DEVELOPMENT AND CLINICAL APPLICATIONS OF IMPROVED ASSAYS FOR DETECTION AND QUANTITATION OF HEPATITIS C VIRUS

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ABSTRACT

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DEVELOPMENT AND CLINICAL APPLICATIONS OF IMPROVED ASSAYS FOR DETECTION AND QUANTITATION OF HEPATITIS C VIRUS

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Quantitation of hepatitis C virus (HCV) RNA levels in serum is important for predicting and monitoring patient response to treatment and for studies into the natural history of the virus. The most widely used assays lack sensitivity and have limited dynamic range. Conventional quantitative polymerase chain reaction (PCR)-based methods are highly sensitive but difficult to optimise. Recently, methods have become available for continuous monitoring of PCR product formation ('real-time' PCR). This permits measurement of PCR products during the exponential reaction phase, allowing accurate quantitation over a large dynamic range. For this project, a real-time PCR-based assay was developed for detection and quantitation of HCV RNA using the TaqMan 5' nuclease assay and the Applied Biosystems PRISM 7700 Sequence Detection System.

TaqMan primers and probes were designed to amplify part of the 5' untranslated region and detected HCV genotypes 1 - 5 with equal efficiency. An HCV standard RNA was developed and used to generate standard curves for quantitation. An internal control RNA was produced and used to monitor efficiency of RNA extraction, reverse transcription and amplification.

The performance of the assay was evaluated using the World Health Organisation International Standard for HCV RNA. The assay quantified the International Standard to within a mean of 27% from the published load and had a limit of detection of 50 international units/ml. The assay had a wide dynamic range (50 - 10^8 copies/ml) and had intra and inter-assay coefficients of variation of 23.8% and 29.4%, respectively. The assay was compared with the Quantiplex HCV RNA 2.0 assay (Bayer) and viral loads given by the two assays correlated strongly.

The quantitative assay was used to study HCV load in 53 symptomatic and 57 asymptomatic chronically infected individuals. No difference in load was found between the two populations. When the groups were combined, a weak correlation was found between viral load and the level of liver fibrosis. Longitudinal variation in load was also studied in 19 asymptomatic patients. Of these, eight showed an increase in load, ten showed a decrease and one showed no overall change in load. Viral load varied by $<1 \log_{10}$ copies/ml in the majority of patients (68%).

HCV loads were also studied in eight HCV-positive patients treated with a combination of pegylated interferon and ribavirin (Roche). End of treatment virological and biochemical response rates were 87.5% and 75%, respectively and the overall end of treatment response rate was 75%. Highest rates of decline in viral load were seen in patients infected with genotype 3 virus.

The assay was also used to quantify HCV RNA in cultured peripheral blood mononuclear cells from HCV-positive patients. The cells were treated with immunomodulatory compounds (cyclosporin A, hydrocortisone, phytohaemagglutinin) and HCV RNA levels measured. HCV RNA levels decreased in all treated and untreated cells over the study period (seven to 14 days). The addition of the immunomodulatory compounds had no detectable effects on virus levels.

To my family and Georgina for their
encouragement, support and patience.

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ABBREVIATIONS

A	adenine
Å	Angstrom unit
ALT	alanine aminotransferase
Amp^R	ampicillin resistance
Asp	aspartic acid
ATP	adenosine triphosphate
A_x	absorbance of x nm wavelength light
b	base
bDNA	branched DNA
bp	base pair
BSA	bovine serum albumin
BT	blood transfusion
C	cytosine
ca.	circa
CCD	charge-coupled device
cDNA	complementary DNA
CIAP	calf intestinal alkaline phosphatase
CPE	cytopathic effect
C_T	threshold cycle
CTL	cytotoxic T lymphocyte
CTP	cytidine triphosphate
CV	coefficient of variation
°C	degrees centigrade
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dd	dideoxy-
dGTP	deoxyguanosine triphosphate
dH_2O	de-ionised water
D. melanogaster	*Drosophila melanogaster*
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
ds	double-stranded

DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
dUTP	deoxyuridine triphosphate
Δ	change in
E	envelope
EBV	Epstein-Barr virus
ECL	electrochemiluminescence
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetra-acetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether) N, N, N', N'-tetra-acetic acid
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbant assay
ER	endoplasmic reticulum
EtBr	ethidium bromide
ETR	end of treatment response
FAM	6-carboxyfluorescein
Fig.	figure
FRET	fluorescence resonance energy transfer
g	gram
G	guanine
GEq	genome equivalent
GITC	guanidinium isothiocyanate
GTP	guanosine triphosphate
HAI	histological activity index
HAV	hepatitis A virus
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HEX	hexachloro-6-carboxyfluorescein
His	histidine
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
hr	hour
HVR	hypervariable region
IC	internal control

i.e.	that is
IFN	interferon
IL	interleukin
IPTG	isopropyl-β-thiogalactopyranoside
IRES	internal ribosome entry site
ISDR	IFN sensitivity determining region
IU	international units
IVDU	*intra venous* drug use
JOE	2,7,-dimethoxy-4,5-dichloro-6-carboxyfluorescein
kb	kilobase
kDa	kilo Daltons
L	litre
LB	Luria broth
LDL	low-density lipoprotein
$\log_{10}$	base ten logarithm
m	milli-
M	molar
mA	milliAmp
MCS	multiple cloning site
mg	milligram
MHC	major histocompatibility complex
min	minute
ml	millilitre
mm	millimetre
MMLV	Maloney murine leukaemia virus
MOPS	3-(N-morpholino) propanesulfonic acid
MΩ	mega Ohm
mRNA	messenger RNA
MU	Mega Units
μ	micro-
μl	microlitre
n	number of samples; nano-
NaAc	sodium acetate
NANBH	non-A, non-B hepatitis
NASBA	nucleic acid sequence-based amplification

nfH_2O	nuclease-free water
NGI	National Genetics Institute
NIBSC	National Institute for Biological Standards and Controls
NK	natural killer
nm	nanometre
No.	number
NR	non-response
NS	non-structural
nt	nucleotide
NTP	nucleoside triphosphate
OD	optical density
ORF	open reading frame
P	probability
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PEG-IFN	pegylated IFN
PHA	phytohaemagglutinin
PHYLIP	Phylogeny Inference Package
PKR	double-stranded RNA-activated protein kinase
PTB	polypyrimidine-tract-binding protein
QC	quantitation control
r	correlation coefficient
RFLP	restriction fragment length polymorphism
RIBA	recombinant immunoblot assay
R_n	normalised reporter fluorescence level
RNA	ribonucleic acid
RNase	ribonuclease
ROX	6-carboxy-X-rhodamine
rpm	revolutions per minute
rRNA	ribosomal RNA
RT	reverse transcription
SD	standard deviation
SDS	sodium dodecyl sulphate; Sequence Detection System
Ser	serine

sec	seconds
S. cerevisiae	*Saccharomyces cerevisiae*
SR	sustained response
ss	single-stranded
SUHT	Southampton University Hospital Trust
T	thymine
TAE	Tris-acetate-EDTA buffer
TAMRA	6-carboxytetramethylrhodamine
TBE	Tris-borate-EDTA buffer
TE	Tris-EDTA-buffer
TEMED	N,N,N',N'-tetramethylethenediamine
TET	tetrachloro-6-carboxyfluorescein
Th	T helper
T_m	melting temperature
TMA	transcription mediated amplification
Tris	Tris(hydroxymethyl)aminoethane
tRNA	transfer RNA
U	unit; uracil
UDCA	ursodeoxycholic acid
UHQ	ultra high quality water
UNG	uracil-N-glycosylase
UK	United Kingdom
USA	United States of America
UTP	uridine triphosphate
UTR	untranslated region
UV	ultraviolet
v/v	volume per unit volume
V	Volt
w/v	weight per unit volume
w/w	weight per unit weight
W	Watt
WHO	World Health Organisation
xg	times gravity
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
>	greater than

$<$	less than
$\geq$	greater than or equal to
$\leq$	less than or equal to
3’	three-prime
5’	five-prime

1. Introduction

1.1. Hepatitis C virus

1.1.1. Discovery of the virus

Hepatitis (inflammation of the liver) is caused by a range of pathogens, toxic substances or immunological abnormalities. Viral infection is the most common cause and hepatitis-causing viruses have been found to belong to diverse groups of both RNA and DNA viruses. The first hepatitis viruses to be discovered were the hepatitis A and B viruses. Hepatitis A virus (HAV) causes acute hepatitis and is spread via the faecal-oral route. Hepatitis B virus (HBV) is a cause of both acute and chronic hepatitis and is transmitted mainly via the parenteral and sexual routes (Ganem, 1996).

The development of diagnostic tests for HAV and HBV allowed the extent of infection with these agents to be investigated. This, followed by the removal of most of the HBV-infected donors from blood transfusion supplies revealed that 90% of cases of transfusion-associated hepatitis were not caused by HAV or HBV (Houghton, 1996). The cause of so-called non-A, non-B hepatitis (NANBH) was found to be a small enveloped virus (Bradley *et al.*, 1985), but the etiological agent remained unknown until the discovery of hepatitis C virus (HCV) by Choo *et al.* in 1989. The group used serum from patients clinically diagnosed with NANBH to screen *Escherichia coli* λgt11 cDNA expression libraries derived from total nucleic acid extracted from plasma of experimentally infected chimpanzees. It was shown that the cloned cDNA was derived from a ssRNA molecule of approximately 10,000nt in length that encoded an antigen specifically associated with NANBH in chimpanzees and humans. Subsequently, an enzyme-linked immunosorbant assay (ELISA) for circulating antibody was developed, allowing studies of the epidemiology of NANBH (Kuo *et al.*, 1989).

1.1.2. Classification

Comparative analysis of the genomes of several HCV strains showed the virus to be closely related to the pestivirus and flavivirus genera of the *Flaviviridae* family (reviewed by Simmonds, 1999). Subsequently, HCV was classified as a member of the genus hepacivirus in the *Flaviviridae* family along with the closely related viruses, GB virus-A, -B and -C (Robertson *et al.*, 1999).

1.1.3. Virion structure

Study of the structure of the HCV virion (reviewed by Houghton, 1996) has been hampered by limited yields of virus from most infections and the lack of an efficient culture system. The HCV virion has been reported as being 55 - 65nm in diameter according to immunoelectron microscopy, and 30 - 38nm according to results of filtration experiments. The density of the majority of viral particles in sucrose gradients is reported as 1.09 - 1.11g/ml. A denser fraction (approximately 1.17g/ml) may correspond to neutralised virus associated with immunoglobulins. A lighter fraction (1.04 - 1.06g/ml) may be virus associated with low-density lipoprotein (LDL), which affects the buoyant density and infectivity of the particles. Treatment of virions with chloroform or non-ionic detergent inactivates the virus, indicating that the virus is enveloped. This produces RNA-containing particles with buoyant densities of 1.17 - 1.25g/ml, thought to be the 33nm viral nucleocapsids.

1.1.4. Genome organisation

The molecular biology of HCV has recently been reviewed by Bartenschlager and Lohmann (2000) and Clarke (1997). HCV has a positive-stranded RNA genome of approximately 9600nt. The genome has a single open reading frame (ORF) encoding a 3011 amino acid polyprotein which is proteolytically cleaved into the viral structural and non-structural (NS) proteins (Fig.1.1). The ORF is flanked by the 5' and 3' untranslated regions (UTRs).

(i) 5' UTR

The 5'UTR is 342nt in length (Han *et al.*, 1991) and is capable of forming extensive secondary and tertiary structures (Wang *et al.*, 1995). The region is the most conserved of the HCV genome with 90 - 100% sequence identity between isolates (Bukh *et al.*, 1992; Smith *et al.*, 1995). The 5'UTR of most HCV genotypes contains four or five AUG codons, which are not used for translation initiation. Instead, it has been shown that the 5'UTR can regulate translation in a cap-independent manner, suggesting the presence of an internal ribosome entry site (IRES) (Honda *et al.*, 1996; Tsukiyama-Kohara *et al.* 1992).

(ii) 3'UTR

The HCV 3'UTR consists of a 27 - 66nt variable sequence following the stop codon, a poly(U) region and the 98nt 3'X tail (Yanagi *et al.*, 1999; Kolykhalov *et al.*, 2000). The poly(U) region is extremely variable in length even between viruses infecting the same individual, whereas the 3'X tail is highly conserved (98 - 100% sequence identity) (Kolykhalov *et al.*, 1996).

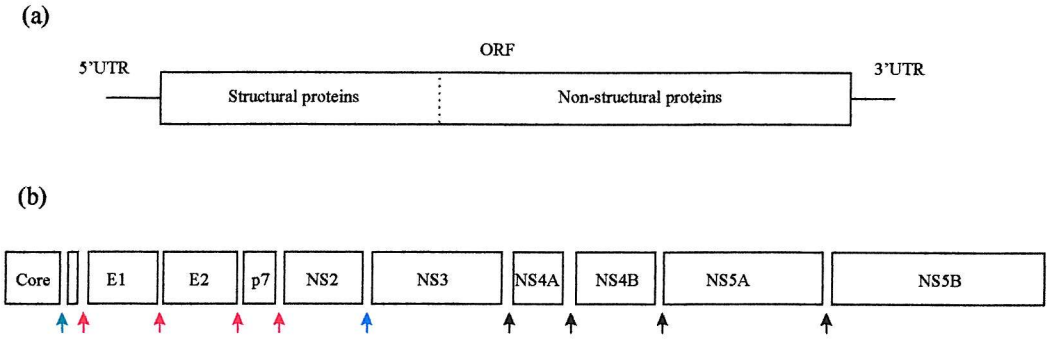


Fig.1.1 (a) HCV genome organisation.
(b) Polyprotein processing.
Arrows show the cleavage sites of host cell signalases (red), the NS2-3 protease (blue), the NS3 protease (black) and an unknown cellular protease (green). Modified from Bartenschlager and Lohmann (2000).

1.1.5. Virus-encoded proteins

(i) Core protein

The highly basic core protein is thought to form the viral nucleocapsid. Several different forms of the core protein have been identified. The p21 protein has been identified *in vitro* and *in vivo* and is believed to be derived from the p23 form. A third species, p19 is the major product from expression in mammalian cell expression. The p19, p21 and p23 proteins are localised in the cell cytoplasm (Moradpour *et al.*, 1996; Yasu *et al.*, 1998). A fourth species, p16 is localised to the nucleolus, possibly due to its ability to bind ribosomes which are assembled in the nucleolus (Santolini *et al.*, 1994). All forms of the core have distinct hydrophobic regions (amino acids 121 - 151 and 170 - 191) which are involved in the association of p21 and p19 with the endoplasmic reticulum (ER) membrane.

(ii) E1 and E2

The viral envelope glycoproteins, E1 (gp35) and E2 (gp70) are highly glycosylated type 1 transmembrane proteins. They are cleaved from the viral polyprotein at amino acids 383 and 746, respectively. E1 and E2 form two types of stable heterodimeric complexes: a disulphide form consisting of misfolded aggregates and a non-covalently linked heterodimer corresponding to the pre-budding complex. Folding and assembly of the heterodimers occurs in the ER and is dependent on association with calnexin which acts as a chaperone. A smaller protein, p7 is sometimes found at the carboxy terminus of E2. The function of p7 is as yet unknown, but it is not thought to be an essential structural component (Baumert *et al.*, 1998).

The E1 and E2 coding regions of the genome are highly variable, with E2 containing hypervariable regions (HVRs) 1 and 2 (amino acids 390 - 410 and 474 - 480, respectively). The 20 - 30 amino terminal amino acids of E2 (HVR1) vary by approximately 36% between individuals infected from a common source (Simmonds, 1999; De Francesco, 1999).

(iii) NS2

The HCV NS2 protein is a transmembrane protein with its carboxy terminus translocated into the ER lumen and its amino terminus in the cytosol. Immunoprecipitation studies have shown that NS2 is closely associated with the structural proteins. The protein has no homology with known classes of metalloproteases, but mutational analysis has revealed that the carboxy terminus is involved in polyprotein processing which is inhibited by EDTA and stimulated by Zn^{2+} ions.

(iv) NS3

The NS3 protein is approximately 70kDa in size and has protease, helicase and NTPase activities (reviewed by Wardell *et al.*, 1999). The protein is the main viral protease involved in polyprotein processing and the amino terminus contains a conserved set of residues (His-1083, Asp-1107, Ser-1165) which make up the catalytic triad characteristic of known serine proteases. The protein's carboxy terminus contains the NTPase and RNA helicase domains which are Mg^{2+} dependent and probably involved in RNA replication. The purified enzyme has been shown to bind RNA and unwind RNA/RNA, RNA/DNA and DNA/DNA duplexes in the 3' to 5' direction using any NTP or dNTP as an energy source. Sequence analysis has shown the protein to be a member of the DEAD-box family of helicases.

