lancet 1: 622-627.

MacCallum FO. (1947). In: Scientific Discussions (infective hepatitis). Lancet 2: 435.

Magrin S., Craxi A., Fabiano C., Fiorentino G., Marino L., Almasio P., Pinzello GB., Palazzo U., Vitale M., Maggio A., Bucca G., Gianguzza F., Shyamala V., Han JH., Pagliaro L. (1992). Serum hapatitis C virus (HCV)-RNA and response to alpha-interferon in anti-HCV positive chronic hepatitis. Journal of Medical Virology 38: 200-206.

Malaguarnera M., Restuccia S., Trovato G., Siciliano R., Motta M., Trovato BA. (1995). Interferon-alpha treatment in patients with chronic hepatitis C - a metaanalytic evaluation. Clinical Drug Investigations 9: 141-149.

Mallet F., Hebrard C., Brand D., Chapuis E., Cros P., Allibert P., Besnier J.M., Barin F., Mandrand B (1993). Enzyme-linked oligosorbent assay for detection of polymerase chain reaction-amplified human immunodeficiency virus type 1. Journal of Clinical Microbiology 31 (6): 1444-1449.

Manson WS., Seal G., Summers J. (1980). Virus in Pekin ducks with structural and biological related to human hepatitis B virus. Journal of Virology 36: 829-836.

Marion P., Oshiro L., Regnery D., Scullard G., Robinson W. (1980). A virus of Beechey ground squirrels that is related to hepatitis B virus of humans. Proceedings of the National Acadamy of Sciences U.S.A 77: 2941-2945.

Martell M., Gomez J., Esteban JI., Sauleda S., Quer J., Cabot B., Esteban R., Guardia J. (1999). High-throughput real-time reverse transcription-PCR quantitation of hepatitis C virus RNA. Journal of Clinical Microbiology. 37(2): 327-32.

Martinot-Peignoux M., Marcellin P., Pouteau M., Castelnau C., Boyer N., Poliquin M., Degott C., Descombes I., Le Breton V., Milotova V., *et al.* (1995). Pretreatment serum hepatitis C virus RNA levels and hepatitis C virus genotype are the main and independent prognostic factors of sustained response to interferon alfa therapy in chronic hepatitis C. Hepatology 22 (4 Pt 1): 1050-1056.

Masuko K., Mitsui T., Iwano K., Yamazaki C., Okuda K., Meguro T., Murayama N., Inoue T., Tsuda F., Okamoto H., Miyakawa Y., Mayumi M. (1996). Infection with hepatitis GB virus C in patients on maintenance hemodialysis. New England Journal of Medicine 334 (23): 1485-90

Maynard JE., Hartwell WV., Berquist KR. (1971). Hepatitis associated antigen in chimpanzees. Journal of Infectious Disease 123: 660-664.

M^{c}Nab GM., Alexander JJ., Lecatsas G., Bey EM., Urbanowicz JM. (1976). Hepatitis B surface antigen produced by a human hepatoma cell line. British Journal of Cancer 34: 509-515.

Mellor J., Walsh EA., Prescott LE., Jarvis LM., Davidson F., Yap PL., Simmonds P (1996). Survey of type 6 group variants of hepatitis C virus in Southeast Asia by using a core-based genotyping assay. Journal of Clinical Microbiology 34 (2): 417-23

Mellor J., Haydon G., Blair C., Livingstone W., Simmonds P. (1998) Low level or absent *in vivo* replication of hepatitis C virus and hepatitis G virus/GB virus C in peripheral blood mononuclear cells. Journal of General Virology 79: 705–714.

Meyer zum Büschenfelde KH, Lohse AW, Gerken G, Treichel U, Löhr HF, Mohr H, Große A, and Diens HP (1995): The role of autoimmunity in hepatitis C infection. Journal of Hepatology 22 (Suppl. 1): 93-96.

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 Acadamy of Science U.S.A. 87 (6): 2057-2061.

Minor P., Pipkin P., Thorpe R., Thomas D. (1990). Antibody to hepatitis C virus in plasma pools. Lancet 336:188.

Mizokami M., Gojobori T., Lau JY. (1994). Molecular evolutionary virology: its application to hepatitis C virus. Gastroenterology. 107 (4): 1181-1182.

Morgan AJ. (1996). Vaccination against Epstein-Barr virus infection. In: DNA Tumour Viruses: Oncogenic Mechanisms. Eds: Barbanti-Brodano G., Bendinelli M., Friedman H. New York: Plenum Press, 395-419.

Moshage H., Yap SH. (1992). Primary cultures of human hepatocytes: a unique system for studies in toxicology, virology, parasitology and liver pathophysiology in man. Journal of Hepatology 15: 404-413.

Muerhoff AS., Leary TP., Simons JN., Pilot Matias TJ., Dawson GJ., Erker JC., Chalmers ML., Schlauder GG., Desai SM., Mushahwar IK. (1995). Genomic organization of GB viruses A and B: two new members of the Flaviviridae associated with GB agent hepatitis. Journal of Virology 69 (9): 5621-5630.

Mullis K., Faloona F., Scharf S., Saiki R., Horn G., Erlich H. (1986). Specific enzymatic amplification of DNA *in vitro*: the polymerase chain reaction. Cold Spring Harbour Symposium of Quantitative Biology. 51 (Pt 1): 263-273.

Mullis KB., Faloona FA. (1987). Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. Methods in Enzymology 155: 335-350.

Mullis KB. (1991). The polymerase chain reaction in anaemic mode: how to avoid cold oligodeoxyribonuclear fusion. PCR methods and applications 1: 1-4.

Murakawa K., Esumi M., Kato T., Kambara H., Shikata T. (1992). Heterogeneity within the non-structural protein 5-encoding region of hepatitis C viruses from a single patient. Gene 117: 229-232.

Mushahwar IK., Zuckerman JN. (1998). Clinical Implications of GB Virus C. Journal of Medical Virology 56: 1-3.

Mutimer DJ., Harrison RF., O'Donnell KB., Shaw J., Martin BA., Atrah H., Ala FA., Skidmore S., Hubscher SG., Neuberger JM *et al.* (1995). Hepatitis C virus in the asymptomatic British blood donor. Journal of Viral Hepatitis 2 (1): 47-53.

Nishiguchi S., Kuroki T., Nakatani S., Morimoto H., Takeda T., Nakajima S., Shiomi S., Seki S., Kobayashi K., Otani S. (1995). Randomised trial of effects of interferon-alpha on incidence of hepatocellular carcinoma in chronic active hepatitis C with cirrhosis. Lancet 346 (8982): 1051-1055.

Ohto H., Terazawa S., Sasaki N., HinoK., Ishiwata C., Kako M., Ujiie N., Endo C., Matui A., Okamoto H., Mishiro S., and the Vertical Transmission of Hepatitis C Virus Collaborative Study Group. (1994). Transmission of hepatitis c virus from mothers to infants. New England Journal of Medicine 330:744-750.

Okamoto H., Okada S., Sugiyama Y., Kuria K., Lizuka H., Machida A., Miyakawa Y., Mayumi M. (1991). Nucleotide sequence of the genomic RNA of hepatitis C virus 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 SI., 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: Applications to clinical surveys and tracing infectious sources. Journal of General Virology 73: 673-679.

Okanoue T., Sakamoto S., Itoh Y., Minami M., Yasui K., Sakamoto M., Nishioji K., Katagishi T., Nakagawa Y., Tada H., Sawa Y., Mizuno M., Kagawa K., Kashima K. (1996). Side effects of high dose interferon therapy for chronic hepatitis C. Journal of Hepatology 25: 283-291.

Olive DM. (1997). *Qβ* Replicase Assays for the Clinical Detection of Infectious Agents. In: Nucleic Acid Amplification Technologies: application to Disease Diagnosis by Lee H., Morse S and Olsvik Ø. BioTechniques Books, Eaton Publishing, Natick, Massachusetts, USA.: 101-113.

Orito E., Mizokami M., Suzuki K., Ohba K., Ohno T., Mori M., Hayashi K., Kato K., Iino S., Lau JY. (1995). Loss of serum HCV RNA at week 4 of interferon-alpha therapy is associated with more favorable long-term response in patients with chronic hepatitis C. Journal of Medical Virology 46 (2): 109-115.