(v) NS4A and B

The NS4 region of the polyprotein is processed to give two proteins, NS4A and NS4B. NS4A is a small (approximately 8kDa) protein which is an essential cofactor of NS3 for efficient polyprotein processing. The function of NS4B (27kDa) is as yet unknown.

(vi) NS5A and B

The NS5 region of the polyprotein is processed to produce two proteins, NS5A and NS5B. NS5A is a phosphoprotein, the degree of phosphorylation differentiating its two forms, p56 and p58. Both are phosphorylated by an unidentified cellular kinase at serine residues after polyprotein processing and the level of phosphorylation is enhanced by the presence of NS4A. This phosphorylation is dependent on association between NS4A and NS5A, or requires the

expression of NS5A in the context of a NS3-5A polyprotein. The regions surrounding the phosphorylation sites are well conserved and the proline-rich nature of the flanking sequence suggests that a proline-directed kinase is involved in NS5A phosphorylation. Both forms of NS5A are localised to the nuclear periplasmic membrane. The function of NS5A is not yet known but it may be involved in regulation of replication and may also play a role in resistance of infected cells to interferon (IFN) (see Section 1.1.8).

The sequence of NS5B is highly conserved between HCV isolates and other members of the Flaviviridae family. It contains the GDD amino acid motif characteristic of all known RNA-dependent RNA polymerases, indicating that the protein is the viral replicase. NS5B has been detected at the nuclear periplasmic membrane, suggesting that NS5A and NS5B may be closely associated components of a membrane-bound replication complex.

1.1.6. Virus replication

Study of HCV biology has been hampered by the lack of an efficient cell culture system. The liver is the main site of HCV replication (reviewed by Negro and Levrero, 1998), as demonstrated by *in situ* hybridisation, immunohistochemistry and detection of negative-strand replication intermediate in hepatocytes. The presence of negative-strand HCV RNA in extrahepatic sites including peripheral blood mononuclear cells (PBMCs) and bone marrow has been reported. However the existence of extrahepatic replication remains controversial because of the low specificity of assays for detection of negative-strand HCV RNA (discussed in Chapter 8). The stages in the replication of HCV have recently been reviewed by Bartenschlager and Lohmann (2000).

(i) Attachment and entry

Little is known about the early events of HCV replication. Virus attachment to host cells is believed to be via the E2 glycoprotein, as binding is prevented by E2-specific antisera (Farci *et al.*, 1996). It is thought that E1 may be the fusion protein due to the presence of a region of hydrophobic amino acids analogous to flavivirus fusion peptides (Flint *et al.*, 1999). Fusion of cultured cells has recently been demonstrated and appears to require both E1 and E2 and is dependent on low pH (Takikawa *et al.*, 2000).

CD81, a tetraspanin expressed on various cell types including hepatocytes, is a putative cellular receptor for HCV (Pileri *et al.*, 1998). This was based on strong binding of E2 and virus particles by the protein and blocking of virus binding by anti-envelope antibodies from vaccinated chimpanzee plasma. The major extracellular loop of the protein is well conserved between humans and chimpanzees which are also susceptible to infection. However, it is

thought that factors other than CD81 are required for entry into the cell (Takikawa *et al.*, 2000). In addition, cells that do not express CD81 have been shown to be susceptible to HCV infection (Hamaia *et al.*, 2001). Binding to LDL receptors may also be involved (Agnello *et al.*, 1999).

(ii) Protein translation

The HCV polyprotein is translated directly from the genomic positive-sense RNA of HCV at the rough ER membrane in the host cell cytoplasm. The genome is not capped as the virus does not possess a methyl transferase enzyme and so translation is initiated by the IRES. Domain 1 of the IRES (nt 5 - 20) consists of a single stem-loop, which is believed to have a regulatory role, as its deletion has a stimulatory effect on translation. Transcription is initiated at the AUG codon at nt 342 and ribosomes are thought to bind close to this codon with little or no scanning. The IRES binds specifically to the 40S ribosomal subunit and does not require any other transcription factors. However, several cellular factors including polypyrimidine-tract-binding protein (PTB), the La antigen, and heterogeneous nuclear ribonucleoprotein L stimulate translation. Translation has been found to be greatest in the mitotic phase and lowest in the quiescent phase of the cell cycle *in vitro*, suggesting that cellular factors may be involved in regulation of HCV protein translation (Honda *et al.*, 2000).

(iii) Protein processing

At the rough ER, the polyprotein is cleaved co- and post-translationally by host cell signalases and proteases (Fig.1.1). It is thought that the carboxy terminal hydrophobic region plays a critical role in translocation of the viral structural proteins into the ER. Hydrophobic regions precede each of the C/E1, E1/E2, E2/p7, and p7/NS2 cleavage sites, suggesting cleavage by host cell signal peptidases. Post-translational cleavage by an unidentified cellular enzyme removes the E1 signal sequence from the core protein. The NS2/NS3 junction is self-cleaved by the NS2-3 protease. The NS3/4A, NS4A/B, NS4B/5A and NS5A/B junctions are cleaved by the NS3 protease. Binding of NS4A to NS3 enhances the proteolytic activity by stabilising the NS3 structure, increasing the metabolic stability and anchoring the enzyme to the ER membrane. The processed HCV proteins are believed to form a stable complex associated with intracellular membranes.

(iv) RNA replication

The mechanism of HCV RNA replication is not yet well understood. The HCV polyprotein cleavage products, and probably cellular proteins, form a replicase complex at intracellular

membranes. NS5B is presumed to be the main enzyme involved in RNA replication but other proteins are thought to be involved. These include the NS3 helicase which may act to unwind the genomic RNA; NS5A which may be involved in regulation of replication through its phosphorylation state; PTB and glyceraldehyde-3-phosphate dehydrogenase which bind the 3'UTR; and cellular proteins p87 and p130 which bind to the X-tail.

It is not known whether RNA synthesis is initiated by primer extension, copy-back priming or by a primer independent mechanism. It is also not known how template specificity is achieved, as NS5B has been shown to bind to many RNA templates.

(v) Virion assembly and release

Little is know about the mechanisms involved in assembly and release of HCV virions. Virus particle formation may be initiated by binding of the core protein with the 5' half of the genomic RNA. This appears to repress translation and therefore may be a switch between translation/replication and virion assembly. It is not yet known whether the core protein forms a distinct nucleocapsid or a less structured ribonucleoprotein complex with the RNA genome. Recently, recombinant core proteins have been shown to self-assemble into nucleocapsid-like particles in the presence of RNA (Kunkel *et al.*, 2001).

Proteins E1 and E2 have been shown to be retained in the ER, suggesting that the HCV envelope is acquired by budding through ER membranes and viral particles are released via the constitutive secretory pathway.

1.1.7. Pathogenesis

The range of clinical diseases and effects on liver histology caused by hepatitis C virus are described in Chapter 6. The relative contributions of viral cytopathic effect (CPE) and host immune responses towards HCV-related liver disease are not yet fully understood.

(i) Effects of host immune response

Corticosteroids have a temporary beneficial effect on infection, and ribavirin can improve liver histology and serum transaminase levels without reducing viral load. Furthermore, serum and intrahepatic viral loads are not related to the degree of liver disease. These findings suggest that that the host immune response plays a major role in the pathogenesis of HCV infection (reviewed by Boyer and Marcellin, 2000; Jenny-Avital, 1998). Immune response to HCV infection has been studied in both acutely and chronically infected patients. HCV-specific helper and cytotoxic T lymphocytes (CTLs) able to recognise structural and non-structural HCV proteins have been detected in liver infiltrates. The predominant type 1 helper T cell

(Th1) response is believed to enhance necro-inflammatory lesions by upregulation of CTL generation and natural killer (NK)-cell activation. Therefore, it is thought that HCV antigens expressed on hepatocytes by MHC class I molecules are recognised by CTLs, resulting in destruction of the infected cell. HCV-specific CTLs from liver infiltrates have been found to secrete interferon (IFN) -γ and other proinflammatory cytokines and the level of interleukin (IL)-2 and IFN-γ correlates with the degree of portal fibrosis and inflammation. The continuous necro-inflammatory process is inefficient at clearing infection and is thought to be the main cause of fibrosis progression. However, there is not always a close correlation between the number of necro-inflammatory lesions and progression of fibrosis, suggesting the involvement of cofactors.

(ii) CPE

There is also evidence to suggest some degree of HCV CPE. Improvements in liver histology due to IFN therapy correlate with reduced serum HCV load, indicating involvement of viral replication in pathogenesis (Shiffman *et al.*, 1997). The core protein has been shown to alter cellular metabolism of triglycerides, suggesting a possible link with liver steatosis (Barba *et al.*, 1997).

The mechanisms involved in the development of hepatocellular carcinoma (HCC) during HCV infection are also not well understood (reviewed by Colombo, 1999). The core protein may play a role in oncogenesis as it has been shown to transform primary rat embryo fibroblasts in conjunction with H-ras oncoprotein. Truncated NS3 protein has also been shown to transform mouse fibroblasts, leading to HCC in immunodeficient mice. HCV proteins may also be involved in inhibiting apoptosis of infected cells as part of the viral persistence strategy (see below), which may participate in oncogenesis.

1.1.8. Immune responses and viral persistence

Patients may remain chronically infected with HCV even though an immune response is mounted against the virus (reviewed by Boyer and Marcellin, 2000). The main mechanism by which HCV evades the immune system is believed to be its high degree of genetic variability. The RNA-dependent polymerases of RNA viruses are highly error prone and lack proof-reading capabilities (Simmonds, 1999). HCV also has a very high reproduction rate (about 10^{10} - 10^{12} virions per day) and a short *in vivo* half life (approximately 4 - 7hr) (Neumann *et al.*, 1998; Zeuzem *et al.*, 1996). These factors result in an estimated spontaneous nucleotide substitution rate of 1.4 - 2.0x10^{-3} substitutions per nt per year (in NS5B and E1, respectively) (Smith *et al.*, 1997). Therefore, the viral population within any infected host is a highly

heterogeneous quasispecies. Sequence variability is not equal across the genome. The E1, E2, NS4 and NS5A regions are most variable and the UTRs and the core region are most conserved (Simmonds, 1999).

Neutralising antibodies are produced during HCV infection (Shimizu *et al.*, 1994) and appear to prevent binding of E2 to CD81 (Pileri *et al.*, 1998). It is thought that HCV infection persists due to the rapid generation of viruses with amino acid sequence variation within the recognised epitopes (Farci *et al.*, 1994). Immune selection explains the presence of HVRs 1 and 2 within the E2 gene (Hijikata *et al.*, 1991; Weiner *et al.*, 1999). The high mutation rate may also result in formation of defective interfering particles which bind to potentially neutralising antibodies, preventing them from reaching replicative particles (Alter, 1995).

Helper T cells are thought to have an important role in clearance of HCV infection. Th1 cells produce IL-2 and IFN-γ which stimulate the development of the cellular host antiviral immune response. Type 2 helper T (Th2) cells secrete IL-4 and IL-10 which up-regulate antibody production and down-regulate the Th1 response. Individuals with acute HCV infection that clear the virus have a strong Th1 response, but a weak or absent Th2 response. Patients that fail to clear the virus and go on to develop a chronic infection mount a weak Th1 response and a strong Th2 response. This suggests that the cellular arm of the immune system is more effective than the humoural response at clearing HCV infection.

The reasons for the imbalance of Th1 and Th2 responses in HCV-infected patients are not known, but differences in patient human leukocyte antigen (HLA) type are associated with viral clearance (Thio *et al.*, 2001). Detection of HCV RNA in cells of the haematopoietic lineage suggests that the virus may be capable of interfering with the host immune response (Negro and Levrero, 1998). The HCV core protein has been shown to interact with the cytoplasmic tail of the lymphotoxin-β receptor which is involved in the development of peripheral lymphoid organs. It has also recently been reported that the HCV core protein suppresses IL-12 and nitric oxide production in macrophage cell lines, suggesting a possible role in reducing the Th1 response in chronically infected patients (Lee *et al.*, 2001a).

HCV may also affect the response of infected cells to the immune mechanisms by interfering with the initiation of apoptosis (reviewed by De Francesco, 1999; Boyer and Marcellin, 2000). The core protein has been reported to suppress apoptosis in cell culture. Consistent with this, the protein has been shown to suppress the transcription of the tumour suppressor protein, p53 (Ray *et al.*, 1997), and interfere with IFN-induced signal transduction (Basu *et al*,. 2001). NS3 is also thought to bind p53 and inhibit expression of p53-induced genes (Kwun *et al.*, 2001). It has also been proposed that NS5A may be involved in the resistance to IFN-α. Sequence analysis of HCV-sensitive and resistant HCV isolates have

revealed amino acid differences in the so-called IFN sensitivity determining region (ISDR) (amino acids 2209 - 2248). The protein has been shown to inhibit the dsRNA-activated protein kinase (PKR), a factor involved in the initiation of apoptosis in response to IFN-α. This may also be an important mechanism involved in resistance to IFN therapy, however this remains unclear. The E2 protein has also been shown to block the inhibitory effect of PKR on cellular transcription (Taylor *et al.*, 1999). Confusingly, there are also conflicting data which shows that HCV infection increases the rate of apoptosis in transfected hepatocytes (Kalkeri *et al.*, 2001).

1.1.9. HCV genotypes

HCV isolates have been classified into genotypes and subtypes (reviewed by Simmonds, 1999) on the basis of their genetic relatedness as assessed by sequence analysis of specific, relatively conserved regions of the genome (5'UTR, core, NS4, NS5B). Routine genotyping of HCV isolates is performed by more rapid and convenient methods including polymerase chain reaction (PCR) using type-specific primers, restriction fragment length polymorphism (RFLP) analysis of PCR products, and analysis of PCR products using type-specific probes (e.g. Inno-Lipa line-probe assay, Innogenetics). The use of these genotyping methods which are based on analysis of single regions of the HCV genome relies on the fact that there has been no reported evidence of recombination between HCV genotypes. Sequence analysis of the NS5B region has led to the description of six genotypes, each consisting of one or more closely related subtypes.

1.1.10. Epidemiology

World-wide there are an estimated 170 million people chronically infected with HCV (Viral Hepatitis Prevention Board, 1999). Prevalence is highest in the Middle East and North Africa, with especially high rates of infection in Egypt, Japan, Spain, Saudi Arabia and southern Italy (Houghton, 1996).

The prevalence of the different genotypes differs with geographical region. The predominant genotypes are 1a, 1b and 3a in north-western Europe, 1 and 2 in southern Europe, 1b, 2 and 6 in the Far East, 1a and 1b in North America, 3b in India, 4 in North Africa and the Middle East, and 5 in South Africa (Harris *et al.*, 1999).

HCV is transmitted mainly via the parenteral route, with high rates of anti-HCV positivity among blood and blood product recipients and *intra venous* drug users (reviewed by Alter *et al.*, 1997 and Di Bisceglie, 1998). Intra-nasal cocaine snorting was found to be a risk factor in the USA, probably due to the occurrence of epistaxis. Health-care workers are at slightly

increased risk compared with the general population. Rare cases of transmission via infected surgeons, human bites and transplantation of infected organs have also been reported.

Sexual transmission is thought to occur at a very low rate (reviewed by Wejstal, 1999). Several studies have shown a higher prevalence of anti-HCV antibodies in homosexuals, heterosexuals with multiple partners, prostitutes and their clients, and among patients with sexually transmitted diseases than in control groups. HCV incidence in monogamous, stable, heterosexual partners of infected individuals is extremely low.

A vertical transmission rate of 6% has been reported (reviewed by Zanetti *et al.*, 1999). The virus was not detected in the babies' cord blood but was in babies' blood samples taken after birth, suggesting that infection took place around the time of birth or within the first few weeks of life. The rate of vertical transmission is higher (15%) in mothers co-infected with human immunodeficiency virus (HIV) (Thomas *et al.*, 1998).

Infection via transfusion and organ transplantation has been virtually eliminated in developed countries by the introduction of donor screening around 1991. Prevalence remains high in *intra venous* drug users and among individuals with sexually transmitted infections, multiple sexual partners, or in lower socio-economic groups.