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.

Osmond DH., Padian NS., Sheppard SW., Glass S., Shiboski SC., Reingold A. (1993). Risk factors for hepatitis C virus seropositivity in heterosexual couples. Journal of the American Medical Association 269: 361-365.

Paccagnini S., Principi N., Massironi E., Tanzi E., Romano L., Muggiasca M.L., Ragni M.C., Salvaggio L. (1995). Perinatal transmission and manifestation of hepatitis C virus infection in a high risk population. Pediatric Infectious Disease Journal 14 (3): 195-9

Pasternack R., Vuorinen P., Miettinen A (1997). Evaluation of the Gen Probe Chlamydia trachomatis transcription mediated amplification assay with urine specimens from women. Journal of Clinical Microbiology 35 (3): 676-678.

Payan C., Bloch F., Gaultier I., Belec L. (1995). Single-step reverse transcription-polymerase chain reaction for hepatitis C virus RNA with DNA enzyme immunoassay hybridization. Journal of Virological Methods 53 (2-3): 167-175.

Phillips AN., Eron JJ., Bartlett JA., Rubin M., Johnson J., Price S., Self P., Hill AM. (1996). HIV 1 RNA levels and the development of clinical disease. North American Lamivudine HIV Working Group. AIDS 10 (8): 859-865.

Pilot-Matias T., Carrick R., Coleman P., Leary T., Surowy T, Simons J., Meurhoff A., Buijk S., Chalmers M., Dawson G., Desai S., Mushahwar I. (1996). Expression of the GB virus C E2 envelope glycoprotein using the semliki forest virus vector system and its utility as a serological marker. Virology 225: 282-292.

Power JP., Lawlor E., Davidson F., Holmes EC., Yap PL., Simmonds P. (1995). Molecular epidemiology of an outbreak of infection with hepatitis C virus in recipients of anti-D immunoglobulin. Lancet 345 (8959): 1211-1213.

Poynard T., Bedossa P., Chevallier M., Mathurin P., Lemonnier C., Trepo C., Couzigou P., Payen JL., Sajus M., Costa JM., *et al.* (1995). A comparison of three interferon alfa-2b regimens for the long-term treatment of chronic non-A, non-B hepatitis. Multicenter Study Group. New England Journal of Medicine 332 (22): 1457-1462.

Poynard T., Marcellin P., Lee SS., Neiderau C., Minuk GS., Ideo G., Bain V., Heathcote J., Zeuzem S., Trepo C., Albrecht J., International Hepatitis Therapy Group. (1998). Randomised trial of interferon α2b plus ribavirin for 48 weeks or for 24 weeks verses interferon α2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. Lancet 352: 1426-1432.

Pozzato G., Kaneko S., Moretti M., Crocè LS., Franzin F., Unoura M., Bercich L., Tiribelli C., Crovatto M., Santini G., Kobayashi K. (1994). Different genotypes of hepatitis C virus are associated with different severity of chronic liver disease. Journal of Medical Virology 43: 291-296.

Prince AM. (1968). An antigen detected in the blood during the incubation period of serum hepatitis. Proceedings of the National Academy of Sciences U.S.A. 60: 814-821.

Prince AM., Brotman B., Grady GF., Kuhns WJ., Hazzi C., Levine RW., Millian SJ. (1974). Long-incubation post-transfusion hepatitis without serological evidence of exposure to hepatitis-B virus. Lancet 2: 241-246.

Provost PJ., Hilleman MR. (1979). Propagation of human hepatitis A virus in cell culture *in vitro*. Proceedings of the Society of Experimental Biology 160: 213-221.

Reesink HW., Wong VCW., Ip HMH., van der Poel CL., van Exel-Oehlers PJ., Lelie PN. (1990). Mother to infant transmission and hepatitis C virus. Lancet 335: 1216-1217.

Renault PF., Hoofnagle JH. (1989). Side effects of alpha interferon. Seminars in Liver Disease 9: 273-277.

Reichard O., Andersson J., Schvarcz R., Weiland O. (1991). Ribavirin treatment for chronic hepatitis C. Lancet 337 (8749): 1058-1061.

Reichard O., Yun ZB., Sonnerborg A., Weiland O. (1993). Hepatitis C viral RNA titers in serum prior to, during, and after oral treatment with Ribavirin for chronic hepatitis C. Journal Medical Virology 41 (2): 99-102.

Reichard O., Foberg U., Fryden A., Mattsson L., Norkrans G., Sonnerborg A., Wejstal R., Yun ZB., Weiland O. (1994). High sustained response rate and clearance

of viraemia in chronic hepatitis C after treatment with interferon alpha 2b for 60 weeks. Hepatology 19: 280-285.

Reichard O., Norkrans G., Fryden A., *et al.* (1998). Randomised, double blind, placebo controlled trial of interferon α 2b with and without Ribavirin for chronic hepatitis C. Lancet 351:83-87.

Reyes GR., Purdy MA., Kim JP., Luk KC., Young LM., Fry KE., Bradley DW. (1990). Isolation of a cDNA from the virus responsible for enterically transmitted non-A, non-B hepatitis. Science 247 (4948): 1335-1339.

Rizzetto M., Shih JW., Gerin JL. (1980). The hepatitis B virus-associated delta antigen: isolation from liver, development of solid-phase radioimmunoassays for delta antigen and anti-delta and partial characterization of delta antigen. Journal of Immunolology 125 (1): 318-324.

Rizzetto M., Verme G. (1985). Delta hepatitis--present status. Journal of Hepatology 1 (2): 187-193.

Roberts P. (1996). Virus safety of plasma products. Reviews in Medical Virology 6: 25-38.

Rodriguez Cuartero A., Garcia Vera E., Gomez Cerro A. (1994). Hepatitis C virus and Sjogren's syndrome. Infection 22 (6): 415-416.

Ryff JC (1995). Usefulness of interferon for treatment of hepatitis C. Journal of Hepatology 22 (Suppl. 1): 101-109.

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

Saiki RK., Scharf S., Falloona F., Mullis KB., Horn GT., Ehrlich HA., and Arnheim N. (1985). Enzymatic amplification of β-globin genomic sequences and restriction site analysis for the diagnosis of sickle cell anaemia. Science 230: 1350-1354.

Sakugawa H., Nakasone H., Nakayoshi T., Kinjo F., Saito A., Yakabi S., Zukeran H., Miyagi Y., Taira R., Koja K., *et al.* (1995). High proportion of false positive reactions among donors with anti-HCV antibodies in a low prevalence area. Journal of Medical Virology 46 (4): 334-8

Saldanha J., Minor P. (1994). A sensitive PCR method for detecting HCV RNA in plasma pools, blood products, and single donations. Journal of Medical Virology 43: 72-76.

Saldanha J., Minor P. (1996). Collaborative Study to Assess the Suitability of an HCV RNA Reference Sample for Detection of an HCV RNA in Plasma Pools by PCR. Vox Sanguinis 70: 148-151.

Sallie R., King R., Silva E., Tibbs C., Johnson P., and Williams R. (1994). Community prevalence of hepatitis C viraemia: a polymerase chain reaction study. Journal of Medical Virology 43 (2): 111-114.

Sambrook J., Fritsch EF., Maniatis T. (1989). Self Priming. In: Molecular Cloning: A Laboratory Manual - Second Edition. Book 2, Chapter 8.14. Cold Spring Harbor Laboratory Press.

Schalm SW., Brouwer JT., Chemello L., Alberti A., Bellobuono A., Ideo G., Schwartz R., Weiland O (1996). Interferon-Ribavirin combination therapy for chronic hepatitis C. Digestive Disease Science 41 (12 Suppl): 131S-134S.

Schmidt B., Korn K., Fleckenstein B. (1996). Molecular evidence for transmission of hepatitis G virus by blood transfusion.Lancet 30; 347 (9005): 909.

Schvarcz R., Yun ZB., Sönnerborg A., Weilland O. (1995). Combined treatment with interferon alpha-2b and Ribavirin for chronic hepatitis C in patients with a previous non-response or non-sustained response to interferon alone. Journal of Medical Virology 46: 43-47.

Self CH. (1985). Enzyme amplification- a general method applied to provide an immunoassisted assay for placental alkaline phosphatase. Journal of Immunological Methods 76 (2): 389-393.

Semple MG., Kaye S., Loveday C., Tedder RS. (1993). HIV-1 plasma viraemia quantification: a non-culture measurement needed for therapeutic trials. Journal of Virological Methods 41 (2): 167-179.

Sharp D. (1996). Vancouver AIDS meeting highlights combination attack on HIV. Lancet 348 (9020): 115

Sherker AH., Twu JS., Reyes GR., Robinson WS. (1993). Presence of viral replicative intermediates in the liver and serum of patients with infected with hepatitis C virus. Journal of Medical Virology 39: 91-96.

Shev S., Widell A., Foberg U., Fryden A., Hermodsson S., Lindh G., Lindholm A., Mansson S., Weiland O., Norkrans G. (1995). HCV genotypes in Swedish blood donors as correlated to epidemiology, liver disease and hepatitis C virus antibody profile. Infection 23: 253-257.

Shimizu TK., Yoshikura H. (1995). *In-vitro* Systems for the Detection of Hepatitis C Virus Infection. Viral Hepatitis Reviews 1 (1): 59-65.

Shindo M., Di Bisceglie A.M., Cheung L., Shih J.W., Cristiano K., Feinstone S.M., Hoofnagle J.H. (1991). Decrease in serum hepatitis C viral RNA during alpha-interferon therapy for chronic hepatitis C. Annals of Internal Medicine 115 (9): 700-704.

Shindo M., Di Bisceglie A.M., Akatsuka T., Fong T-L., Scaglione L., Donets M., Hoofnagle J H., Feinstone S.M. (1994). The physical state of the negative strand of hepatitis C virus RNA in serum of patients with chronic hepatitis C. Proceedings of the National Academy of Sciences of the U.S.A. 91: 8719-8723.

Siegl G., Frosner GG. (1978). Characterization and classification of virus particles associated with hepatitis A. II. Type and configuration of nucleic acid. Journal of Virology 26 (1): 48-53.

Simmonds P., Balfe P., Peutherer JF., Ludlam CA., Bishop JO., and Leigh-Brown AJ. (1990a). Human immunodeficiency virus-infected individuals contain provirus in small numbers of peripheral mononuclear cells and at low copy numbers. Journal of Virology 64: 864-872.

Simmonds P., Zhang LQ., Watson HG., Rebus S., Ferguson ED., Balfe P., Leadbetter GH., Yap PL., Peutherer JF., Ludlam CA. (1990b). Hepatitis C quantification and sequencing in blood products, haemophiliacs, and drug users. Lancet 336, 1469-1472.

Simmonds P., Holmes EC., Cha TA., Chan SW., McOmish F., Irivine B., Beall E., Yap PL., Kolberg J., Urdea M. (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. (1995). Variability of hepatitis C virus. Hepatology 21 (2): 570-583.

Simons JN., Leary TP., Dawson GJ., Pilot Matias TJ., Muerhoff AS., Schlauder GG., Desai SM., Mushahwar IK. (1995a). Isolation of novel virus-like sequences associated with human hepatitis. Nature Medicine. 1 (6): 564-569.

Simons JN., Pilot Matias TJ., Leary TP., Dawson GJ., Desai SM., Schlauder GG., Muerhoff AS., Erker JC., Buijk SL., Chalmers ML., *et al.* (1995b). Identification of

two flavivirus-like genomes in the GB hepatitis agent. Proceedings of the National Acadamy of Sciences U S A. 92 (8): 3401-3405.

Simons JN., Desai SM., Schultz DE., Lemon SM., Mushahwar IK. (1996). Translation initiation in GB viruses A an C: evidence for internal ribosome entry and implications for genome organisation. Journal of Virology. 70 (9): 6126-6135.

Smedile A., Farci P., Verme G., Caredda F., Cargnel A., Caporaso N., Dentico P., Trepo C., Opolon P., Gimson A., Vergani D., Williams R., Rizzetto M. (1982). Influence of delta infection on severity of hepatitis B. Lancet 302 (8305): 945-7

Smith DB., Mellor J., Jarvis LM., Davidson F., Kolberg J., Urdea M., Yap PL., Simmonds P. (1995). Variation of the hepatitis C virus 5' non-coding region: implications for secondary structure, virus detection and typing. Journal of General Virology 76: 1749-1761.

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 (Pt 6): 1093-102.

Summers J., Smolec JM., Snyder R. (1978). A similar virus to human hepatitis B virus associated with hepatitis and hepatoma in woodchucks. Proceedings of the National Academy of Sciences U.S.A. 75: 4533-4537.

Suzuki T., Tanaka E., Matsumoto A., Urushihara A., Sodeyama T. (1995). Usefulness of Simple Assays for Serum Concentration of Hepatitis C Virus RNA and HCV Genotype in Predicting the Response of Patients with Chronic Hepatitis C to Interferon α2a Therapy. Journal of Medical Virology 46: 162 168.

Tabor E. (1989). Non Human primate models for non-A, non-B hepatitis. Cancer Detection and Prevention 144: 221-225.

Takamizawa A., Mori C., Fuke I., Manabe S., Murakami S., Fujiita 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.

Tameda Y., Kosaka Y., Tagawa S., Takase K., Sawada N., Nakao H., Tsuda F., Tanaka T., Okamoto H., Miyakawa Y., Mayumi M. (1996). Infection with GB virus C (GBV-C) in patients with fulminant hepatitis. Journal of Hepatology 25 (6): 842-847.

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

Tanaka E., Kiyosawa K., Matsumoto A., Kashiwakuma T., Hasegawa A., Mori H., Yanagihara O., Ohta Y. (1996). Serum levels of hepatitis C virus core protein in patients with chronic hepatitis C treated with interferon alfa. Hepatology 23 (6): 1330-1333.

Telfer PT., Garson JA., Whitby K., Grant PR., Yardumian A., Hoffbrand AV., Wonke B. (1997). Combination therapy with interferon alpha and Ribavirin for chronic hepatitis C virus infection in thalassaemic patients. British Journal of Haematology 98: 850-855.

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.

Theodore D, Lemon SM. (1997). GB virus C, hepatitis G virus, or human orphan flavivirus? Hepatology 25 (5): 1285-1286.

Thomas SL., Newell ML., Peckam CS., Aedes AE., Hall AJ. (1998). A review of hepatitis C (HCV) vertical transmission: risks of transmission to infants born with and without HCV viraemia and human immunodeficiency virus infection. International Journal of Epidemiology 27: 1208-1217.

Tine F., Magrin S., Craxi A., Pagliaro L. (1991). Interferon for non-A, non-B chronic hepatitis. A meta-analysis of randomised clinical trials. Journal of Hepatology 13 (2): 192-199.

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 Science of the U.S.A. 91 (23): 11022-11026.

Tsuda F., Hadiwandowo S., Sawada N., Fukuda M., Tanaka T., Okamoto H., Miyakawa Y., Mayumi M. (1996). Infection with GB virus C (GBV-C) in patients with chronic liver disease or on maintenance hemodialysis in Indonesia. Journal of Medical Virology 49 (3): 248-252.

Tsukuma H., Hiyama T., Tanaka S., Nakao M., Yabuuchi T., Kitamura T., Nakanishi K., Fujimoto I., Inuoe A., Yamazaki H., Kawashima T. (1993). Risk factors for hepatocellular carcinoma among patients with chronic liver disease. New England Journal of Medicine 328: 1797-1801.

Ulrich PP., Romeo JM., Lane PK., Kelly I., Daniel LJ., Vyas GN. (1990). Detection, semiquantitation, and genetic variation in hepatitis C virus sequences amplified from the plasma of blood donors with elevated alanine aminotransferase. Journal of Clinical Investigation 86 (5): 1609-1614.

Urdea MS., Warner BD., Running JA., Stempien MM., Clyne J., Horn T. (1988). A comparison of non radioisotopic hybridisation assay methods using fluorescent, chemiluminesent and enzyme labeled synthetic oligodeoxyribonucleotide probes. Nucleic Acids Research 16: 937 956.