1.1.11. Prevention and control of HCV infection

(i) Prevention of transmission

Public health programs have been implemented in developed countries to attempt to reduce infection rates of blood-borne viruses (reviewed by Lavanchy, 1999). The risk of acquiring HCV through transfusion of blood and blood product has been dramatically cut in developed countries by the introduction of screening programmes and viral inactivation processes. Screening of blood donations also allows HCV-infected individuals to be identified, their disease progression evaluated and given counselling and treatment if appropriate (current treatments for HCV infection are discussed in Chapter 7). *Intra venous* drug use (IVDU) remains a major risk factor in developed countries. Prevention of illegal drug use and education and rehabilitation programmes are used to try to reduce infection rates.

However, many developing countries do not have such programmes in place and blood or blood products are not screened for HCV and contaminated medical equipment is used. Traditional practices such as circumcision, bloodletting, tattooing and piercing also pose a risk of infection in many parts of the world. Introduction of educational programmes, utilisation of single-use or properly sterilised equipment, screening and therapy of health-care workers has

been recommended. The risk of sexual transmission may be reduced by education about safe-sex practices.

(ii) Vaccination

The major goal for prevention of HCV infection is the development of an effective vaccine. However, HCV vaccine research has been hampered by the high rate of genomic variation, the lack of an efficient cell culture system for viral replication, and an incomplete understanding of the immune response to infection. An ideal vaccine would prime cross-neutralising anti-envelope antibodies, wide helper and inflammatory CD4+ T cell responses and a wide CTL response (Abrignani and Rosa, 1998).

There have been a number of attempts made at producing a vaccine for HCV. Most studies have relied on injection of mice or chimpanzees with either recombinant proteins or DNA. Choo *et al.* (1994) showed that chimpanzees vaccinated with recombinant envelope proteins developed high serum titres of anti-E2 antibodies and were protected from subsequent challenge with homologous isolates. Farci *et al.* (1996) demonstrated that anti-E2 HVR1 antibodies could neutralise homologous isolates of HCV *in vitro* and protect chimpanzees from subsequent infection. However, no protection was elicited against heterologous virus populations.

Forns *et al.* (2000) used a DNA vaccine expressing E2 protein to immunise chimpanzees. Anti-E2 antibodies were produced and both CTL and $CD4^+$ cell responses were detected. When challenged with homologous monoclonal HCV, the animals became infected. However, the infection was cleared early, in contrast to the control animal which became chronically infected. Therefore, even if a vaccine does not provide sterilising immunity, it might prevent progression to chronicity. Promisingly, Bassett *et al.* (2001) recently showed that chimpanzees that had previously cleared HCV infection, produced strong T-cell proliferative responses to rapidly clear infection with homologous and heterologous virus upon rechallenge.

Strategies used to overcome the high level of genetic variability include the use of conserved HCV proteins such as the core protein as antigen (Tokushige *et al.*, 1996). Several researchers have attempted to overcome the problem by selection of synthetic HVR epitopes that are cross reactive with large numbers of patient sera and antibodies (termed mimotopes). These have been shown to induce cross-reacting antibodies against a range of HVR1 variants (Puntoriero *et al.*, 1998).

1.1.12. Diagnostic assays

Patients with hepatitis C are usually asymptomatic, and when symptoms are present they are often non-specific and difficult to interpret. Diagnostic tests for HCV are therefore used for confirmation of clinical diagnosis and blood product screening (reviewed by Gretch, 1997). There are two types of HCV diagnostic assays available: serological assays for detection of anti-HCV antibodies or HCV antigens, and nucleic acid-based assays for detection or quantitation of HCV RNA (quantitative assays are discussed in Chapters 5).

Detection of HCV-specific antibodies is performed by enzyme immunoassays (EIAs). The first-generation EIA (EIA-1) contained a single recombinant HCV antigen derived from NS4. The second-generation EIA (EIA-2) contained recombinant antigens derived from the core, NS3 and NS4 genes and was more sensitive and specific then the EIA-1. The third-generation test (EIA-3) contains reconfigured core and NS4 antigens plus a recombinant NS5 antigen and has improved sensitivity and specificity.

The first-generation EIA test had low sensitivity and specificity and so recombinant immunoblot assays (RIBAs) were developed for use as confirmatory tests. These use the same recombinant antigens as the EIA tests, but in immunoblot form.

Use of serological assays for diagnosis of hepatitis C is hampered by the fact that an individual may not seroconvert until up to 31 weeks after infection (Chien *et al.*, 1992). To overcome these problems, assays have been developed to detect directly or quantify virus circulating in the bloodstream. The use of a core antigen ELISA is reported to detect HCV in patient sera during the so-called window period (Lee *et al.*, 2001b). However, the most widely used methods detect viral genomic RNA.

Most qualitative HCV assays are based on reverse transcription (RT)-PCR (reviewed by Powlotski, 1999). There have been many in-house RT-PCR methods described for detection of HCV RNA but commercially produced assays have been better standardised and are more widely used. The Amplicor HCV assay (Roche) involves RT and PCR being performed in a single-tube using the DNA polymerase of *Thermus thermophilus* (*Tth*). The use of biotinylated primers allows amplicons to be detected by incubation with horseradish peroxidase-labelled avidin and colourimetric substrate. The first generation of the assay had a detection limit of 1000 copies/ml, but was less sensitive for genotype 2 and 3 than genotype 1 HCV. The second generation assay has a limit of detection of 50 IU/ml and equal sensitivity for all genotypes (Lee *et al.*, 2000). An automated version (Cobas Amplicor v2.0) of the assay is also available.

The VERSANT TMA assay recently released by Bayer is a transcription-mediated amplification (TMA) assay capable of detecting HCV at <10 IU/ml (Sawyer *et al.*, 2000). The assay involves three steps that are performed in a single tube: (a) Target capture where viral

particles are lysed, HCV RNA is bound by capture oligonucleotides and the viral-probe complex is bound to magnetic microparticles. (b) Amplification of target RNA by autocatalytic, isothermal production of RNA transcripts using reverse transcriptase and T7 RNA polymerase. (c) Detection of RNA amplicons by hybridisation protection assays with amplicon-specific acridinium ester-labelled DNA probes.

1.2. Aims of project

Accurate quantitation of HCV load is important for many aspects of HCV research and patient management. At the outset of this project HCV quantitative assays lacked sensitivity and dynamic range (reviewed in Chapter 5). The aims of this project were to develop an improved assay for quantitation of HCV load using new technology for real-time PCR detection and to apply the assay to three important areas of HCV research:

(i) Study of viral load profiles in chronically infected patients.
(ii) Monitoring responses to a new combination therapy for hepatitis C.
(iii) Measurement of HCV levels in a cell culture system.

2. Materials and Methods

2.1. Materials

Suppliers are listed in Appendix I (Table 10.1).

2.1.1. Water

De-ionised water (dH_2O) was prepared by reverse osmosis using an Elgan water purification system. Further purification was achieved by reverse osmosis to an electrical resistance of 18MΩ. This produced water of a high purity (ultra high quality water, UHQ H_2O), suitable for use in DNA manipulation procedures. Nuclease-free H_2O (nfH_2O) was purchased from Ambion.

2.1.2. Buffers and solutions

Agarose gel loading buffer (6x):
15% (w/v) Ficoll (type 400) polymer, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, in dH_2O. Filtered through a 0.2μm pore filter.

Ethidium bromide (EtBr) stock:
1mg/ml ethidium bromide in sterile dH_2O. Stored at 4°C, protected from light.

Stock dNTP mixes:
100mM stocks of dATP, dTTP, dCTP and dGTP (Promega) were mixed in equimolar amounts and diluted in UHQ H_2O to give the desired concentration.

TE buffer (1x):
10mM Tris-HCl pH 8.0, 1 mM disodium ethylenediaminetetra-acetic acid (EDTA) in UHQ H_2O.

TBE buffer (10x):
10.8% (w/v) Tris-base, 0.74% (w/v) disodium EDTA, 5.5% (w/v) orthoboric acid in dH_2O. The pH was brought to 8.3 with 5M HCl.

TAE buffer (50x):
0.2M Tris-base, 0.1M acetic acid, 0.5M disodium EDTA in UHQ H_2O. pH was brought to 7.8 with 5M HCl.

RNA gel running buffer (10x):
0.2M 3-(N-morpholino) propanesulfonic acid (MOPS), 0.5M sodium acetate (NaAc), 0.01M disodium EDTA. Filtered through a 0.2μm pore filter. Stored at 4°C, protected from light.

2.1.3. Enzymes

Enzymes and buffers used for the manipulation of DNA were obtained from Promega and Life Technologies unless otherwise stated.

2.1.4. Chemicals

Standard laboratory reagents were obtained from Merck and Sigma-Aldrich unless otherwise stated and were of AnalaR grade or equivalent. Bacto-tryptone, Bacto-yeast extract and Bacto-agar were supplied by Difco. Hydrocortisone was from Glaxo Wellcome.

2.1.5. Mammalian cell culture medium

Supplemented RPMI:
RPMI 1640 cell culture medium was supplemented with glutamine (2mM), penicillin and streptomycin (each at 100U/ml) (Life Technologies) and 5% (v/v) human serum (Biowhittaker).

2.1.6. *E. coli* growth media

Luria broth (LB):
1% (w/v) Bacto-tryptone, 0.5% (w/v) Bacto-yeast extract, 0.5% (w/v) NaCl in dH_2O. The pH was adjusted to 7.5 with 5M HCl and the broth divided into 250ml aliquots and autoclaved.

LB agar:

1.5% (w/v) agar in LB. Autoclaved in 250ml aliquots. Melted agar was allowed to cool to approximately 55°C before pouring into petri-dishes.

LB agar plus ampicillin:

250ml of autoclaved LB agar was allowed to cool to approximately 55°C before addition of 250µl of 100mg/ml ampicillin

LB agar plus ampicillin, X-gal and IPTG:

200µl of 50mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and 1.25ml of 100mM isopropyl-β-thiogalactopyranoside (IPTG) was added to 250ml of molten LB agar plus ampicillin.

2.1.7. *E. coli* Top10 strain characteristics

E. coli Top10 (Invitrogen) was used for cloning. This is of the genotype: F-, *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), ϕ80*lac*ZΔM15, Δ*lac*X74, *deo*R, *rec*A1, *ara*D139, Δ(*ara leu*) 7697, *gal*U, *gal*K, *rps*L (Str^R), *end*A1, *nup*G (Grant *et al.*, 1990).

2.1.8. Plasmid vectors

pGEM-3Z is a 2743nt *E. coli* plasmid and was supplied by Promega. This vector expresses the ampicillin resistance gene (Amp^R) which encodes β-lactamase, and the (*lacZ*) gene which encodes *lacZ* α-peptide. A multiple cloning site (MCS) within the *lacZ* gene allows insertion of foreign DNA and screening of transformants.

2.1.9. Oligonucleotides

Oligonucleotides used in this project are listed in Appendix II (Table 10.2). and were supplied by Oswel DNA Service, Scandinavian Gene Synthesis AB, Cruachem or were synthesised in-house on an Expedite nucleic acid synthesis system (Millipore). TaqMan probes were supplied purified by high performance liquid chromatography.

2.1.10. *Drosophila melanogaster* DNA

D. melanogaster genomic DNA was supplied by Novagen. This was extracted from Sevelin strain embryonic nuclei and supplied in TE buffer.

2.1.11. Clinical specimens

Unless otherwise stated, HCV strains used in the study were from chronically infected blood donors enrolled in a longitudinal study of HCV infection (SUHT Liver Unit, unpublished data, 1998). All patients were antibody positive for HCV by RIBA-2 (Chiron).

Isolates were named with the prefix Sot (for Southampton), a number and the genotype as a suffix (e.g. Sot6-1a). Cloned DNA from each sample was named by giving the prefix p (e.g. pSot6-1a). *In vitro* transcribed RNA from cloned isolate DNA was given the prefix r (e.g. rSot6-1a).

2.1.12. WHO International Standard

The WHO International Standard for HCV RNA for genomic amplification technology assays (96/790, Saldanha *et al.*, 1999) was supplied by the National Institute for Biological Standards and Controls (NIBSC) as lyophilised serum samples. These were reconstituted in nfH_2O to a concentration of $1x10^5$ IU/ml.

2.2. Bacterial methods

2.2.1. Maintenance of *E. coli* Top 10

E. coli Top 10 was maintained on LB agar plates. Strains containing the cloning vector pGEM-3Z were selected for and maintained on LB plus ampicillin agar plates. Inoculated plates were incubated at 37°C overnight. The plate was then sealed with Parafilm (Merck) and stored at 4°C for up to one month before re-plating.

For long term storage, *E. coli* strains were frozen as 15% glycerol stocks. An overnight culture of the clone was made in LB plus ampicillin broth. Of this, 850µl was mixed with 150µl of sterile glycerol in a sterile 2ml screw-topped tube (Sarstedt) and stored at -80°C.

To revive a frozen strain, material was scraped from the top of the frozen stock with a culture loop and spread onto an LB plus ampicillin plate. This was incubated overnight at 37°C.

2.2.2. Preparation of competent *E. coli* cells

Competent *E. coli* Top 10 cells were prepared according to a method modified from Sambrook *et al.* (1989). LB broth (20ml) was inoculated with *E. coli* Top10 and incubated at 37°C overnight on an orbital shaker (Adolf Kuhner AG Schweiz) at 180rpm. A 1ml sample of the culture was then added to 24ml of LB broth in a 250ml conical flask and incubated at

37°C at 180rpm. After every 30min, 700µl of the culture was transferred to a plastic cuvette (Sarstedt) of path-length 10mm. The density of the culture was estimated by measuring its absorption at 550nm wavelength (A_{550}) using a spectrophotometer (Hitachi). LB broth was used as a blank sample. When the A_{550} reached 0.5 - 0.6 the culture was chilled on ice for 20min to stop growth.

The following steps were then carried out at 4°C using chilled pipette tips and containers. The culture was transferred to a universal container and centrifuged for 10min at 850xg. The supernatant was removed and the pellet resuspended in 10ml of ice-cold 0.1M $MgCl_2$. The suspension was centrifuged for 10min at 850xg and the supernatant removed. The pellet was then resuspended gently in 1ml of 0.1M $CaCl_2$. The suspension was incubated on ice for 1hr before transformation.

2.2.3. Transformation of competent *E. coli*

A 100µl sample of competent *E. coli* Top10 was added to 10µl of a completed DNA ligation reaction mix (0.6ng/µl ligation product) in a microfuge tube. The following were also added to 100µl of cells as transformation controls:

(a) Uncut plasmid DNA (0.6ng/µl)	10µl
(b) UHQ H_2O	10µl
(c) Cut, unligated plasmid (0.6ng/µl)	10µl
(d) Cut, ligated plasmid (0.6ng/µl)	10µl

These were incubated on ice for 30min with occasional agitation. The tubes were then incubated at 42°C for 2min and placed on ice for 30min. The suspension was allowed to come to room temperature before pipetting onto LB agar plates containing ampicillin, X-gal and IPTG. These were incubated overnight at 37°C.

The following day, the plates were inspected for white colonies indicating recombinant clones. Blue colonies indicated transformants containing plasmid with no insert.

2.3. Mammalian cell methods

2.3.1. Isolation and washing of PBMCs from whole blood

PBMCs were separated from defibrinated patient blood within 4hr of collection. Unclotted blood was diluted 1:1 in supplemented RPMI, 40ml carefully layered onto 10ml of Lymphoprep (Nycomed) and centrifuged at 500xg for 30min without braking. The layer of

separated PBMCs was removed by pipetting and transferred to a fresh tube. The cells were then suspended in 50ml of supplemented RPMI, centrifuged at 250xg for 10min and the supernatant discarded. The pellet was resuspended in supplemented RPMI as previously, centrifuged at 150xg for 10min and the supernatant discarded. The cells were then resuspended in 5ml of supplemented RPMI and quantified (as in Section 2.3.3). Day zero cell samples (1ml) were centrifuged at 250xg for 5min, supernatant and cells were separated and stored at -80°C.

2.3.2. Culture of PBMCs

Isolated, washed PBMCs were diluted to the appropriate density in supplemented RPMI and 2ml placed in wells of a 24-well cell culture plate. Cultures were incubated at 37°C in a 5% CO_2 incubator.

Cells were harvested by resuspending the cells in the culture medium by gentle pipetting. The cell concentration was determined as in Section 2.3.3, the suspension transferred to a fresh tube and centrifuged at 250xg for 10 min. Cell pellets and supernatants were then stored at -80°C.