Van der Poel CL., Cuypers HT., Reesink HW., Weiner AJ., Quan S., Di Nello R., Van Boven JJ., Winkel I., Mulder Folkerts D., Exel Oehlers PJ., et al. (1991). Confirmation of hepatitis C virus infection by new four-antigen recombinant immunoblot assay. Lancet. 337 (8737): 317-319

Van Doorn LJ., van Belkum A., Maertens G., Quint W., Kos T., Schellekens H. (1992). Hepatitis C virus antibody detection by a line immunoassay and (near) full length genomic RNA detection by a new RNA-capture polymerase chain reaction. Journal of Medical Virology 38: 298-304.

Van Doorn LJ., Kleter B., Pike I., Quint W. (1996). Analysis of hepatitis C virus isolates by serotyping and genotyping. Journal of Clinical Microbiology 34 (7): 1784-1787.

Viazov S., Riffelman M., Sarr S., Ballauff A., Meisel H., and Roggendorf M. (1997). Transmission of GBV-C/HGV from drug-addicted mothers to their babies. Journal of Hepatology 27 (1): 85-90.

Vrati S., Giri DK., Parida SK., Talwar GP. (1992). An epidemic of non-A, non-B hepatitis in south Delhi: epidemiological studies and transmission of the disease to rhesus monkeys. Archives in Virology 125 (1-4): 319-326.

Wales NM., Smith JR. (1996). Human immunodeficiency virus and pregnancy. Current Opinions in Obstetrics and Gynaecology 8 (6): 454-7.

Wang JT, Chen PJ, Liu DP, Sheu JC, Wang TH, Chen DS. (1998). Prevalence and infectivity of hepatitis G virus and its strain varient, the GB agent, in volunteer blood donors in Taiwan. Transfusion 38 (3): 290-295.

Walker GT., Little MC., Nadeau JG., Shank DD. (1992). Isothermal *in vitro* amplification of DNA by a restriction enzyme/DNA polymerase system. Proceedings of the National Acadamy of Science U.S.A. 89: 392-396.

Weiner A., Geysen HM., Christopherson C., Hall JE., Mason TJ., Saracco G., Bonino F., Crawford K., Marion CD., Crawford KA., Brunetto M., Barr PJ., Miyamura T., McHutchinson J., and Houghton M (1992). Evidence for immune selection of hepatitis C virus (HCV) putative envelope glycoprotein variants: Potential role in chronic HCV infections. Proceedings of the National Academy of Science of the U.S.A. 89: 3468-3472.

Whitby K., Garson JA. (1995). Optimisation and evaluation of a quantitative chemiluminescent polymerase chain reaction assay for hepatitis C virus RNA. Journal of Virological Methods 51 (1): 75-88.

Wonke B., Hoffbrand AV., Brown D., Dusheiko G. (1990). Antibody to hepatitis C virus in multiply transfused patients with thalassaemia major. Journal of Clinical Pathology 43 (8): 638-640.

Wreghitt TG., Gray JJ., Allain JP., Poulain J., Garson JA., Deaville R., Maple C., Parameshwar J., Calne RY., Wallwork J., and Alexander GJM (1994). Transmission of hepatitis C virus by organ transplantation in the United Kingdom. Journal of Hepatology 20: 768-772.

Wright TL., Hollander H., Pu X., Held MJ., Lipson P., Quan S., Polito A., Thaler MM., Bacchetti P., Scharschmidt BF. (1994). Hepatitis C in HIV infected patients with and without AIDS: prevalence and relationship to patient survival. Hepatology 20: 1152-1155.

Yamada G., Takatini M., Kishi F., Takahashi M., Doi T., Tsuji T., Shin S., Tanno M., Urdea MS., Kolberg JA. (1995). Efficacy of interferon alpha therapy in chronic hepatitis C patients depends primarily on hepatitis C virus RNA level. Hepatology 22 (5): 1351-1354.

Yanagi M., Purcell RH., Emerson SU., Bukh J. (1997). Transcripts from a single full length cDNA clone of hepatitis C virus are infectious when directly transfected into

the liver of a chimpanzee. Proceedings of the National Acadamy of Science U.S.A. 94(16): 8738-8743.

Young KKY, Resnick RM, and Myers TW (1993). Detection of Hepatitis C Virus RNA by a Combined Reverse Transcription Polymerase Chain Reaction Assay. Journal of Clinical Microbiology 31 (4): 882-886.

Yoshioka K., Kakumu S., Wakita T., Ishikawa T, Itoh Y., Takayanagi M., Highashi Y., Shibita 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 HT., Reesink HW., Winkel IN., Gerken G., Lelie PN. (1993). Reliability of polymerase chain reaction for detection of hepatitis C virus. Lancet 341 (8847): 722-724.

Zacher V., Thomas RA., Goustin AS. (1993). Absolute quantification of target DNA: a simple competitive PCR for efficient analysis of multiple samples. Nucleic Acids Research 21 (8): 2017-2018.

Zanetti AR., Tanzi E., Paccagnini S., Principi N., Pizzicolo G., Caccamo ML., D'Amico E., Cambie G., Vecchi L. (1995). Mother to infant transmission of hepatitis C virus. Journal of Paediatrics 127: 278-280.

Zebala JA., Barany F. (1993). Implications for the ligase chain reaction in gastroenterology. Journal of Clinical Gastroenterology 17 (2): 171-175.

Zeuzem S., Schmidt J.M., Lee J.H., Ruster B., Roth W.K. (1996). Effect of interferon alfa on the dynamics of hepatitis C virus turnover in vivo. Hepatology 23 (2): 366-371.

Zuccotti GV., Ribero ML., Giovanni M., Fasola M., Riva E., Portera G., Biasucci G., Decarlis S., Profeta ML., Tagger A. (1995). Effect of hepatitis C genotype on mother to infant transmission of virus. Journal of Paediatrics 127: 278-280.

Zuckerman AJ., Zuckerman JN. (1995): Prospects for hepatitis C vaccine. Journal of Hepatology 22 (Suppl. 1): 97-100.

Zuckerman AJ. (1996). Alphabet of hepatitis viruses. Lancet 347: 558-559

Zuckerman MA., Aitken C., Whitby K., Deaville R., Sanders E., Glynn MJ., Swain CP., and Garson JA. (1997). Acute hepatitis C viral infection during pregnancy: failure of mother to infant transmission. Journal of Medical Virology 52: 161-163.

Zurlo MG., De Stefano P., Borgna Pignatti C., Di Palma A., Piga A., Melevendi C., Di Gregorio F., Burattini MG., Terzoli S. (1989). Survival and causes of death in thalassaemia major. Lancet 2 (8653): 27-30.

Publications Arising from this Thesis

A. PAPERS

BRILLANTI S., GARSON J., FOLI M., WHITBY K., DEAVILLE R., MASCI C., MIGLIOLI M., LUIGI BARBARA L.

A pilot study of combination therapy with Ribavirin plus interferon alfa for interferon alfa - resistant chronic hepatitis C.

Gastroenterology 107: 812 - 817. 1994

WHITBY K., GARSON JA.

Optimisation and evaluation of a quantitative chemiluminescent polymerase chain reaction assay for hepatitis C virus RNA.

Journal of Virological Methods 51 : 75-88. 1995.

GARSON JA, BRILLANTI S, WHITBY K, FOLI M, DEAVILLE R, MASCI C, MIGLIOLI M, BARBARA L.

Analysis of clinical and virological factors associated with response to alpha interferon therapy in chronic hepatitis C.

Journal of Medical Virology 45: 348-353. 1995.

WONKE B., TELFER P., GARSON JA., WHITBY K., GRANT P., ROWNTREE C., HOFFBRAND AV.

Alpha-interferon alone and in combination with Ribavirin for hepatitis C infection inmultiply transfused patients with thalassemia major - the UK experience.

Proceedings of Bone Marrow Transplantation. 19(S2): 163-165. 1997

WHITBY K., GARSON JA.

A single tube two compartment RT-PCR system for the ultrasensitive quantitative detection of HCV-RNA.

Journal of Virological Methods 66: 15-18. 1997

GARSON JA., WHITBY K., WATKINS P., MORGAN A.
Lack of susceptibility of the cottontop tamarin to hepatitis C infection.
Journal of Medical Virology 52: 286-288. 1997

ZUCKERMAN M., AITKEN C., WHITBY K., DEAVILLE R., SANDERS E., GLYNN M., SWAIN P., GARSON JA.
Acute hepatitis C viral infection during pregnancy - failure of mother to infant transmission
Journal of Medical Virology 52 : 161 - 163. 1997.

GARSON JA., UHNOO I., WHITBY K., BRACONIER J.H., DEAVILLE R., DUBERG A., WALLMARK E., WISTRÖM J., SPACEY B.
Virological, biochemical and histological effects of human lymphoblastoid interferon in Swedish patients with chronic hepatitis C.
Journal of Viral Hepatitis 4: 325-331. 1997.

TELFER P., GARSON JA., WHITBY K., GRANT P., HOFFBRAND A., WONKE B.
Combination therapy with interferon alpha and Ribavirin for hepatitis C virus (HCV) infection in multiply transfused thalassaemic patients.
British Journal of Haematology 98(4): 850-855. 1997.

GARSON JA., LUBACH D., PASSAS J., WHITBY K., GRANT PR.
Suramin Blocks Hepatitis C Binding To Human Hepatoma Cells *In Vitro*.
Journal of Medical Virology 57: 238-242. 1999.

B. POSTERS

WHITBY K., GARSON J., BARET A.

A microtitre format quantitative PCR assay for HCV RNA employing xanthine oxidase generated chemiluminescence.

Federation of European Microbiological Societies (FEMS) Symposium on the hepatitis C virus and its infection. June 29 - July 1, 1993, Istanbul, Turkey. Abst PP 137, p 76.

BRILLANTI S., GARSON J., WHITBY K., FOLI M., DEAVILLE R., MASCI C., MIGLIOLI M., BARBARA L.

HCV genotypes and HCV RNA titers in chronic hepatitis C patients treated with interferon-alfa.

Meeting of the American Association for the Study of Liver Diseases, New Orleans, May, 1994. Gastroenterology 106 (4): Abstract# A870. 1994.

TELFER PT., WHITBY K., GARSON JA., HOFFBRAND AV., WONKE B.

Alpha interferon alone and in combination with Ribavirin for hepatitis C virus (HCV) infection in multiply transfused thalassaemic patients.

Annual Meeting of the American Society for Haematology, Seattle USA. 1995.

Blood 86 (10) S1, p1924; 1995.

WHITBY K., GARSON JA., MORGAN A.

Is the cottontop tamarin a suitable animal model for hepatitis C virus infection?

Hepatitis Workshop of the Annual Meeting of the Society for General Microbiology, Warwick, March 28th, 1996.

TELFER PT., WHITBY K., GRANT P., GARSON JA., HOFFBRAND A., WONKE B

Combination therapy with interferon alpha and Ribavirin for hepatitis C virus (HCV) infection in multiply transfused thalassaemic patients.

Annual Meeting of the American Society for Haematology, Orlando, Florida, USA. 1996

Blood 88 (10) Pt1, S1, p2096; 1996.

WHITBY K., FERNS RB., BAILEY M., HOWELL D., GARSON JA., BARBARA J., TEDDER RS.
Rate of transmission of GBV-C/HGV following blood transfusion in North London.
1997 Meeting of the British Blood Transfusion Society. Warwick University. Sept 5-7

WHITBY K., ATKINS M., KINGDOM E., MANSELL M., GARSON JA., FERNS B.
GBV-C/HGV infection amongst patients in a London haemodialysis unit.
Inaugural Meeting of the European Society for Clinical Virology. Bologna, Italy, September 7-10, 1997. Progress in Clinical Virology III. Abstract P28.

GARSON J., GILSON R., WHITBY K., HAWLEY S., ATKINS M.
Pilot study of lamivudine in chronic hepatitis C virus infection.
Journal of Hepatology 28 (suppl 1) page 206. 1997. Data presented at EASL 1998.

C. PAPERS IN PREPARATION

WHITBY K., FERNS RB., GARSON JA., BAILEY M., HOWELL D., BARBARA J., TEDDER RS.
Transfusion Transmission of GBV-C.

WHITBY K., ATKINS M., KINGDOM E., MANSELL M., GARSON JA., FERNS B. GBV-C infection amongst patients in a London haemodialysis unit.

Appendix I

"Nested" PCR for HCV RNA (based on the method of Garson *et al.*, 1990)

This represents the method from which the developments described in this thesis evolved

A RNA extraction

1. Preparation of reagents and controls

- Collect samples and control aliquots from *frozen*. Leave to thaw.
- While samples thaw, prepare RNAzol solution and other reagents

RNAzol Stock Solution A.

Prepared in a fume cupboard.

47.82g guanadinium thiocyanate

2.50ml 1M sodium citrate pH 7.0

1.00g Sarkosyl (N-lauroylsarcosine, sodium salt)

Place reagents in a beaker, add approximately 50ml sterile Baxter water and dissolve in 37°C waterbath.

Transfer to a 100ml volumetric flask.

Fill to mark with sterile Baxter water.

Transfer to a Duran bottle.

Add 0.72ml β-mercaptoethanol.

Stored at 4°C for 1 month.

RNAzol Solution.

Prepared in a fume cupboard.

800µl per sample.

1 volume RNAzol stock solution A

1 volume saturated phenol - stored below water

0.1 volume 2M sodium acetate pH 4.0

Add reagents to a universal and mix.

Chloroform Mixture.

Prepared in a fume cupboard.

1ml iso-amyl-alcohol

24ml chloroform

Add chloroform to iso-amyl-alcohol in a glass universal

Stored at room temperature.

- Collect and label conical Sarstedt tubes as required for the TEST samples in the run.

NB The maximum number of tubes that can be accommodated in the cold room centrifuge is 24. The number of controls for each run is two negatives and three positives (one "A" 10^{-1} and two "A" 10^{-2}). Thus the maximum of test samples per run is 19.

2. Extraction procedure

- for test samples:

  To each tube add: 800µl RNAzol solution [CLEAN pipette].

  200µl test sample [DIRTY pipette, fresh tips].

  (If any sample contains less than 200µl, make up the volume to 200µl using water).

- for controls:

  To thawed aliquots (200µl) add: 800µl RNAzol [CLEAN pipette, fresh tips].

- To all tubes add 100µl chloroform [CLEAN pipette, fresh tips]. Secure caps and shake all tubes thoroughly for 15 seconds.
- Place on ice for 15 minutes.

- Centrifuge for 15 minutes at 14,000 rpm in cold room (or refrigerated centrifuge).
- Label a duplicate set of fresh sarstedt tubes.
- Transfer 620ml of the RNA-containing upper phase from the original tube (avoiding the DNA/protein interphase) to the fresh tube [DIRTY pipette, fresh tips]. The remaining fluid from the original tube is tipped into a closed vessel prior to disposal. The original tube is discarded.
- Add an equal volume (620µl) of propan-2-ol to each tube [CLEAN pipette, fresh tips] followed by 3µl of glycogen (20µg/µl) [CLEAN pipette, fresh tips].
- Vortex and place at -20°C overnight.
- Centrifuge tubes at 14,000 rpm for 15 minutes (refrigerated). A line marked across cap and tube, and aligned to the outer edge of the rotor during spinning, helps to mark the position of the pellet which at this stage is not easily seen in some cases.
- Wash pellets with ice cold 75% ethanol.
  (a) Remove propan-2-ol avoiding disturbance of the pellet. Discard into a closed interim storage vessel. [DIRTY pipette, fresh tips].
  (b) Add 1ml of 75% ethanol (from -20°C storage) to each tube. [CLEAN pipette, fresh tips].
  (c) Vortex so that pellet comes away from tube wall.
  (d) Centrifuge at 14,000 rpm for 8 minutes (in cold room, or refrigerated centrifuge).
  (e) Remove ethanol [DIRTY pipette, fresh tips]. Discard ethanol into universal bottle - dispose of via sink.
  (f) Add 1ml 75% ethanol from -20°C. [CLEAN pipette, fresh tips].
  (g) Vortex and spin at 14,000 rpm for 8 minutes (cold).
- Pipette off ethanol from second wash and leave tubes uncapped for 10-15 minutes to dry pellets. [DIRTY pipette, fresh tips]. Carefully pipette off any remaining ethanol.
- Add 10µl Baxter water to each tube to resuspend pellet. [CLEAN pipette, fresh tips]. Vortex and spin briefly.