2.3.3. Quantitation of PBMCs

Cells were quantified using a counting chamber and light microscopy. A 10µl sample of the cell suspension was added to the counting chamber (Bio-Stat). The cells in each of four small (11.11nl) grid squares were counted using a light microscope and the cell concentration determined using the formula:

Number of cells/ml = Mean number of cells per small grid square x 90,000.

2.4. Nucleic acid isolation and purification

2.4.1. Gene Clean II kit

DNA was extracted from agarose gels or solutions using the Gene Clean II kit (Bio 101) according to the manufacturers instructions. DNA molecules longer than 200nt in length could be purified using the kit. The method relies on the binding of DNA to silica particles at salt concentrations above 3M (Vogelstein and Gillespie, 1979).

For extraction of DNA from an agarose gel slice, the required band was cut from the gel and weighed to estimate the volume of the slice. NaI solution (3 volumes; 6M) was added to the gel slice in a microfuge tube. For extraction of DNA shorter than 500bp, a 1/10 volume of

TBE modifier solution was added to the NaI. This lowered the pH of the NaI/Glassmilk/DNA mixture to approximately pH6.5, at which smaller DNA species bind more efficiently (O'Day *et al.*, 1996). The mixture was then incubated at 55°C until the gel was fully dissolved. The 55°C incubation stage was omitted when extracting DNA from solutions.

Glassmilk suspension (5µl) was added to solutions containing 5µg of DNA or less. A further 1µl of glassmilk was added for each 0.5µg of DNA above 5µg. The solution was then vortex-mixed and incubated for 5min at 55°C for DNA of 200 - 500nt in length, or at room temperature for larger fragments. The tube was agitated every min during incubation to keep the particles in suspension. The tube was then centrifuged at 14,000xg for 10sec and the supernatant removed.

The pellet was vortex-mixed and resuspended in 200µl of NEW wash solution (NaCl, tris-base and ethanol in water). The tube was then centrifuged for 5sec at 14,000xg and the supernatant discarded. This was repeated twice more and a fine-tip pipette used to remove as much supernatant as possible. The tube was then incubated uncapped at room temperature for 10min to dry the pellet. The pellet was resuspended in 10µl of UHQ H_2O and incubated for 5min at 55°C. The sample was mixed and centrifuged at 14,000xg for 30sec and the supernatant transferred to a fresh tube. The pellet was resuspended in a further 10µl of UHQ H_2O and the tube centrifuged as previously. The supernatant was removed and combined with the supernatant from the previous step.

2.4.2. Nucleon QC kit

The Nucleon QC kit (Scottlab) was used to purify DNA fragments larger than 75nt from dNTPs, oligonucleotides and short DNA species. The procedure was carried out according to the manufacturer's instructions. SX resin (600µl) was added to a Nucleon spin-column. The column was centrifuged at 8,000xg for 6sec to bed down the resin. The eluted resin suspension buffer was discarded and 10 - 100µl of DNA solution was added to the highest point of the resin. The column was then centrifuged at 8,000xg for 5sec to elute the purified DNA.

2.4.3. QIAprep Spin Miniprep Kit

Small-scale extraction of plasmids from *E. coli* clones was performed using the QIAprep Miniprep Kit (Qiagen). The extraction method was adapted from the manufacturer's instructions and gave yields of up to 20µg of DNA. A 5ml overnight culture of *E. coli* in LB broth plus ampicillin was centrifuged at 18,000xg for 5min. The supernatant was discarded and the pellet resuspended in 250µl of buffer P1. The cells were lysed by addition of 250µl of

buffer P2 which was mixed gently with the suspension by inverting the tube six times. The suspension was observed for clearing before addition of 350µl of buffer N3 to neutralise lysis. The tube was inverted immediately six times to mix. The suspension was then centrifuged for 10min at 18,000xg and the supernatant decanted into a spin-column. The spin-column was centrifuged for 1min and the eluate discarded.

To wash the bound DNA, 750µl of buffer PE was added to the spin-column. This was centrifuged for 1min at 18,000xg and the eluate discarded. The column was centrifuged for a further 1min to remove residual buffer, placed in a fresh microfuge tube and dried for 3min in a vacuum desiccator. UHQ H_2O (50µl) was added and the spin-column incubated for 1min at room temperature. The column was then centrifuged at 18,000xg for 1min to elute plasmid DNA.

2.4.4. Qiagen Plasmid Midiprep Kit

The Qiagen Plasmid Midiprep Kit was used to purify up to 100µg of cloned plasmid DNA from *E. coli* strains. A single colony was inoculated into 2ml of LB plus ampicillin broth which was incubated at 37°C for 8hr on an orbital shaker at 300rpm. A 50µl sample of this culture was then added to 25ml of LB plus ampicillin broth and incubated at 37°C for 12 - 16hr with 300rpm shaking. The culture was then centrifuged for 30min at 850xg at 4°C. The supernatant was removed and the pellet resuspended in 4ml of buffer P1 (50mM Tris-HCl, pH8.0; 10mM EDTA; 100µg/ml RNase A). To promote lysis of cells, 4ml of buffer P2 (200mM NaOH; 1% (w/v) SDS) was added and mixed with the suspension by inverting six times. The suspension was incubated for 5min at room temperature. Chilled buffer P3 (3M potassium acetate, pH5.5) (4ml) was then mixed with the lysate by inverting the tube six times. The tube was incubated on ice for 15min and centrifuged for 30min at 850xg at 4°C. The supernatant was filtered through a 0.2µm filter. Meanwhile, 4ml of buffer QBT (750mM NaCl; 50mM MOPS, pH7.0; 15% (v/v) isopropanol; 0.15% (w/v) Triton X-100) was added to a Qiagen tip and allowed to drain though by gravity. The reaction filtrate was then added to the Qiagen tip and allowed to drain through. Two 10ml volumes of buffer QC (1.0M NaCl; 50mM MOPS, pH7.0; 15% (v/v) isopropanol) were passed through the tip to wash the bound DNA. The DNA was then eluted in 5ml of buffer QF (1.25M NaCl; 50mM Tris-HCl, pH 8.5; 15% (v/v) isopropanol). DNA was precipitated with 5.5ml of isopropanol and the tube centrifuged for 30min at 850xg at 4°C. The supernatant was carefully decanted and the pellet washed with 2ml of 70% (v/v) ethanol in UHQ H_2O. The sample was then centrifuged for 10min and the supernatant discarded. The pellet was dried for 10min at 37°C and dissolved in 1ml of TE.

2.4.5. QIAamp Viral RNA Mini Kit

HCV RNA was purified from patient serum using the QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. Stored serum samples were thawed on ice. To 140µl of serum, 560µl of buffer AVL (contains guanidinium isothiocyanate, GITC) was added in a microfuge tube. The sample was vortex-mixed and incubated at room temperature for 10min to release RNA from viral particles. The sample was then vortex-mixed and briefly centrifuged. Ethanol (560µl) was added, the tube vortex-mixed and briefly centrifuged. Half of the mixture (630µl) was added to a spin-column sitting in a collection tube and centrifuged at 6000xg for 1min. The eluate was discarded and another 630µl of the mixture added. The tube was then centrifuged and the eluate discarded as previously. High salt content buffer AW1 (500µl) was added and the column centrifuged at 6000xg for 1min. The eluate was discarded and 500µl of buffer AW2 added. The column was centrifuged at 20,000xg for 3min and the eluate discarded. The spin-column was then placed in a microfuge tube. RNA was eluted by addition of 50µl of nfH_2O to the column, incubation at 80°C for 10min and centrifugation at 6,000xg for 1min.

2.4.6. RNeasy Mini Kit

(i) Purification of RNA from in vitro transcription reactions

RNA was purified from *in vitro* transcription reactions using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The RNA sample volume was adjusted to 100µl with nfH_2O and 350µl of GITC containing buffer RLT was added. Ethanol (250µl) was added and the sample mixed well by pipetting. The sample was applied to an RNeasy spin-column which was then centrifuged for 15sec at 8,000xg. The eluate was discarded and 500µl of high salt content buffer RPE added. The column was then centrifuged for 15sec at 8,000xg to wash. A further 500µl of buffer RPE was added and the column centrifuged at 20,000xg for 2min. The column was then placed into a siliconised microfuge tube. NfH_2O (50µl) was added to the centre of the column membrane and the column centrifuged at 8,000xg for 1min to elute the RNA. The elution step was repeated with a further 50µl of nfH_2O and the eluates combined.

(ii) Extraction of RNA from cultured PBMCs

RNA was extracted from PBMCs using the RNeasy kit (Qiagen) according to the manufacturer's instructions. Cells were suspended in 350µl of buffer RLT to which 10^5 copies of internal control (IC) RNA (described in Chapter 4) had been added. The resulting lysate

was homogenised by centrifugation through a QIAshredder (Qiagen) at 20,000xg for 2min. The sample was then mixed with 350µl of 70% (v/v) ethanol. The mixture was transferred to an RNeasy spin-column, centrifuged for 15sec at 8,000xg and the eluate discarded. Buffer RW1 (700µl) was added, the column centrifuged as previously and the eluate discarded. This process was repeated with 500µl of buffer RPE. A further 500µl of RPE was added, the column centrifuged at 20,000rpm for 2min and placed in a siliconised RNase-free microfuge tube. The RNA was eluted in 50µl of nfH_2O by centrifugation at 8000xg for 1min.

2.4.7. Purification of DNA using phenol/chloroform/isoamyl alcohol

Linearised DNA templates were purified for *in vitro* transcription reactions as described in the MEGAscript *in vitro* transcription kit protocol (Ambion). To each DNA sample, one volume of phenol/chloroform/isoamyl alcohol (Ambion) (pH7.9) was added. The mixture was emulsified by vortex-mixing and centrifuged at 12,000xg for 15sec. The aqueous phase was transferred to a fresh microfuge tube and one volume of chloroform added. The sample was vortex-mixed and centrifuged at 12,000xg for 15sec. The aqueous phase was transferred to a fresh microfuge tube and precipitated with ethanol.

2.4.8. Ethanol precipitation of DNA

Precipitation of DNA from solutions was performed according to a method described by Sambrook *et al.* (1989). A 1/10 volume of 3M NaAc (pH5.2) and two volumes of ice-cold ethanol were mixed with the sample. The mixture was incubated on ice for 1hr and centrifuged at 21,000xg for 10min. The supernatant was removed and the pellet washed with 1ml of 70% (v/v) ethanol. The sample was then centrifuged at 21,000xg for 10min. The supernatant was removed and the pellet dried for 10min at room temperature. The pellet was then resuspended in nfH_2O.

2.4.9. Purification of RNA using phenol/chloroform/isoamyl alcohol

RNA was purified from *in vitro* transcription reactions by a phenol/chloroform extraction method described in the MEGAscript *in vitro* transcription kit protocol (Ambion). Phenol/chloroform/isoamyl alcohol (pH6.6) (one volume) was added to the RNA sample. The sample was then vortex-mixed and centrifuged for 15sec at 12,000xg. The aqueous phase was then transferred to a fresh microfuge tube and one volume of isopropanol was added. The sample was vortex-mixed, incubated at -20°C for 1hr and centrifuged for 10min at 21,000xg. The supernatant was removed and the RNA pellet allowed to dry at 40°C for 10min. The RNA was then resuspended in nfH_2O.

2.5. Cutting DNA with restriction enzymes

Site-specific cutting of DNA was performed using restriction enzymes. These enzymes are of bacterial origin and recognise short sequences of dsDNA. They cut within, or near these sequences resulting in either overhanging (cohesive) or blunt ends at the cutting site. A typical restriction enzyme digestion consisted of:

10x Buffer (specific to enzyme)	2µl
DNA (150ng/µl)	6µl
BSA (1mg/ml)	2µl
Restriction Enzyme (10U/µl)	0.5µl
UHQ H_2O	to 20µl

Reagents were mixed and then incubated at the optimum reaction temperature of the enzyme, typically 37°C for 4hr.

Digestion with two enzymes simultaneously was performed by including the two restriction enzymes at equal concentrations. A single reaction buffer was chosen such that both enzymes cut with high efficiency according to the manufacturer's information. Restriction enzymes were then inactivated by incubation at 70°C for 15min.

2.6. Dephosphorylation of 5' ends of plasmid DNA

The 5' ends of plasmid DNA linearised in a restriction enzyme reaction were dephosphorylated to prevent re-ligation. The enzyme, calf intestinal alkaline phosphatase (CIAP) was used in the following reaction:

DNA (45ng/µl)	20µl
CIAP buffer (10x)*	5µl
CIAP (0.001U/µl)	1µl
UHQ H_2O	to 50µl

*(500mM Tris-HCl (pH9.3), 10mM $MgCl_2$, 1mM $ZnCl_2$, 10mM spermidine)

The reactants were incubated at 37°C for 30min. To stop the reaction, 2µl of 0.5M EDTA was added and the tube incubated at 65°C for 20min.

2.7. Ligation of plasmid and insert DNA

Ligation of plasmid and insert DNA with complementary cohesive ends was performed using T4 DNA ligase. Reactions with vector to insert ratios of 1:3, 1:1 and 3:1 were performed as follows:

Vector	xng
Insert	yng
Ligase Buffer (10x)*	1µl
T4 DNA ligase (3U/µl)	0.5µl
UHQ H_2O	to 10µl

*(300mM Tris-HCl (pH7.8), 100mM $MgCl_2$, 100mM DTT, 10mM ATP)

The reaction was incubated for 16hr at 4°C.

2.8. Reverse transcription (RT)

RT involves the production of complementary DNA (cDNA) from an RNA template. The process is performed by a reverse transcriptase enzyme. RT proceeds in the 5' to 3' direction from an oligonucleotide primer annealed to the RNA template. RT can be primed using a sequence-specific primer to produce cDNA products with a common 5' end. Alternatively, RT can be primed using short oligonucleotides with random sequence (random hexamer primers) which prime at different sites on the RNA molecule (Gerard *et al.*, 1997).

Ribonuclease activity was inhibited by the inclusion of RNasin. Bovine serum albumin (BSA) and Dithiothreitol (DTT) were included in reaction mixes to stabilise reverse transcriptase enzyme structure. Reactants were assembled as a master mix to minimise contamination risks.

2.8.1. First stand cDNA synthesis with Superscript RNase H⁻ reverse transcriptase

Superscript RNase H⁻ reverse transcriptase is a cloned RNase H deficient version of the Maloney murine leukaemia virus (MMLV) reverse transcriptase (Kotewicz *et al.*, 1988). RT was primed using random hexamer oligonucleotides or by a target-sequence specific primer.

(i) Random primed RT

Random hexamer (1.5mM) (0.5µl) was added to 11.5µl of viral RNA in a microfuge tube and incubated at 90°C for 5min to denature RNA secondary structure. The mixture was then placed on ice to prevent strand re-annealing. To this, the following were added:

Superscript buffer (5x)*	5µl
DTT (0.1M)	2.5µl
dNTP mix (10mM)	1.25µl
BSA (1mg/ml)	2.5µl
RNasin (40U/µl)	0.5µl
Superscript (200U/µl)	1.25µl

*(250mM Tris-HCl (pH8.3), 375mM KCl, 15mM $MgCl_2$)

The 25µl reaction mixture was incubated at 42°C for 60min. The tube was then centrifuged briefly and incubated at 95°C for 5min to inactivate the reverse transcriptase.

(ii) Specifically primed RT

Target-specific priming of RT by Superscript RNase H⁻ reverse transcriptase was performed as in Section 2.8.1.(*i*) except that 0.8µl of primer (25µM) were used. The reaction volume was kept at 25µl by including 0.6µl of 20mM dNTPs and making the volume up to 25µl with UHQ H_2O.

2.8.2. First stand cDNA synthesis with AMV reverse transcriptase

AMV reverse transcriptase is purified from avian myeloblastosis virus (Verma *et al.*, 1977). RT was primed with a sequence-specific primer as for Superscript RNase H⁻ reverse

transcriptase. To the annealed RNA and primer mix the following were added:

AMV RT buffer (5x)*	5µl
KCl solution (250mM)	5µl
dNTP mix (25mM)	0.5µl
RNasin (40U/µl)	0.5µl
AMV reverse transcriptase (2.5U/µl)	1.7µl

* (500mM Tris-HCl (pH8.3), 2mM DTT, 50% (v/v) glycerol, 0.2% (w/v) Triton X-100)

The 25µl reaction was incubated at 42°C for 60min. RT was then terminated as for Superscript RNase H⁻ reverse transcriptase reactions.

2.8.3. First stand cDNA synthesis with *Tth* DNA polymerase

Tth DNA polymerase is a heat-stable enzyme purified from *Thermus thermophilus* which can polymerise DNA using RNA or DNA templates (Röttimann *et al.*, 1985). RT was primed with a sequence-specific primer as for Superscript RNase H⁻ reverse transcriptase. To the annealed RNA and primer mix the following were added:

Reaction buffer (10x)*	2.5µl
$MnCl_2$ solution (10mM)	2.5µl
dNTP mix (2mM)	2.5µl
RNasin (40U/µl)	0.5µl
Tth DNA polymerase (5U/µl)	1.5µl
UHQ H_2O	to 25µl

* (100mM Tris-HCl (pH 8.3), 900mM KCl)

The 25µl reaction was incubated at 60°C for 30min. RT was then terminated by addition of 1µl of 0.5M EGTA.

2.8.4. First strand cDNA synthesis with Sensiscript reverse transcriptase

Sensiscript reverse transcriptase (Qiagen) is a heterodimer of undisclosed origin expressed in *E. coli*. RT reactions were primed using a sequence-specific primer as for Superscript RNase

H⁻ reverse transcriptase. To the annealed RNA and primer mix the following were added:

Sensiscript RT buffer (5x)*	2.5µl
dNTP mix (5mM)	2.5µl
RNasin (10U/µl)	1.25µl
Sensiscript reverse transcriptase (2.5U/µl)	1.25µl

* (500mM Tris-HCl (pH8.3), 2mM DTT, 50% (v/v) glycerol, 0.2% (w/v) Triton X-100)

The reaction was brought to 25µl with nfH_2O and incubated at 42°C or 37°C for 60min. RT was then terminated as for Superscript RNase H⁻ reverse transcriptase reactions.

2.9. *In vitro* transcription using the MEGAscript T7 kit

The MEGAscript T7 *in vitro* transcription kit (Ambion) makes use of the bacteriophage T7 RNA polymerase to produce RNA from template DNA downstream of a T7 promoter as described by Tabor and Richardson (1985).

Proteinase K was used to degrade proteins in preparations of linearised plasmid DNA for use in *in vitro* transcription reactions. Reactions were made up as follows:

Plasmid DNA	10µg
SDS (20% v/v)	2µl
Proteinase K (20mg/ml)	2µl
UHQ H_2O	to 200µl

Reactions were incubated at 37°C for 1hr. DNA was then purified by phenol/chloroform/isoamyl alcohol extraction followed by ethanol precipitation.

In vitro transcription was performed according to the kit protocol. A typical reaction

mixture took the form:

Reaction buffer (10x)	2µl
ATP (75mM)	2µl
CTP (75mM)	2µl
GTP (75mM)	2µl
UTP (75mM)	2µl
Template DNA (0.5µg/µl)	2µl
T7 RNA polymerase enzyme mix	2µl
nfH_2O	to 20µl

The reaction was incubated for 6hr at 37°C. Template DNA was degraded by addition of 1µl of DNase I (2U/µl) followed by incubation at 37°C for 15min. The reaction was then terminated by addition of 115µl of nfH_2O and 15µl of ammonium acetate stop solution (5M ammonium acetate, 100mM EDTA).

Dilution of *in vitro* transcribed RNA was performed in nfH_2O including 0.2µg/ml *Saccharomyces cerevisiae* tRNA (Sigma) as a carrier molecule. Siliconised microfuge tubes were used (Ambion) to reduce RNA adherence to tube surfaces.

2.10. Storage of nucleic acids

HCV RNA extracted from patient serum was stored at -80°C in UHQ H_2O. *In vitro* transcribed RNA was stored at -80°C in RNA storage solution (1mM sodium citrate) (Ambion) including 10mM DTT, 200U/ml RNasin, and 0.2µg/ml *S. cerevisiae* tRNA (Sigma). Plasmid DNA was stored for up to two months at 4°C in TE buffer. For long-term storage plasmid DNA was stored at -20°C in TE buffer.

2.11. Polymerase Chain Reaction (PCR)

The PCR method of amplifying DNA was first described by Saiki *et al.* (1985). The method involves repeated cycles of the following:

1. Heat denaturation of the DNA at 94 - 96°C

2. Annealing of two oligonucleotide primers to their complementary sequences on opposite strands of the template DNA at a temperature 5 - 10°C below the primers' estimated melting temperature (T_m).
3. Extension of the annealed primers with a DNA polymerase at the optimum temperature of the enzyme.

Successive cycles of denaturation, primer annealing and extension results in (theoretically exponential) accumulation of the target sequence bounded by and including the two primers. The use of thermostable DNA polymerases such as *Taq* polymerase from *Thermus aquaticus* has allowed automation of the cycle (Saiki *et al.*, 1988).

Taq polymerase was used for general purpose PCRs including the detection of HCV RNA in patient sera. Bio-X-act (Bioline) was used for applications demanding high-fidelity amplification. Bio-X-act is a mixture of polymerases that possesses 5'-3' polymerase activity and 3'-5' exonuclease activity which reduce misincorporations during primer extension. The mix also contains an additive which eliminates the sequence-dependent removal of 3'-terminal nucleotides from growing chains by pyrophosphorolysis.

To control for PCR contamination with template molecules, negative control reactions were performed where UHQ H_2O was included instead of template DNA. Reactions including known target-positive DNA were included as positive controls.

2.11.1. Conventional single-round PCR

Two oligonucleotide DNA primers were designed to anneal to opposite strands and at opposite ends of the region of DNA to be amplified. These were included in the following typical reaction mixture:

OptiBuffer (x10)	5µl
Forward primer (50ng/µl)	5µl
Reverse primer (50ng/µl)	5µl
dNTP mix (2mM)	5µl
$MgCl_2$ solution (50mM)	1.5µl
Bio-X-act DNA polymerase (Bioline) (4U/µl)	0.25µl
UHQ H_2O	to 50µl

The reagent mix was added to PCR tubes (Applied Biosystems) on ice. The target sample (2µl) was then added to each PCR tube. The PCR was carried out on a GeneAmp PCR

System automated thermocycler 9600 (Applied Biosystems) using 40 cycles of the following incubation steps:

1. 20sec at 94°C.
2. 20sec at the primers' T_m-10°C.
3. 72°C for approximately 1min per kb of amplicon.

A hot-start strategy was used to minimise non-specific amplification. This involved preparing and keeping reactions on ice until the thermocycler had reached 94°C before inserting the reaction tubes.

2.11.2. Nested PCR

Nested PCR is a more sensitive form of PCR involving two rounds of PCR performed using two nested pairs of primers. It was typically used to amplify HCV cDNA prepared from clinical samples. The first round of PCR, using the outer pair of primers, was set-up as above with the following reactants:

Taq buffer (10x)*	2µl
dNTP mix (2mM)	2µl
Outer forward primer (50ng/µl)	2µl
Outer reverse primer (50ng/µl)	2µl
$MgCl_2$ solution (25mM)	1.6µl
Taq DNA polymerase (5U/µl)	0.1µl
HCV cDNA	1µl
UHQ H_2O	to 20µl

*(100mM Tris-HCl (pH9), 500mM KCl, 1% w/v Triton X-100)

Thermocycling (25 cycles) was performed as for single-round PCR and 1µl of product transferred to a PCR tube containing the second-round reagents. These were as for the first-round but included the inner primer-pair instead of the outer primers. Amplification was then performed as above but for 30 cycles.

2.11.3 TaqMan PCR

TaqMan PCR was carried out using the TaqMan Core Reagent kit (Applied Biosystems). AmpliTaq Gold DNA polymerase was used. This enzyme is inactive until a 95°C incubation

stage, so reducing non-specific amplification (Perkin Elmer, 1996). Uracil-N-glycosylase (UNG) was included to prevent carry-over contamination of PCRs with products of previous reactions. Inclusion of dUTP instead of dTTP in the PCR allows degradation of contaminating PCR products during a 50°C incubation stage (Longo *et al.*, 1990). The standard (β-actin) reaction was assembled as follows:

Reaction buffer A (10x)*	5µl
dATP (10mM)	1µl
dCTP (10mM)	1µl
dGTP (10mM)	1µl
dUTP (10mM)	1µl
$MgCl_2$ solution (25mM)	7µl
Forward primer (3µM)	5µl
Reverse primer (3µM)	5µl
Probe (2µM)	5µl
AmpliTaq Gold (5U/µl)	0.25µl
UNG (1U/µl)	0.5µl
UHQ H_2O	to 49µl

*(500mM KCl, 100mM Tris-HCl, 0.1M EDTA, 600nm passive reference 1, pH8.3)

Reagents were assembled as a master mix before distributing to optical reaction tubes with optical caps (Applied Biosystems). DNA (1µl) was then added to each reaction tube. PCR was performed on a PRISM 7700 Sequence Detection System (SDS) (Applied Biosystems). The tubes were initially incubated at 50°C for 2min to activate the UNG. The incubation temperature was then held at 95°C for 10min to activate the AmpliTaq Gold. The temperature was then cycled 50 times through 15sec at 95°C (denaturation) and 1min at 60°C (primer annealing and extension).

Data was collected and analysed using the Sequence Detection System program (Applied Biosystems) on a Macintosh computer (Apple). Amplification plots were created for each cycle (see Section 3.1.5(iii)). An amplification threshold was determined by the program but could also be user-defined. The threshold cycle (C_T) was then calculated for each positive sample.

2.11.4. Precautions taken to avoid PCR contamination

Due to its high sensitivity, PCR is vulnerable to contamination with previously amplified target molecules. The following measures were taken to avoid contamination (Kwok and Higuchi, 1989):

1. Reactions were prepared, incubated and analysed in separate laboratories.
2. Second-round nested PCRs were prepared in a separate laboratory from that used for the first-round set-up.
3. Reactions were set-up in a PCR hood which was flooded with ultraviolet (UV) light for 5min after use.
4. Amplified material was not handled in the PCR set-up laboratory.
5. Pipettes used for PCR set-up were not used for other purposes.
6. Aerosol resistant pipette tips were used during reaction set-up.
7. Reactions were assembled as a master mix to minimise reagent manipulations.

2.12. Agarose gel electrophoresis

Agarose gel electrophoresis involves separation of nucleic acids in an agarose gel matrix by application of an electrical current. The gel is submerged in a buffer solution which acts as an electrolyte, as well as maintaining the pH of the system. Nucleic acids are separated on the basis of charge, size and complexity and, being of negative charge, migrate towards the anode. Separation is based mainly on size, however, structural differences, such as supercoiling of plasmids affect migration. The rate at which a molecule passes through the gel matrix is inversely proportional to its size.

Plasmids migrate through agarose gels at different rates depending on their structure. A linearised plasmid is able to migrate at a faster rate than its circular form. The supercoiled form of a plasmid is able to move through a gel at a faster rate than its open circle form. Nucleic acids are visualised by staining with EtBr which intercalates and fluoresces under UV light (Sharp *et al.*, 1973).

2.12.1. Separation of DNA by agarose gel electrophoresis

Agarose gel electrophoresis was performed according to a method modified from Sambrook *et al.* (1989). Different types and strengths of gel were used for analysing different sizes of DNA (Table 2.1). Agarose powder was added to 60ml of stirred TAE buffer to give the desired

concentration. The suspension was stirred for 10min to hydrate the agarose powder. This was then heated, with frequent mixing, in a microwave oven until the powder had completely dissolved. The solution was then allowed to cool for 7min in a waterbath at 60°C. A well-forming comb was placed in the gel casting-tray of a horizontal gel electrophoresis apparatus (BioRad). The agarose solution was then poured into the casting tray to a depth of 5mm and allowed to set for 30min.

Table 2.1 Agarose type and concentration used for different DNA lengths.

Agarose type*	Nucleic acid size range (nt)	Agarose concentration (percentage w/v)
NuSieve 3.1 Agarose	100 - 500	4.0
Metaphor Agarose	100 - 600	3.0
SeaKem LE Agarose	800 - 23000	0.7

*Supplied by Flowgen.

When set, the gel was covered to a depth of approximately 5mm with TAE buffer and the comb carefully removed. DNA samples were then mixed with an appropriate volume of 6x loading dye and UHQ H_2O to give a final volume of 10µl. This was pipetted into separate wells of the gel. For estimation of band size and concentration, 150ng of 1kb marker DNA (1µg/ml of 12,216bp, 11,198bp, 10,180bp, 9,162bp, 8,144bp, 7,1632bp, 6,108bp, 5,090bp, 4,072bp, 3,054bp, 2,036bp, 1,636bp, 1,018bp, 515bp, 506bp, 396bp, 344bp, 298bp, 220bp, 201bp, 154bp, 134bp and 75bp. DNA; Life Technologies) was loaded into flanking lanes. This contained of dsDNA of lengths: The gel tank was connected to a Power-Pac 300 power source (BioRad) and run at 100V until the bromophenol blue dye front reached about 1cm from the end of the gel.

The gel stained in a 1µg/ml solution of EtBr in dH_2O on a Lab-line rotary mixer (Jencons) for 20min. The EtBr solution was then aspirated off, replaced with dH_2O and the gel was de-stained for 30min. The gel was then viewed under 302nm light on an UV transilluminator (Ultra-Violet Products) and photographed on Polaroid 667 film using a Polaroid camera system.

2.12.2. Separation of RNA species by formaldehyde-agarose gel electrophoresis

Formaldehyde-agarose gel electrophoresis of RNA was performed according to a method modified from Sambrook *et al.* (1989). Formaldehyde was included to denature secondary RNA structures. Due to the toxicity of formaldehyde vapours, pipetting of formaldehyde, and running and staining of gels was performed in a fume-cupboard.

For analysis of *in vitro* transcription products, a 2% agarose gel was prepared. High-strength agarose powder (BioRad) (3g) was added to 107ml of stirred UHQ H_2O. The suspension was stirred for 10min to re-hydrate the agarose powder. This was then heated, with frequent mixing, in a microwave oven until the powder had completely dissolved and 15ml of 10x RNA gel running buffer was added. The solution was allowed to cool to 50°C in a waterbath. Formaldehyde (25% v/v solution) (25ml) was then added to the molten gel. A well-forming comb was placed in the gel-casting tray of a horizontal gel electrophoresis apparatus (BioRad). The agarose solution was then poured into the casting tray to a depth of 5mm and allowed to set for 1hr. The gel was then covered to a depth of approximately 5mm with 1x RNA gel running buffer and the comb removed.

RNA samples (5 - 20µg) were prepared by incubating at 95°C for 5min to denature secondary structures. The following were then added:

RNA gel running buffer	4µl
Deionised formamide (Ambion)	10µl
Formaldehyde (40% v/v solution)	3.5µl

The mixture was incubated at 65°C for 5min and placed on ice for a further 5min. Agarose gel loading buffer (6x) (4µl) was then added and the sample loaded into wells of the gel. For estimation of RNA length in formaldehyde agarose gels, RNA Century size marker (1mg/ml of 500nt, 400nt, 300nt, 200nt and 100nt RNA; Ambion) was used. The gel tank was connected to a Power-Pac 300 power source (BioRad) and run at 100V for 4hr.

The gel was stained in a 1µg/ml solution of EtBr in dH_2O on a rotary mixer for 30min and de-stained in dH_2O for 30min. The gel was then viewed and photographed as for agarose gels.

2.13. Elution of DNA from agarose gels

To isolate a particular DNA species from an agarose gel, the agarose gel was viewed on a transilluminator and the required DNA band cut out using a sterile scalpel. Excess gel was trimmed from the slice, which was then placed in a microfuge tube. To the gel slice, 20µl of UHQ H_2O was added. The tube was then incubated at 50°C for 5min to elute the DNA. The sample was vortex-mixed and briefly centrifuged. The tube was incubated, vortex-mixed and centrifuged twice more. The supernatant, containing eluted DNA was then removed.

For re-amplification of PCR products from a gel slice, the eluate was diluted 1/20 in UHQ H_2O. A sample of this was then included in the PCR.

2.14. Measurement of nucleic acid concentration by absorption spectroscopy

Determination of the concentration of nucleic acids in a solution was achieved by measuring the absorption of 260nm wavelength light (A_{260}) by the solution using a spectrophotometer (Hitachi) (Ausubell *et al.*, 1994). The nucleic acid solution was diluted 100-fold in UHQ H_2O and placed in a quartz cuvette (Hellma) of path-length, 10mm for analysis. A quartz cuvette was used so as to allow light of short wavelengths to pass through. The spectrophotometer was zeroed with a water blank before determining the A_{260} value of the nucleic acid solution. The concentration of the solution was then calculated from the following equations:

An A_{260} reading of 1.0 = 50µg/ml of dsDNA.
An A_{260} reading of 1.0 = 37µg/ml of ssDNA.
An A_{260} reading of 1.0 = 40µg/ml of ssRNA.