- Proceed to cDNA synthesis reaction. If this is not to be done immediately, store at -70°C until required.

B. cDNA synthesis

NB: a CLEAN pipette has ***never*** been exposed to any of the following: clinical material; or any material derived from clinical material e.g. RNA, cDNA, cDNA libraries as plasmids or phage, etc; any synthetic RNA or DNA constructs amplifiable by PCR; or any material containing or exposed to sources of PCR products.
DIRTY pipettes fail to meet the criteria for CLEAN pipettes.

- Collect and label enough sterile sarstedt tubes for the number of samples and controls in the run, plus an extra tube for an RNA control.
- Prepare sufficient volumes of mix 1 and mix 2 for the number of tubes required, leaving out of mix 2 the RNA guard and reverse transcriptase until mix 2 is ready to be used (these should be kept at -20°C until time of addition). Vortex and spin briefly. [CLEAN pipettes].

Ingredients of cDNA synthesis mixtures one and two:

Mix one ingredients	Mix two ingredients
(volume per tube)	(volume per tube)
100mM HEPES pH 6.9 (1.0µl)	1M Tris. HCl pH 7.5 (1.0µl)
300µM Random Hexamers (0.5µl)	1M KCl (1.5µl)
RNase Free Water (3.5 µl)	250 mM $MgCl_2$ (0.24µl)
	1M DTT (0.2µl)
	dNTPs 25 mM each dA, dG, dC and dTTP. (0.4µl)
	RNA guard (0.6µl)
	MMLV Reverse Transcriptase (0.5µl)
	RNase Free Water (5.56µl)

- Add 5µl of mix 1 to each of the newly-labeled fresh sarstedt tubes [CLEAN pipette]. Discard remaining mix 1.
- Add 5µl of the resuspended RNA solution from each original reaction tube to the corresponding mix 1 tube [DIRTY pipette, fresh tips] (Mix up and down with pipette). Place RNA/mix 1 mixture on ice. Store original reaction tubes with remaining RNA solution at -70°C.
- Complete mix 2 by adding RNA guard and RT [CLEAN pipette]. Vortex and spin briefly.
- Add 10µl of mix 2 to tubes containing RNA/mix 1 mixture [CLEAN pipette, fresh tips]. Vortex and spin briefly.
- Incubate reaction tubes at 37°C for 90 minutes.
- Ensuring that all caps are tightly closed, boil reaction tubes for 5 minutes.
- Dry tops of tubes, ensure caps are tightly closed again and plunge tubes deeply into ice (tubes must go from hot water into ice without delay). Leave tubes on ice for 2 minutes.
- Go on to PCR reactions. If not proceeding immediately, store at -20°C.

C. PCR reactions

A "nested" PCR is performed in two stages (rounds one and two).

Round one

- Collect and label sufficient PCR tubes for the run (samples and controls plus RNA and PCR positive control).

- Prepare round 1 PCR reaction mix sufficient for the number of tubes required. [CLEAN pipettes]. Vortex and spin briefly.

  Round one PCR reaction mix ingredients: (volume per tube)

  Sterile water (29.8µl)

  10 x buffer (5.0µl)

  1.25mM dNTP's (8.0µl)

  PT1 primer 100ng/µl (1.0µl)

  PT2 primer 100ng/µl (1.0µl)

  Taq polymerase 5u/µl (0.2µl)

- Add 45µl of PCR mix 1 to each tube [CLEAN pipette].
- Add 2 (pastette) drops of mineral oil to each tube.
- Briefly spin cDNA synthesis tubes. Then:

  (a) Add 5µl of each cDNA synthesis reaction mixture to the corresponding PCR mix 1 tube; through the mineral oil. Flush out tip to ensure complete ejection of fluid [DIRTY pipette, fresh tips].

  (b) Add 4µl of water to the PCR positive control tube.

  (c) Freeze remaining cDNA synthesis product (-20°C).

- Transfer PCR mix tubes to PCR room.
- Add 1µl of PCR positive control to the designated tube, through the mineral oil [Designated PCR product pipette].
- Place tubes in a thermal cycling machine. The cycling programme consists of an initial heat denaturation step (94°C for 4 minutes), followed by 35 cycles of 95°C for 1 minute; 45°C for 1 minute, with a 72° for 7 minutes extension to the final cycle.

<u>Round two</u>

- Collect and label the required number of PCR tubes.
- Prepare sufficient round 2 PCR mix for the number of tubes to be tested. Vortex and spin briefly.

PCR round two reaction mix ingredients (volume per tube):

Sterile water (17.3µl)

10x buffer (2.5µl)

1.25mM dNTPs (4.0µl)

PT3 primer 100ng/µl (0.5µl)

PT4 primer 100ng/µl (0.5µl)

Taq polymerase 5u/µl (0.2µl)

- Add 24µl of PCR mix 2 to each tube. [CLEAN pipette].
- Add 1 pastette drop of mineral oil to each tube.
- Transfer tubes to PCR room.
- Add 1µl of 1st round product to the corresponding tube of PCR mix 2, through the mineral oil. [Dedicated PCR product pipette, fresh, aerosol-resistant tips].
- Place tubes in thermal cycling machine. For the second round no initial denaturation step is needed. Run 30 cycles (95°C, 1 min; 45°C, 1 minute).

D. Agarose gel electrophoresis

PCR products from the second round are electrophoresed on a 3% (w/v) agarose gel. A 60ml gel allows analysis of 26 samples (in fact it allows analysis of more) which is the number generated by the proposed maximum run size of 19 samples (plus controls, RNA control and PCR control).

- Prepare gel

		60ml gel	(100ml gel)
(a)	Place into a suitable container:	1.8g agarose	(3.0g)
		60ml deionised water	(100ml)
		1.2ml 50x TAE buffer	(2ml)

  (b) microwave to dissolve

  (c) cool to 60°C (or just cool enough to handle) and add 3µl of 10mg/ml ethidium bromide (5µl for 100ml gel).

- Prepare gel plate. Rinse and wipe plate and combs with 70% ethanol. Dry. Seal plate ends with tape. Insert combs into plate. Pour agarose into plate and leave to set.
- Remove tape from set gel, carefully remove combs.
- Prepare and load PCR product samples.

  (a) Pipette 2µl of 6x "orange G" sample buffer into the wells of a microtitre plate; 1 well per sample, plus one well for DNA MW marker(s).

  (b) Add 10µl of PCR product to the 2µl of "orange G" in a well, mix thoroughly and add the whole volume to the appropriate well in the gel. Repeat for all samples and controls.

  (c) Add 10µl running buffer to the DNA MW markers well(s) and 1µl of ΦX 174 *Hae* III MW markers. Mix. (Multiply volumes if more than one MW marker well is to be run). Load DNA markers into relevant well(s) of gel.

- When gel is completely loaded place carefully into electrophoresis tank containing running buffer (≈ 970ml).
- Electrophorese at 100V for approximately one hour.

- After electrophoresis, gel is photographed to provide a permanent record of results.

Appendix II

Quantitative RT-PCR.

The following protocol was written for use by the Diagnostic Virology Laboratory (UCLMS) and is based on the QRT-PCR method described in this thesis.

Reagents.

- RNAzol Stock Solution A.

  Prepared in a fume cupboard.

47.82g	guanadinium thiocyanate
2.50ml	1M sodium citrate pH 7.0
1.00g	Sarkosyl (N-lauroylsarcosine, sodium salt)

  Place reagents in a beaker, add approximately 50ml sterile Baxter water and dissolve in 37°C waterbath.

  Transfer to a 100ml volumetric flask.

  Fill to mark with sterile Baxter water.

  Transfer to a Duran bottle.