Measurement of the absorption of 280nm light (A_{280}) by a nucleic acid solution allowed the A_{260}/A_{280} ratio of the solution to be calculated. An A_{260}/A_{280} ratio of 1.9 - 2.0 indicated a high purity RNA solution. An A_{260}/A_{280} ratio of 1.8 - 1.9 indicated a high purity DNA solution. Impurities that absorb at A_{280} such as proteins and phenol lower this ratio (Ausubell *et al.*, 1994). Absorbance at 230nm (A_{230}) was used as a measure of GITC contamination. A_{260}/A_{230} ratios of less than 1.5 indicated contamination (Brisco *et al.*, 1997).

2.15. Quantitation of RNA by RiboGreen fluorimetry

The RiboGreen method (Molecular Probes) makes use of a fluorescent, cyanine dye for quantifying RNA in solution. When bound to RNA, the dye exhibits 1000-fold greater fluorescence than unbound dye. The excitation maximum and emission maximum of RiboGreen reagent bound to RNA are at approximately 500nm and 525nm, respectively. Fluorescence can be measured using a fluorescence microplate reader, spectrofluorometer, or filter fluorometer. The fluorescence level of an RNA solution is compared with a standard curve produced by measurement of fluorescence from a dilution series of ribosomal RNA

(rRNA). The advantages of this method over A_{260} measurement are its approximately 1000-fold greater sensitivity and its reduced sensitivity to contaminants such as salts, proteins, phenol, chloroform and free nucleotides (Molecular probes product data).

RNA was quantified according to the manufacturer's instructions. The RiboGreen reagent was diluted 200 fold in TE. The kit RNA standard (100μg/ml 16s and 23s rRNA mixture) was diluted in TE to 0.04, 0.2, 1 and 2μg/ml. Samples (100μl) of each dilution and a TE blank were then added to triplicate wells of black, flat-bottomed polystyrene microstrips (Labsystems). To each standard curve sample, 100μl of working concentration RiboGreen reagent was added. The reactions were mixed and incubated at room temperature for 3min. They were then analysed using a Fluostar fluorescence microplate reader (Tecan). The mean fluorescence values (minus the mean blank fluorescence value) of the triplicate standard readings were plotted against RNA concentration to produce a standard curve.

Measurement of A_{260} was performed to give an approximation of test sample RNA concentration. Sample RNA was diluted in TE to three concentrations (0.5, 1 and 1.5μg/ml based on the A_{260} measurement) within the standard curve range. Triplicate RiboGreen measurements were then performed for each dilution as described for the standard RNA. Triplicate TE blank readings were also performed. The mean blank fluorescence value was subtracted from the mean of each triplicate sample dilution reading. The normalised fluorescence level was compared to the standard curve to give the sample concentration at each dilution. The mean of the three dilutions was used as the RNA sample concentration.

2.16. Automated DNA Sequencing

DNA sequencing was performed using an automated version of the dideoxy chain-termination method developed by Sanger *et al.* (1977). The method involves multiple cycles of DNA denaturation, primer annealing, and chain-elongation steps. Samples are heated to 96°C to denature template DNA. The temperature is then held at the annealing temperature of a target-specific oligonucleotide primer. Incubation at 60°C promotes elongation of the primer strand by a thermostable DNA polymerase enzyme.

The reaction mix contains fluorescently labelled dideoxy- (dd) ATP, ddCTP, ddTTP, and ddGTP, as well as dNTPs. When the polymerase incorporates a ddNTP, chain-elongation is terminated. This results in the accumulation of copy-strand fragments of all possible lengths. As each ddNTP is labelled with a different rhodamine derivative fluorescent dye, the terminal ddNTP can be identified. This is achieved by heat denaturing the products and subjecting

them to polyacrylamide gel electrophoresis (PAGE) on an automated sequence detector. Fragments migrate through the gel with a rate inversely proportional to their length. The automated sequencer scans the gel with a laser which excites each dye. Resultant fluorescence is detected by a photomultiplier tube and the data processed by computer to deduce the sequence of bases.

2.16.1. Sequencing reaction

The Thermo Sequenase DNA dye terminator cycle sequencing pre-mix kit (Amersham) was used for the sequencing reaction. This makes use of T7 DNA polymerase, modified to allow incorporation of ddNTPs and to increase stability at high temperatures. The components of each sequencing reaction were assembled in PCR tubes (Applied Biosystems) as follows:

Thermo Sequenase Pre-mix*	4µl
Primer (50ng/µl)	0.5µl
DNA	15ng of linear DNA or 0.1 - 1µg of plasmid DNA
UHQ H_2O	to 10µl

*125mM Tris-HCl (pH9.5), 5mM $MgCl_2$, 1.25mM dITP, 0.25mM each of dATP, dCTP, dATP, ddATP (R6G labelled), ddCTP (R110 labelled), ddGTP (ROX labelled), ddTTP (TAMRA labelled), Thermo Sequenase DNA polymerase, *Thermoplasma acidophilum* thermostable inorganic phosphatase, Nonidet P40, Tween 20, 6.25% glycerol.

The cycle sequencing reaction was carried out on a GeneAmp PCR System automated thermocycler 9600 (Applied Biosystems). A program of 25 cycles of the following incubation conditions was used:

1. Denaturation at 96°C for 10sec for linear DNA, or for 30sec for plasmid DNA.
2. Incubation at a temperature that allowed annealing of the primers to template DNA (typically 50 - 60°C), for 10sec for linear DNA, or 15sec for plasmid DNA.
3. Polymerisation at 60°C for 4min.

2.16.2. Purification of sequencing reaction products

Sequencing reaction products were purified for analysis as follows. To each sample, 25µl of ice-cold ethanol and 1µl of 3M NaAc (pH 5.2) were added in a microfuge tube. The samples were incubated on ice for 10min and centrifuged at 22,000xg at 10°C for 15min. The supernatant was removed and the DNA pellet washed with 200µl of ice-cold 70% (v/v)

ethanol in dH_2O. The samples were then centrifuged as previously. The supernatant was aspirated and the pellets vacuum dried for 2 - 5min.

2.16.3. Polyacrylamide gel preparation

An 8% polyacrylamide gel was used to analyse the sequencing reaction products.
The following constituents were mixed and filtered through a 0.22μm pore filter (Nalgene) to remove particulate matter:

Ultrapure Sequagel-6 (National Diagnostics)	32ml
10x TBE	5.28ml
UHQ H_2O	2.72ml

To catalyse polymerisation, 200μl of 10% (v/v) ammonium persulphate and 20μl of N,N,N',N'-tetramethylethenediamine (TEMED) were added. Using a 10ml pipette, the mixture was poured between two glass plates separated by plastic spacers creating a gap of 2mm. A plastic well-former was inserted into the top of the gel to create a straight edge. Clips were placed around the top and sides of the plates and the gel was allowed to solidify for 4hr.

2.16.4. Sequence detection

The sample pellets were resuspended in 4μl of formamide loading dye (Amersham) and incubated at 90°C for 2min to denature the DNA. The samples were then immediately placed on ice to prevent re-annealing.

The prepared sequencing gel was mounted on a 373A automated sequencer (Applied Biosystems) and the well-former removed. A plastic shark's-tooth comb was placed in the top of the gel, between the plates, to create 24 separate wells. The upper and lower reservoirs were filled with 1.3x TBE buffer. The wells were flushed out with buffer to remove urea accumulation and the samples were loaded into separate wells. The gel was run at 2500V, 40mA, 28W for 12hr. Data was collected with a Macintosh computer (Apple) using the Data Collection program (Applied Biosystems).

2.16.5. Sequence data analysis

Analysis of sequence data was performed on a Macintosh computer (Apple) using the Data Analysis program (Applied Biosystems). Plots of sequence data were used to identify incorrectly called nucleotides and the area of high quality sequence data. Poor quality data was edited from the beginning and end of the sequence using the SeqEd program of the Lasergene

package (DNA Star). The Lasergene contig alignment program was used to align sequences derived from opposite strands of the same region of DNA. This alignment was used to confirm the accuracy of sequencing.

Multiple alignments of sequences were made on a personal computer using the Clustal W program (Thompson *et al.*, 1994). This aligned sequences and gave a measure of percentage identity with a reference sequence. Interpretation of the Clustal W output was aided using a Microsoft Word version 6 macro that replaced nucleotides identical to the reference strand with a dot. The program FASTA (Pearson and Lipman, 1988) was used to determine percentage identities between test and a library sequences. Unrooted phylogenetic trees were drawn using the Drawtree program in the Phylogeny Inference Package (PHYLIP) version 3.5c (Felsenstein, 1993).

DNA sequences were retrieved from GenBank and EMBL on-line computer databases using the Sequence Retrieval System facilities of the Seqnet laboratory (http://www.seqnet.dl.ac.uk). The FASTA program was used to search DNA databases for sequences of high percentage identity with test sequences.

2.17. Oligonucleotide primer design

Oligonucleotide primers were designed for PCR, RT and DNA sequencing using the following criteria. Primer-pairs were designed to be non-complementary to avoid dimer formation which can reduce reaction efficiency. Pairs of primers for PCR were designed to have approximately the same melting temperature (T_m). Primers were designed to have no large regions of secondary structure which can reduce specificity and efficiency of the reaction.

Primers T_ms were estimated using the expression:

T_m(°C) = (No. of G and C residues x 4) + (No. of A and T residues x 2).

The effective annealing temperature for oligonucleotide primers was calculated as T_m-10°C.

Primers for TaqMan PCR were designed using the Gene Runner (Hastings Software) and Primer Express computer programs (Applied Biosystems). These use the nearest-neighbour thermodynamic values method (Breslauer *et al.*, 1986) to calculate primer T_ms.

2.18. TaqMan probe design

TaqMan oligonucleotide probes were designed using the criteria described by Livak *et al.* (1995):

1. Probes were 20 - 30 bases long to give T_ms of 68 - 70°C.
2. GC content was 40 - 60%.
3. The probes were designed so that the T_ms were 10°C higher than the anneal/extend temperature allowing probes to bind to the template before primer extension.
4. Secondary structures in the probe and target sequence were avoided if possible.
5. Probes were designed so that they did not overlap or hybridise with primer binding sites.
6. Long runs of a single base were avoided.
7. The DNA strand the probe bound to was selected to give the probe sequence with more C than G residues.
8. A maximum of one - two G and C residues were allowed in the 3' five nucleotides of the probe and no GC clamp was included.
9. Placing a G residue at the 5' end was avoided because of reported quenching of reporter fluorescence by this nucleotide (Adam Corner, Applied Biosystems personal communication).

T_ms of TaqMan probes were determined using the Primer Express program (Applied Biosystems). The 5' nucleotides of the probes were labelled with 6-carboxyfluorescein (FAM) and the 3' nucleotide was labelled with 6-carboxytetramethylrhodamine (TAMRA).

2.19. Oligonucleotide de-protection and purification

Oligonucleotides produced in-house were supplied bound to a filter membrane. Before use, oligonucleotides had to be released from the membrane and the protecting trityl groups removed. The filter casing was removed and the membrane placed into 1.5ml of NH_3 in a screw-capped tube (Sarstedt). To this, 50µl of triethylamine was added as a catalyst. The tube was incubated at 80°C for 30min, then cooled on ice for 10min.

The oligonucleotide was precipitated as follows. NaAc (3M, pH5) (30µl) was added to four microfuge tubes and 300µl of the oligonucleotide solution added to each tube. Ethanol (900µl) was added and the tubes were incubated at -20°C overnight. The tubes were then centrifuged for 15min at 3000xg. The supernatant was removed and the pellet washed with 200µl of ice-cold 70% (v/v) ethanol in UHQ H_2O. The tube

was then centrifuged for 2min at 3000xg. The supernatant was removed with a fine-tipped pipette and the pellet dried for 5min in a vacuum desiccator. The pellets were stored at -20°C and resuspended in 200µl of UHQ H_2O when required.

2.20. Separation of serum from patient blood

Blood samples were processed within 2hr of receipt to avoid degradation of viral RNA. Samples were centrifuged in their collection tubes at 700xg for 5min to separate serum from the clot. Multiple 200µl samples of serum were placed in screw-topped tubes (Sarstedt) and stored at -80°C.

2.21. Detection of HCV RNA in patient serum

HCV RNA was extracted from patient serum using the QIAamp viral RNA kit. Random-primed RT was performed using Superscript reverse transcriptase. Nested PCR was carried out using the outer primers, UTR1 and UTR2, and the inner primers, UTR 3 and UTR4 (Green *et al.*, 1998). The first-round PCR was incubated for 25 cycles of 94°C for 20sec, 50°C for 20sec and 72°C for 1sec. The second-round was incubated for 30 cycles of 60°C for 20sec and 60°C for 20sec.

PCR products (88bp) were analysed by 4% NuSieve agarose gel electrophoresis, followed by EtBr staining. The gel was then viewed on a transilluminator.

2.22. Genotyping of HCV isolates by restriction fragment length polymorphism (RFLP) analysis

HCV genotype was determined by a RFLP method developed by McOmish *et al.* (1994). This involved PCR of a section of the 5'UTR from a cDNA sample previously shown to be HCV PCR positive.

Nested PCR of a section of the HCV 5'UTR was performed using outer PCR primers HCV939, HCV209, and inner PCR primers HCV940 and HCV211 (Garson *et al.*, 1990; Okomoto *et al.*, 1990). A 25µl volume first-round PCR was performed using 25 cycles of the following conditions: 94°C for 20sec, 50°C for 20sec, 72°C for 10sec. A 1.25µl sample of the

first-round product was added to the second-round reaction. The second-round PCR was carried out using 35 cycles of the following conditions: 94°C for 20sec, 60°C for 20sec, 72°C for 10sec.

The reaction product (305bp) was gel-purified and primers HCV940 and HCV211 used to re-amplify the product in a 100µl PCR with the same conditions as for the second-round of nested PCR. A 50µl sample of the reaction product was then purified from unincorporated nucleotides and primers using the Nucleon QC kit. The concentration of the eluted DNA was estimated by analysing a 2µl sample by 4% NuSieve agarose gel electrophoresis.

A 600µg sample of the purified PCR product was cut with each of the restriction endonucleases shown in Table 2.2 using the reaction temperatures and buffers stated. The 20µl volume restriction reactions initially included 0.5µl of each enzyme. The reactions were incubated for 1hr. Another 0.5µl of enzyme was then added and the reactions were incubated for a further 1hr.

To each completed restriction reaction, 2µl of 6x agarose gel loading buffer was added and 10µl of the resulting mixture was analysed by 3% Metaphor agarose gel electrophoresis. The product banding pattern was interpreted according to the scheme described by McOmish *et al.* (1994).

Table 2.2 Restriction enzymes, buffers and reaction temperatures used for RFLP analysis of HCV isolates.

Restriction enzymes	Reaction buffer	Incubation temperature (°C)
Rsa I, Hae III	C[1]	37
Hinf I, Mva I	B[2]	37
Scrf I	4[3]	37
Bst UI	2[4]	60

[1]100mM Tris-HCl (pH7.9), 100mM $MgCl_2$, 500mM NaCl, 10mM DTT; [2]60mM Tris-HCl (pH7.5), 60mM $MgCl_2$, 500mM NaCl, 10mM DTT; [3]500mM potassium acetate, 200mM Tris-acetate (pH7.9), 100mM magnesium acetate, 10mM DTT; [4]100mM Tris-HCl (pH7.9), 100mM $MgCl_2$, 100mM NaCl, 10mM DTT.

2.23. Quantitation of HCV load in patient serum and cell culture media

2.23.1. Quantiplex HCV RNA 2.0 assay

HCV RNA 2.0 assays (Bayer) were performed according to the manufacturer's instructions using a bDNA analyser instrument (Bayer). For each specimen, 50µl of serum was added to 150µl of HCV specimen working reagent in duplicate wells of the capture plate provided. The

plate was incubated for 16hr at 53°C in the bDNA analyser. The wells were washed twice with 400µl of wash A solution. To each well, 50µl of label working reagent was then added. The plate was incubated at 53°C for 15min, allowed to cool, then washed twice with 400µl of wash A and thrice with 400µl of wash B. Substrate solution (50µl) was added and the plate incubated at 37°C for 25min. The relative luminescence from each well was recorded and the HCV concentration calculated by the bDNA analyser.