  Add 0.72ml β-mercaptoethanol.

  Stored at 4°C for 1 month.

- RNAzol Solution.

  800µl per sample.

1 volume	RNAzol stock solution A
1 volume	saturated phenol - stored below water
0.1 volume	2M sodium acetate pH 4.0

  Add reagents to a universal and mix.

- Controls.

  High positive quantification standard 'P' $4.0x10^6$ genomes ml^{-1}

  Spiked negative 1 in 100

  Negative

- Chloroform Mixture.

  Prepare in fume cupboard

  1ml iso-amyl-alcohol

  24ml chloroform

  Add chloroform to iso-amyl-alcohol in a glass universal

  Stored at room temperature.

- Propan-2-ol.

  Stored at -20°C.

- Glycogen.

  20mg.ml^{-1} molecular biology grade

  Stored at -20°C.

- 75% ethanol.

  Stored at -20°C.

- Nuclease free water.

  Stored at room temperature

- Reverse Transcription Buffer.

200µl	100mM HEPES pH 6.9
100µl	oligonucleotide PT-2 100ng.μl^{-1}
200µl	1M Tris-HCl pH 7.5
300µl	1M potassium chloride
48µl	250mM magnesium chloride
40µl	1M DDT
80µl	dNTP mix (25mM each dA,dG,dC and dTTP)

- RNA Guard.
  Stored at -20°C.

- Cloned Maloney Murine Leukemia Virus Reverse Transcriptase.
  Stored at -20°C.

- 10x PCR .Buffer.
  Supplied with enzyme.
  Stored at -20°C.

- dNTP's.
  1.25mM
  Stored at -20°C.

- Primer PT3BIO.
  Stored at -20°C.

- Primer PT4.
  Stored at -20°C.

- Exo$^-$*Pfu* DNA Polymerase.
  Stored at -20°C.

- Carbonate/Bicarbonate Buffer.
  pH 9.6
  5µg.ml^{-1} native streptavidin

- Blocking Buffer.

40ml	casein
200ml	2xPBS
400µl	thiomersal
159.6ml	Baxter water

- Coated Plates.

  Stoed at 4°C.

- Sample Diluent Buffer.

5ml	2x PBS
500µl	casein
50µl	$Tween_{20}$
4.45ml	Baxter water

- NaOH 0.15M.

  15ml Baxter water

  450µl 5M NaOH

- TTA.

  10mM Tris.Cl pH 7.5,

  0.5% $Tween_{20}$,

  0.1% sodium azide

- INTAP Probe.

25µl	Alkaline phosphatase labelled probe (INTAP)
5ml	20xSSC
500µl	caesin
50µl	Tween20
4.5ml	Baxter water

- Lumiphos[530]
  Stored at 4°C

Isolation of RNA.

- Add 800µl RNAzol solution to required number of non-skirted Sarstedt tube [CLEAN pipettes, fresh tips]
- Add 200µl of control or test serum/plasma - NOT HEPARIN - to 1 tube each [DIRTY pipettes, fresh tips]
- Add 100µl chloroform mixture [CLEAN pipettes, fresh tips]
- Vortex capped tubes for 15 seconds.
- Place on ice for 15 minutes.
- Centrifuge at 14000 rpm at 4°C for 15 minutes.
- Label required number of non-skirted Sarsted tubes marking one side.
- Add 600µl ice-cold propan-2-ol to each tube [CLEAN pipettes, fresh tips]
- Add 3µl glycogen 20µg/µl [CLEAN pipettes, fresh tips]
- Transfer 600µl of upper phase from original Sarstedt to new Sarstedt [DIRTY pipettes, fresh tips].
- Vortex tubes.
- Leave at -20°C overnight.
- Centrifuge tubes (place all marks in the same orientation) at 14000 rpm at 4°C for 15 minutes.
- Pour off supernatant.
- Add 1ml ice-cold 75% ethanol [CLEAN pipettes, fresh tips]
- Vortex.
- Centrifuge tubes (place all marks in the same orientation) at 14000 rpm at 4°C for 8 minutes.
- Pipette off ethanol using fine tipped pastette.
- Centrifuge at 14000 rpm for 20 seconds.
- Pipette off remaining ethanol [DIRTY pipettes, fresh tips]
- Leave caps off the tubes for 15 minutes to allow pellet to dry.

- Add 10µl RNase free water [CLEAN pipettes, fresh tips]
- Vortex - ensure pellet is dissolved.
- Centrifuge at 14000 rpm for 10 seconds.
- Tubes may be stored at -70°C.)

cDNA Synthesis.

- Label Sarstedt tubes.
- Prepare "reaction mix" [CLEAN pipettes, fresh tips]**.** Each sample requires:

4.84µl	Reverse transcription buffer
9.06µl	Baxter water
0.6µl	RNAguard
0.5µl	MMLV reverse transcriptase

- Vortex "reaction mix".
- Add 15µl "reaction mix" to each tube [CLEAN pipettes, fresh tips]
- Add 5µl RNA solution to appropriate tube and mix by pipetting [DIRTY pipettes, fresh tips]
- Incubate at 37°C for 90 minutes in waterbath.
- Place tubes in a boiling waterbath for 5 minutes.
- Submerge tubes in ice for at least 2 minutes.
- Tubes may be stored at -20°C.

Polymerase Chain Reaction.

- Centrifuge tubes at 14000 rpm for 10 seconds.
- Prepare standard curve [DIRTY pipettes, fresh tips]

  neat control P

  2µl high positive control P + 20µl Baxter water - vortex

  2µl of above + 20µl Baxter water - vortex,

  2µl of above + 20µl Baxter water - vortex

  2µl of above + 20µl Baxter water - vortex

  20µl Baxter water
- Prepare "master mix" - use clean pipettes. Each control dilution/sample requires:

29.2µl	Baxter water
5µl	x10 PCR buffer
8µl	dNTP's
1µl	primer PT3BIO
1µl	primer PT4
0.8µl	exo-*Pfu* DNA polymerase

- Add 45µl "master mix" to each linked eppendorf tube 1 per control dilution/sample [CLEAN pipettes, fresh tips]
- Add 5µl control dilution or sample preparation to appropriate tube
- Cover eppendorf tubes with plastic cover and heat seal.

In PCR room

- Place tubes in thermal cycler GENEAMP 9600.
- Select programme 57:

94°C 4 minutes

94°C 60 seconds ⎫

53°C 30 seconds ⎬ x 35

75°C 2 seconds ⎭

75°C 7 minutes

Running time approximately 1 hour 30 minutes.

- Tubes may be stored at 4°C overnight or long-term at-20°C.

Enzyme Linked Oligonucleotide Assay - ELONA.

- Prepare sample diluent.

In PCR room

- Add 100µl sample diluent to each well of ELONA plate.
- Leave 2 rows blank - add 5µl PCR product to 2 wells each, mix by pipetting:

blank	blank	P	P	neg	neg	S 23	S 23				
blank	blank	$Px10^{-1}$	$Px10^{-1}$	S 16	S 16						
blank	blank	$Px10^{-2}$	$Px10^{-2}$	S17	S 17						
blank	blank	$Px10^{-3}$	$Px10^{-3}$	S18	S 18						
blank	blank	$Px10^{-4}$	$Px10^{-4}$	S19	S 19						
blank	blank	diluent	diluent	S 20	S 20						
blank	blank	spike	spike	S 21	S 21						
blank	blank	neg	neg	S22	S22						

- Cover and incubate for 45 minutes at 45°C in a moist box.

- Prepare NaOH and INTAP probe.
- Aspirate wells using hand washer.
- Add 100µl 0.15M NaOH.
- Leave for 2-5 minutes.
- Aspirate wells and fill with TTA.
- Wash x10 using hand washer and TTA, blot dry on paper towel.
- Add 100µl INTAP probe to each well.
- Cover and incubate for 30 minutes at 45°C in a moist box.
- Wash x10 using hand washer and TTA.
- Soak in TTA for at least 1 minute.
- Wash as in 9.
- Add 100µl lumigen to each well.
- Seal plate with sticky sealers CoStat.
- Incubate at room temperature for 1 hour.
- Read plate on Topcount luminometer - programme 29.

- **PCR ROOM REGULATIONS**

- Restrict access to authorised personnel.
- Only labcoats specifically designated for a given PCR room may be used in that room, and they must be washed separately from other labcoats.
- When entering the following order of dressing should be observed before crossing the yellow line:

  GLOVES

  OVERSHOES

  COAT
- When exiting each overshoe is removed as the yellow line is crossed, then remove your coat, before finally removing gloves.
- Hands are washed before leaving.
- Removal of equipment - by special arrangement only.
- Removal of pens, notebooks, *etc.*, is not permitted.
- Notes, photographs, *etc.*, should be removed in suitable plastic folders, with the exception of autoradiographs, which are oversize and are removed directly from the X-ray processor.
- No reagents should be removed from the PCR room. Material which must be removed (e.g. to transfer from one PCR room to the other) must be contained and transported in a clean sealed container.
- All work should be performed within a lined spill tray.
- All spills should be mopped up immediately. Areas potentially contaminated by PCR product should be swabbed with 0.1M HCl. Gel tanks should be decontaminated in this manner on a regular basis.
- External contractors must be accompanied, gloved, shoed and gowned.
- All departmental safety regulations apply.
- All yellow/blue/white tips, Eppendorf tubes, *etc.*, go into CinBins.
- All waste material from the PCR room will be incinerated in leak-proof bags (Yellow bags). These are placed into a clean yellow bag (double bagging requires two people), sealed with tape, labeled 'VIROLOGY', and placed by lift doors, where the bags are collected and sent to be incinerated.

- Radioactive materials will be handled in the same way as outside the PCR room, all handling being separate from the main lab. Each lab is equipped with a designated sink for the handling of liquid radioactive waste. All solid radioactive waste is stored within the PCR room in designated containers, which when full are removed from site separately from normal PCR waste. Radioactivity disposal records are kept as normal, and are removed from the PCR lab in plastic folders as described above.

PCR REAGENTS LIST

NB: order molecular biology grade reagents where possible and restrict use to work involving PCR

- Sterile "Baxter" water (Cat.No. F7114)
- Water Saturated Phenol (Rathburn Chemicals RP3024)
- 2M Sodium Acetate pH 4.0
- Guanidinium Thiocyanate Salt (Sigma, Cat.No. G-6639),
- 1M Sodium Citrate, pH 7.0
- Sarkosyl (N-Lauroylsarcosine, Na salt, Sigma, Cat.No. L-5125).
- 2-Mercaptoethanol
- 1.5 ml screw capped Sarstedt tube (Cat.No. 72.692-005).
- 24:1 Chloroform: Iso-Amyl Alcohol
- Propan-2-ol
- Glycogen (20 mg/ml, molecular biology grade, Boehringer Mannheim, Cat.No. 901-393)
- 75% Ethanol
- Sterile RNAse free water (18MΩ) [Sigma Cat No. W 4502]
- 100 mM Hepes (pH 6.9)
- 1 M Tris.Cl (pH7.5) (Trizma Base, Sigma T8524)
- 1 M KCl
- 250 mM $MgCl_2$
- 1M Dithiothreitol (DTT)
- dNTP mix (25 mM each dA, dG, dC and dTTP) [Pharmacia Cat No. 27-2035-01]
- RNAguard_ (^40 units/µl, Pharmacia, Cat No. 27-0815-01)
- Cloned Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV Reverse Transcriptase, RNase H Minus, 150-200 units/µl, Promega, Cat No. M5302)
- dNTP set 100mM solutions (dATP, dCTP, dGTP, dTTP) [Phamacia Cat No. 27-2035-01]

- Polyadenosine Ribonucleotide Homopolymer (Pharmacia Cat No. 27-4110-01)
- Exo-*Pfu* DNA polymerase (2.5units/µl) [Stratagene Cat No. 600163]
- *Pfu* buffer (10x) supplied with enzyme
- Immunomodules, Black Frames with F16 Maxisorb (Nunc Cat no 4-75515A)
- 1mg Streptavidin (Promega Z7041)
- Carb/Bicarb buffer pH 9.6. (Sigma C3041)
- Casein blocking buffer [Cambridge Research Biochemicals Cat No SU 07 250]
- Phosphate Buffered Saline (Dulbecco 'A') Oxoid Code No. BR14a
- 10% Thiomersal (BDH 30416)
- Polyoxyethylenesorbitan Monolaurate ($Tween_{20}$) Sigma cat no P 1379.
- 5M NaOH Solution
- TTA (10mM Tris.Cl pH 7.5, 0.05% $Tween_{20}$, 0.1% Sodium Azide)
- Sodium Azide (S 2002).
- 20x SSC (3M NaCl, 300mM tri-Sodium Citrate) Sigma Cat. No. S6639
- Sticky plate sealers [Costar]
- $Lumiphos^{530}$ (Lumigen inc.)

Appendix III

Preparation of Stock Solutions, Buffers and Agarose Gels

Solution	*Method of preparation*	*Comments*
0.5M EDTA	Add 18.61g of disodium ethylenediaminetetra-acetate.2H_2O to 80ml of water and adjust the pH to 8.0 with 2g of NaOH and the volume to 100ml with water.	EDTA will not dissolve until the pH is approximately 8.0. EDTA should not be stored more than 3 months as it tends to precipitate.
10x Tris Tween Azide (TTA)	To 100mM Tris.Cl (pH 7.5) add: 5% $Tween_{20}$ and 1% sodium azide. Stored at room temperature.	Diluted 1 in 10 with deionised water before use as a wash buffer.
1M Dithiothreitol (DTT)	Dissolve 3.09g of DTT in 20ml of 0.01M sodium acetate (pH 5.2). Dispense into 1ml aliquots and store at -20°C.	Do not autoclave DTT or solutions containing DTT.
1M Tris.Cl (pH 7.5)	Dissolve 12.1g of Tris base to 80mls of sterile nuclease free water. Adjust the pH to 7.5 with 6.5ml of concentrated HCl and the volume to 100ml with more water.	1ml aliquots stored at -20°C. The pH of Tris buffers is temperature dependant. Adjust pH at room Dissolve temperature. Only use electrodes marked as suitable for Tris.
20x Salt Sodium Citrate (SSC)	Dissolve 175.3g of NaCl and 88.2g of sodium citrate in 800ml of water. Adjust the pH to 7.0 and the volume to 1 litre.	
250mM $MgCl_2$	Dissolve 5.1g of $MgCl_3.6H_2O$ in 80ml of water. Adjust the volume to 100ml. Dispense into 1ml aliquots and store at -20°C.	The dry salt $MgCl_2$ is extremely hygroscopic. It should be bought in small amounts and stored in an airtight container.
3% agarose for electrophoresis	Add 3g of molecular biology grade agarose (Promega) to 100ml of 1x TAE buffer. Boil for 2 mins then allow to cool to below 70°C before adding 5µl of ethidium bromide stock.	Gels run at a constant 100 volts for 30 mins before being photographed on an ultraviolet transilluminator.

Solution	*Method of preparation*	*Comments*
4% Metaphor agarose for RFLP analysis	Add 4g of Metaphor agarose (NuSieve) to 100ml of 1x TAE buffer. Boil for in short bursts until dissolved, then allow to cool to below 70°C before adding 5µl of ethidium bromide stock.	Metaphor agarose tends to boil over very quickly and so care needs to be taken in its preparation. Metaphor gels are run at 100 volts for 30 minutes before being photographed on an ultraviolet transilluminator. For RFLP analysis the gel is then returned to the tank and run for a further 30 minutes.
50x Tris Acetate EDTA (TAE)	Per Litre: 242g Tris base, 57.1ml glacial acetic acid, 100ml 0.5M EDTA.	Dilute 2ml per 100ml for electrophoresis running buffer or the preparation of agarose gels.
Ethidium Bromide (10g/ml)	Add 1g of ethidium bromide to 100ml of water. Stir on a magnetic stirrer for several hours to ensure it has dissolved. Wrap the container in aluminium foil and store at +4°C.	Ethidium bromide is a powerful mutagen. This solution should be prepared in a fume hood whilst wearing gloves.