2.23.2. TaqMan PCR

HCV RNA was quantified by TaqMan PCR according to the method developed for this project (Chapters 3 - 5). RNA was extracted from patient serum or cell culture medium using the QIAamp Viral RNA Mini Kit as described in Section 2.4.5 but with the following modifications:

(a) RNA was extracted from 280µl of the sample instead of 140µl.

(b) 10^5 IC RNA copies (in 10µl of 0.2µg/ml tRNA solution) were added to 1110µl of lysis buffer for each sample.

(c) Duplicate lysis steps were performed on 140µl of the sample, each using 560µl of the lysis buffer/IC mix.

(d) Following lysis and addition of ethanol, the duplicate samples were combined by centrifugation four times at 6,000xg for 1min through a single spin-column.

To the eluted RNA, 2µl of random hexamer primer was added and RT was performed using Superscript enzyme as described in Section 2.8.1(i) with the following modifications:

(a) RT reactions (75µl) included GeneAmp 10x PCR buffer II (100mM Tris-HCl pH8.3, 500mM KCl; Applied Biosystems) instead of Superscript buffer.

(b) Reactions were incubated at 50°C instead of 42°C.

Duplicate HCV and a single IC 50µl TaqMan PCRs were performed as described in Section 2.11.3 with the following modifications:

(a) Each PCR included 25 µl instead of 1µl of the RT product.

(b) TaqMan PCR buffer A and $MgCl_2$ were included at 0.5x concentration and 2.5mM, respectively.

(c) HCV PCRs included primers TMF1 and TMR2 at 400nM and 200nM, respectively and probe TMP1 at 175nM.

(d) IC PCRs included primers 2DTMF and TMR2 at 300nM and probe 2DTMP at 200nM.

Standard curves were produced by addition of ten-fold serial dilutions ($2x10^1$ - $2x10^7$ copies in 20µl of 0.2µg/ml tRNA solution) of rSot34-3a and 10^5 copies of IC RNA (in 10µl of 0.2µg/ml tRNA solution) to 50µl RT reactions. Duplicate HCV PCRs were performed as described above.

PCR conditions were as described previously (Section 2.11.3). A fluorescence threshold value of 0.05 $\log_{10} \Delta R_n$ was used for all runs. The mean result of the duplicate HCV PCRs (copies/PCR) was used to calculate the serum HCV genome RNA concentration in copies/ml of serum (copies/PCR x 10.71).

A quantitation run was accepted if the standard curve correlation coefficient (r) was ≥0.95 and the mean IC PCR C_T was within three SDs of the mean C_T from all samples run to date. The run was repeated if any of these criteria were not met.

An individual assay was accepted if both of the duplicate HCV PCRs gave results (in copies/PCR) within 50% of their mean, and the IC PCR C_T was within three SDs of the mean IC PCR C_T for that run. If the results of the duplicate HCV PCRs were >50% from their mean, the fluorescence threshold was reset to a different region of the $\log_{10}$-linear part of the amplification plots and the C_Ts recalculated. The assay was repeated if any of these criteria were not met.

2.24. Statistical analysis

Log_{10} transformation, linear regression analysis and calculation of mean and standard deviation (SD) were performed using the Microsoft Excel software. Coefficients of variation (CVs) were calculated by dividing the SD by the mean and multiplying by 100. Other statistical analyses were performed using the SPSS software (release 9.0) (Statistical Package of Services Solutions, SPSS). Differences in mean viral load, changes in load and other clinical variables were analysed by the Mann-Whitney U test for comparison of means. Differences in mean C_T values were compared using the Student's t-test. Correlation was assessed using the Spearman's rank coefficient. Statistical significance was considered if $P<0.05$.

3. Development of a real-time PCR-based assay for detection of hepatitis C virus

3.1. Introduction

3.1.1. Detection of PCR products

Since its development in 1985 by Saiki *et al.*, PCR has become an invaluable tool in many fields of biology and medicine. It has proven especially useful for detection of viral nucleic acids. Conventionally, PCR products have been analysed post-amplification. This has usually involved resolution by agarose or polyacrylamide gel electrophoresis, followed by staining of the DNA with a fluorescent intercalating dye such as EtBr (Saiki *et al.*, 1988). Alternatively, if radiolabelled or fluorescently-labelled primers are used, the products can be analysed by autoradiography (Desjardin *et al.*, 1998) or fluorescence detection (Chehab and Kau, 1989).

PCR products can also be transferred from the gel to a nitrocellulose or nylon membrane by Southern blotting. The DNA is then denatured and hybridised with labelled oligonucleotide probes. Biotinylated or digoxygenin-labelled probes are bound to by streptavidin or anti-digoxygenin antibodies, respectively, conjugated with an enzyme such as alkaline phosphatase or horseradish peroxidase. The activity of the enzyme is detected by a substrate colour change (Jantos *et al.*, 1998; Castelain *et al.*, 1997). Alternatively, probes may be radioactively-labelled, allowing detection by autoradiography (Cha *et al.*, 1991; Hagiwara *et al.*, 1993). PCR products have also been detected without resolution on a gel, by enzyme immunoassay (Sander and Penno, 1999), and fluorescence (Chehab and Kau, 1989) after immobilisation on a solid phase.

Haberhausen *et al.* (1998) used an electrochemiluminescence (ECL) method to detect PCR products. This involved amplification with biotinylated PCR primers and amplicon hybridisation with a ruthenium-tris(bipyridyl)-labelled oligonucleotide capture probe. The hybrid was bound to the surface of streptavidin-coated magnetic beads which were captured by an electrode using a magnet. ECL was then activated by voltage application and detected by luminometer.

Such post-amplification processing is time-consuming, labour-intensive and increases the risk of PCR contamination with amplification products. Methods have recently become available to measure PCR product formation within the reaction tube. Two separate developments have occurred to make this possible:

(i) Development of improved fluorescent dyes and oligonucleotide systems for detection of amplification products.

(ii) Development of instruments for measurement of fluorescence in closed reaction tubes.

These developments can potentially negate the need for routine post-amplification processing of PCR products.

3.1.2. Properties of fluorescent dyes

Fluorescence occurs in polyaromatic hydrocarbons, known as fluorophores or fluorescent dyes. These have excitable π electrons in conjugated double bonds. The fluorescence process involves three stages, excitation by a photon, the excited state, and emission of a photon as fluorescence (Fig.3.1). The energy of the emitted photon is lower than that of the excitation photon, resulting in the emitted photon having a longer wavelength. This difference in wavelength is known as the Stokes' shift. Excitation and emission of a fluorophore is cyclical, unless photobleaching occurs due to high intensity illumination (Johnson *et al.*, 1996).

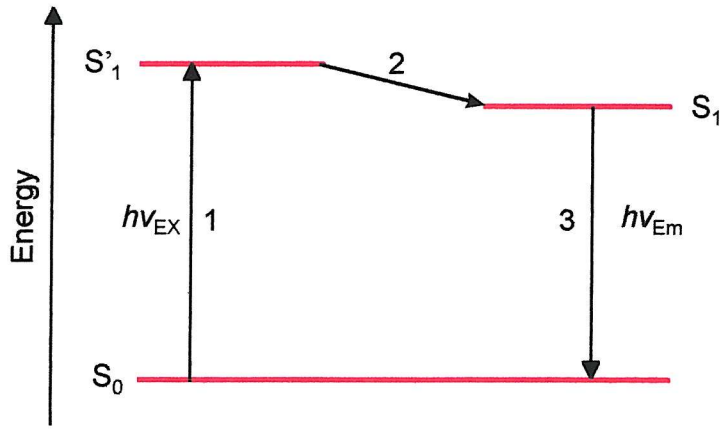


Fig.3.1 Jablonski diagram showing the three stages of the fluorescence process.
1. Excitation of a fluorophore at it's ground state (S_0) occurs when a photon of energy, hv_{EX} (where h = Plank's constant; v_{EX} = frequency of excitation) is absorbed to produce an excited singlet state (S'_1). The excited state exists for a finite time, during which energy is partially dissipated (2) due to interactions with the environment to produce a relaxed singlet state (S_1).
3. The fluorophore then returns to the ground state, emitting a photon of energy, hv_{EM} (where v_{EM} = frequency of emission). Red lines represent electron energy levels. Arrows represent energy level transitions. (Johnson *et al.*, 1996).

3.1.3. Measurement of PCR product accumulation by fluorescent dye intercalation

The first methods for monitoring PCR in closed tube systems involved measuring the increase in fluorescence of fluorophores due to their intercalation into dsDNA product. Higuchi *et al.* (1992; 1993) monitored the intercalation of EtBr into double-stranded PCR products. When the dye is bound to DNA, it is excitable by UV light (at 302nm wavelength) and fluoresces at a maximum wavelength of 605nm. The amount of fluorescence from a reaction increased in direct proportion to product accumulation. Other fluorescent dyes such as SYBR Green have also been used (497nm excitation maximum; 520nm emission maximum) (Woo *et al.*, 1999). The drawback with using fluorescent dye incorporation to monitor PCR product accumulation is the inability to distinguish between the specific product and non-specific amplification products such as primer-dimers.

3.1.4. Monitoring PCR product accumulation with fluorescently-labelled oligonucleotides

More specific methods of PCR product detection have been developed using fluorescently-labelled oligonucleotides. Detection of amplicons is based on changes in fluorescence levels resulting from interaction between different fluorescent dyes. Fluorescence energy is passed between the dyes by a process known as fluorescence resonance energy transfer (FRET), principally due to Förster type energy transfer (Lakowicz, 1983). FRET is a distance-dependent interaction between the electronic excited states of two fluorophores, in which excitation is transferred from a donor molecule to an acceptor molecule without the emission of a photon (Haugland *et al.*, 1996). For FRET to occur:

(a) The donor and acceptor must be in close proximity (10 - 100Å).

(b) The absorbance spectrum of the acceptor must overlap the fluorescence emission spectrum of the donor.

(c) The donor and acceptor transition dipole orientations must be approximately parallel.

Depending on the system used, FRET can result in either the quenching or excitation of fluorescence from a reporter dye, the level of which is used to monitor PCR product formation. Examples of reporter dyes that have been used for monitoring PCR product level include tetrachloro-6-carboxyfluorescein (TET), 2,7,-dimethoxy-4,5-dichloro-6-carboxyfluorescein (JOE), hexachloro-6-carboxyfluorescein (HEX), and 6-carboxyfluorescein (FAM) (Fig.3.2). Each has different emission maximum wavelengths, allowing the possibility of monitoring levels of multiple product in multiplex PCRs.

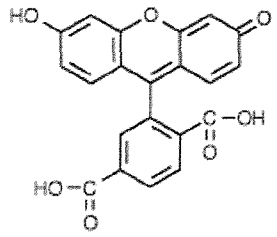
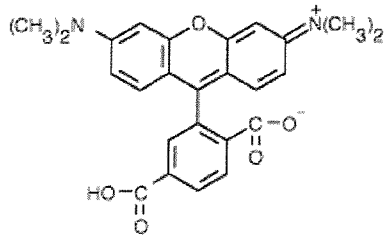
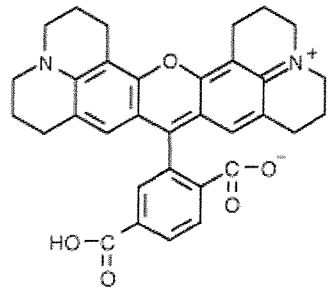
Dye	Structure	Absorbance maximum wavelength (nm)	Emission maximum wavelength (nm)
FAM		492nm	515nm
TAMRA		540nm	560nm
ROX		570nm	590nm

Fig.3.2 Structures and absorbance and emission data for fluorescent dyes used in this project for the TaqMan PCR.
Absorbance and emission data is for 6-FAM dissolved in pH 9.0 aqueous buffer and for 6-carboxy-X-rhodamine (ROX) and 6-carboxytetramethylrhodamine (TAMRA) dissolved in methanol (Haugland *et al.*, 1996).

There are two main strategies for detecting PCR products using FRET between fluorescent dyes:

(i) The sample is illuminated with photons at the excitation wavelength of a reporter dye and the reporter fluorescence measured. In the absence of PCR product, the fluorescence energy of the reporter dye is absorbed by a proximal quencher dye on the same oligonucleotide. PCR product accumulation results in separation of the dyes so as to reduce FRET and increase reporter dye fluorescence. This strategy is employed by TaqMan, molecular beacon and molecular beacon primer assays (Fig.3.3).

(ii) The sample is illuminated with photons at the excitation wavelength of a donor dye. In the absence of PCR product, the donor and reporter dyes are unlinked, preventing significant FRET from occuring. PCR product accumulation brings the dyes together resulting in fluorescence energy being transferred from the donor to the reporter and an increase in reporter fluorescence. This strategy is used in bi-probe and hybridisation probe assays (Fig.3.3).

Several different methods have been employed using fluorescently-labelled oligonucleotides and FRET for closed-tube detection of PCR product formation.

(i) TaqMan assay

The 5' nuclease assay, or TaqMan assay (Fig.3.4) is based on the ability of *Taq* DNA polymerase to displace and cleave oligonucleotides, that are bound to the template DNA strand, from the 5' end during polymerisation (Holland *et al.* 1991). TaqMan probe design is described in Section 2.18. As *Taq* polymerase is unable to digest unbound probe, reporter fluorescence is only detected when both probe binding and amplification of the target DNA occurs. TaqMan assays have been used for detection of a wide range of pathogens, including Epstein-Barr virus (EBV) (Hamilton and Schichman, 1997), human herpes virus-8 (Kennedy *et al.*, 1999), *E. coli* O157:H7 (Oberst *et al.*, 1998) and HCV (Morris *et al.*, 1996).

(ii) Molecular beacon assay

The molecular beacon assay makes use of an oligonucleotide probe labelled with a reporter dye and a quencher dye at opposite ends (Fig.3.3). The probe has terminal regions of self-complementarity flanking sequence complementary to the target. This allows the unbound probe to form a hairpin structure during the transition from the PCR denaturation temperature to the primer annealing temperature bringing the fluorophores into close proximity, resulting in quenching of reporter fluorescence. During this phase of the reaction, the probe may also hybridise to its complementary sequence within amplification products. This prevents

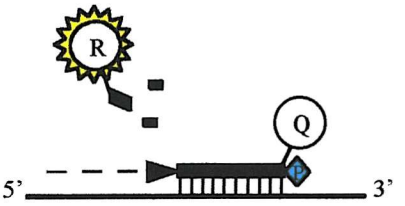
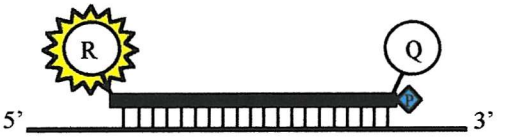
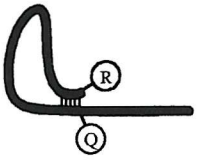
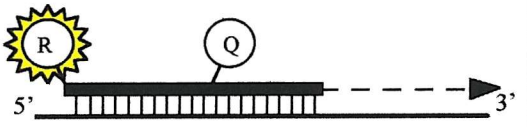
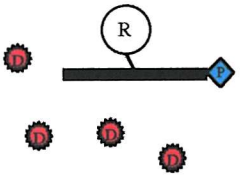
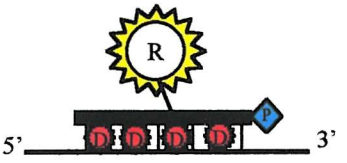
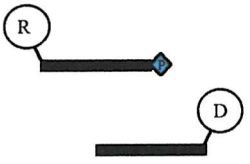
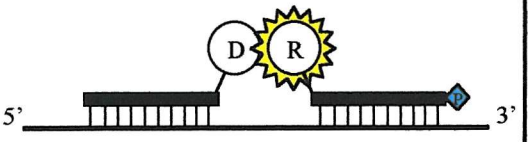
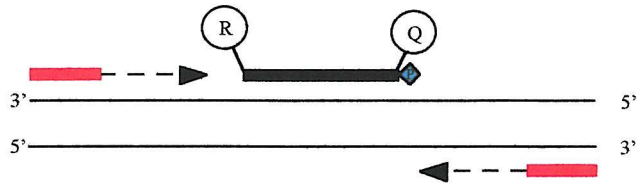
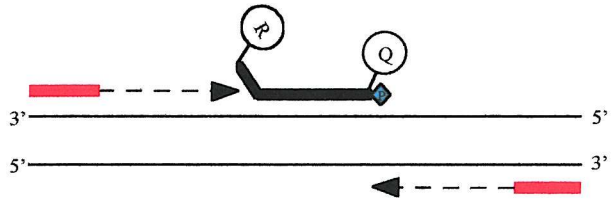
Strategy	Reporter fluorescence undetectable	Reporter fluorescence detectable
TaqMan		
Molecular beacon		
Molecular beacon primer		
Bi-probe		
Hybridisation probe		

Fig.3.3 Diagrammatic representations of methods for real-time detection of PCR product accumulation using fluorescently-labelled oligonucleotides.
Thin solid lines = template DNA; dashed arrow = primer extension; thick lines = labelled oligonucleotide; Q = quencher dye; R = reporter dye; D = donor dye; P = 3' phosphate group.

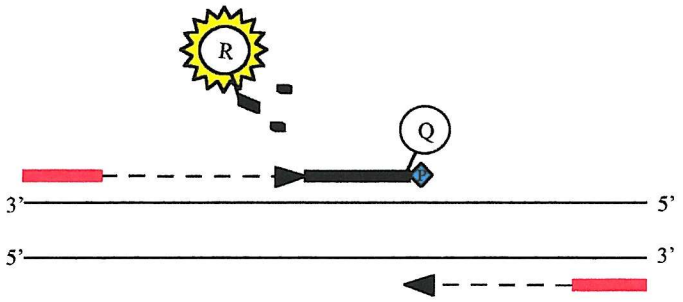
(a) Polymerisation.



(b) Probe displacement.



(c) Probe cleavage.



(d) Polymerisation completed.

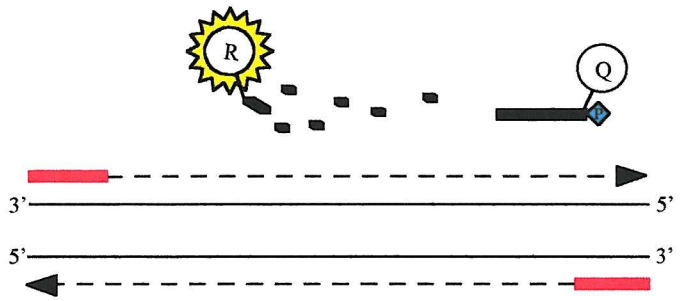


Fig.3.4 Diagrammatic representation of the TaqMan assay.
A TaqMan probe (thick black line) has a reporter dye (R) and a quencher dye (Q) at opposite ends. The probe has a phosphate group (P) at the 3′ end to prevent extension by *Taq* polymerase. During the annealing phase of PCR, the probe binds to one strand of the template DNA (thin black lines) at it's complementary sequence between the PCR primers (red lines). During primer extension (a) the 5′ nuclease activity of *Taq* DNA polymerase displaces (b) and cleaves (c) the probe. When the unbound probe is intact, the flexibility of the oligonucleotide allows the dyes to come into close proximity, resulting in quenching of reporter dye fluorescence. When the probe is cleaved, the dyes are no longer in close proximity, allowing reporter fluorescence to be detected (d). Dashed black line = newly synthesised DNA. Modified from Perkin Elmer TaqMan reagent kit protocol (1996).

formation of the hairpin structure, allowing detection of reporter fluorescence. The probe then dissociates from the template as the temperature is raised for primer extension (Kramer and Tyagi, 1996; Lewin *et al.*, 1999). Molecular beacons have been used to detect adenovirus DNA (Poddar, 1999) and HIV (Lewin *et al.*, 1999).

(iii) Molecular beacon primer assays

The molecular beacon primer strategy involves one of the PCR primers being labelled with reporter and quencher dyes (Jordens *et al.*, 2000). One dye is bound at the 5' end and the other at an internal position (Fig.3.3). The 5' end of the unincorporated primer binds to an internal complementary region as the temperature is decreased for primer annealing. This brings the reporter and quencher dyes into close proximity, resulting in quenching of reporter fluorescence. When a primer is incorporated into a PCR product, the hairpin structure is disrupted by *Taq* polymerase. This increases the distance between reporter and quencher, allowing reporter fluorescence to be detected.

The Amplifluor system (Intergen) is a variation on the molecular beacon primer strategy. PCR is performed with a pair of template-specific primers, one of which has a 5' complementary site for a UniPrimer molecular beacon primer (Nazarenko *et al.*, 1997). During the first cycles of PCR, the UniPrimer anneals to the complementary PCR primer sequence and become incorporated into subsequent reaction products. Assays using this strategy are commercially available for detection of apoptosis-associated genes, prostate-specific antigen and human papilloma virus-16.

(iv) Bi-probe assay

The bi-probe method combines fluorescent dye intercalation with probe annealing. A probe, labelled with a reporter dye, is designed to bind to single-stranded PCR product at a temperature above the T_m of the primers. A donor dye is included in the PCR reaction, which can then intercalate between the probe and product strands, resulting in increased fluorescence from the reporter dye (Fig.3.3). Whaley *et al.* (1999) have used a bi-probe to detect HBV polymerase PCR products and used the melting properties of the probe to detect lamivudine resistance-associated mutations.

(v) Hybridisation probe assay

The hybridisation probe assay involves the use of two probes, one labelled with a reporter dye, the other with a donor dye (Fig.3.3). The probes are designed to hybridise to adjacent sites on one strand of the PCR product at a temperature above the T_m of the primers. This brings the

dyes into close proximity, allowing transfer of fluorescence from the donor to the reporter. The probes then dissociate as the temperature is raised further for primer extension. Fluorescence is measured during the probe hybridisation stage and the reporter dye is only detected if both probes have annealed (Nauck *et al.*, 1999). This strategy has been used successfully to detect various targets, including HBV (Cane *et al.*, 1999).

Of the above strategies, those involving probe annealing theoretically offer higher specificity. Methods involving fluorescent dye intercalation only, or molecular beacon primers, may detect non-specific amplification products. These assays are therefore more difficult to optimise.

3.1.5. Closed-tube detection of PCR products

Various instruments are available for closed-tube detection of fluorescence produced as a result of PCR product formation. These have the following features in common:

(i) A source of photons at the excitation wavelength of the reporter or donor dye.

(ii) Reaction vessels able to transmit photons of the excitation and emission wavelengths of the fluorescent dyes.

(iii) A fluorescent detection system able to measure photons at the assay emission wavelength.

(iv) A system to detect emission photons and produce a readable output.

(v) A computer to control fluorescence monitoring and analyse data.

The first generation of instruments designed for closed-tube PCR product detection measured end-point fluorescence. PCRs were performed on a dedicated thermocycler and fluorescence measured, post-amplification, with machines such as the TaqMan LS-50B PCR Detection System or the PRISM 7200 Sequence Detection System (SDS) (Applied Biosystems).

More sophisticated instruments have now been developed which allow closed-tube measurement of fluorescence from PCR in real-time. Four systems are currently available: the PRISM 7700 SDS, the GeneAmp 5700 SDS (Applied Biosystems), the Light Cycler (Roche); and the iCycler (Bio-Rad).

(i) iCycler and GeneAmp 5700 SDS

The iCycler is a block-based thermocycler to which an optical module for fluorescence detection can be attached. It has built-in data analysis software, making it smaller than the other real-time PCR systems available. Reports of the instrument's use have yet to be published.

The GeneAmp 5700 SDS includes a block-based thermocycler, fluorometer and personal computer. The light source is a tungsten-halogen lamp and fluorescence is detected with a charge-coupled device (CCD) camera across the wavelength range 530 - 580nm.

(ii) Light Cycler

The Light Cycler performs up to 32 PCRs in air-incubated, glass capillary tubes which allows rapid temperature cycling and hence reduced incubation times. Reactions are carried out in low volumes (1 - 10µl), reducing reagent and sample use, but this may compromise sensitivity due to the low sample input volumes possible. The reaction mixture is excited with photons from a blue light-emitting-diode, filtered to produce 450 - 490nm light. Fluorescence is detected by a series of photodiodes. Temperature cycling, reaction monitoring and fluorescence analysis is computer-controlled (Witter *et al.*, 1997). The Light Cycler has been used for detection of *Leptospira biflexa* (Woo *et al.*, 1999), lamivudine resistance-associated mutations in HBV (Cane *et al.*, 1999) and HCV (Schroter *et al.*, 2001).

(iii) PRISM 7700 Sequence Detection System

The PRISM 7700 SDS was designed primarily for the detection of PCR products using the TaqMan 5' nuclease assay. The unit combines a 96 well block-based thermocycler with a fluorescence measurement system and is controlled by a Macintosh computer.

PCRs are excited with light (488nm) from an argon laser. Fibre optic cables collect emitted fluorescence between the wavelengths 500nm and 660nm. The machine can be programmed to collect fluorescence at any stage of the PCR cycle and can complete collection from all wells approximately once every 7sec. Fluorescence is focused onto a spectrograph which separates light on the basis of wavelength into a predictably spaced pattern across a CCD camera. The computer then applies analysis algorithms to the data from the CCD camera.

The level of reporter dye fluorescence is compared with that of a passive reference dye, ROX (Fig.3.2), the fluorescence level of which is unaffected by PCR product level. Volumetric changes are controlled for by dividing the reporter signal by the passive reference signal to give a normalised reporter fluorescence level (R_n). The system automatically plots ΔR_n against PCR cycle number and calculates a fluorescence threshold (Fig 3.5). A reaction is called positive once the reporter fluorescence exceeds the threshold value, and the PCR cycle at which this occurs is known as the threshold cycle (C_T). Positive reactions have a C_T value less than the number of cycles used for the PCR. The ABI 7700 SDS has the advantage of a high throughput (96 reactions per run) and has been used successfully for detection of a range of pathogens, including HCV (Morris *et al.*, 1996), *Yersinia pestis* (Higgins *et al.*, 1998), EBV

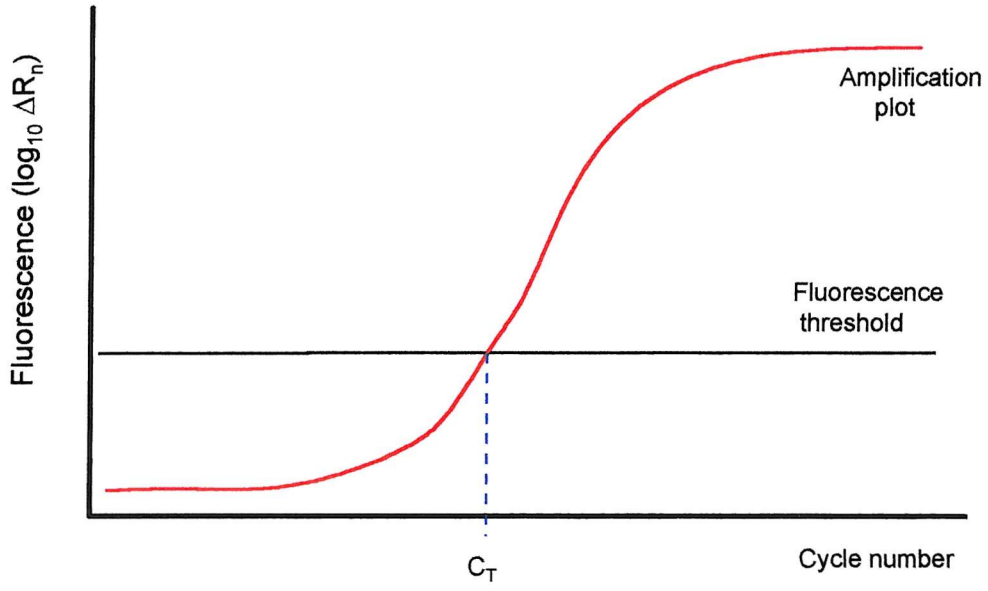


Fig 3.5 Diagrammatic representation of a typical amplification plot produced by the PRISM 7700 SDS.
The software analyses fluorescence from the early cycles and calculates the average and standard deviation of R_n to define baseline fluorescence. The system then multiplies the standard deviation of the background R_n by a default factor of ten to set a threshold fluorescence value. The threshold can also be set manually. ΔR_n, the change in R_n from the baseline fluorescence, is plotted. The cycle number at which fluorescence exceeds the fluorescence threshold is called the threshold cycle (C_T) (illustrated by dashed line).

(Hamilton *et al.*, 1997) and simian immunodeficiency virus (SIV) (Suryanarayana *et al.*, 1998).

Prior to the start of this project, one TaqMan PCR-based assay for detection of HCV RNA had been developed (Morris *et al.*, 1996). This used a luminescence spectrometer to detect fluorescence and involved post-amplification manipulation of PCR products. For this project, the assay could have been modified to measure fluorescence with a closed-tube detection system in real-time. However, the primers used by Morris *et al.* (1996) were designed using 5'UTR sequence data from a study performed on HCV detected in patients from the USA, Japan and Italy (Cha *et al.*, 1991). HCV genotype prevalence varies with geographical region (McOmish *et al.*, 1994). In the USA genotypes 1a and 1b are most common, in Japan 1b prevails, in southern Europe types 1 and 2 predominate (Davidson *et al.*, 1995; Guadagnino *et al.*, 1997), while in the UK 1a and 3a are most prevalent (Harris *et al.*, 1999). The distribution of genotypes identified in the Wessex region (Fig.3.6) reflects that of the UK as a whole.

Therefore, the need remained for a closed-tube TaqMan PCR-based assay for detection of HCV genotypes commonly identified in the UK. For this project, an assay was developed for detection of HCV RNA using the PRISM 7700 SDS to monitor closed-tube TaqMan PCR amplification of HCV cDNA, and was to form the basis of a quantitative assay for HCV RNA.

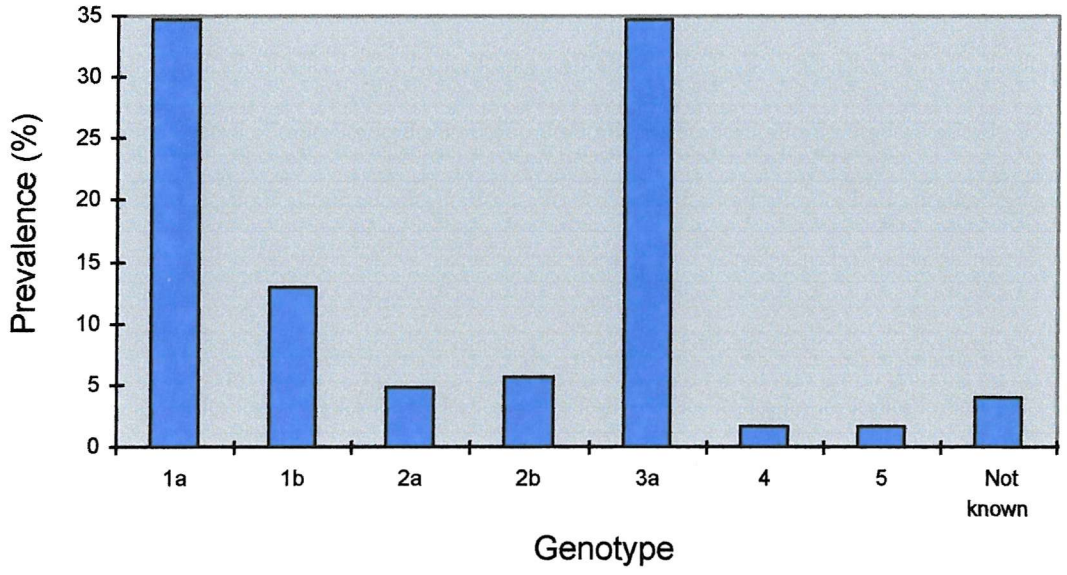


Fig.3.6 Chart showing the relative prevalence of HCV genotypes identified by RFLP in the Wessex area (S. Green, personal communication).

3.2. Design of HCV TaqMan primers and probes

The 5'UTR (341 - 344nt) was chosen as the target for the HCV TaqMan PCR because it is one of the least variable regions of the HCV genome (90 - 100% sequence identity) (Bukh *et al.*, 1992). The terminal 98nt region of the 3'UTR is reportedly less variable (98 - 100% sequence identity) (Kolykhalov *et al.*, 1996), but the 5'UTR offered a greater length of relatively conserved sequence in which to design broadly reactive primers and probes and more sequence data was available for this region.

3.2.1. 5'UTR sequence analysis

Due to the lack of published sequence data for UK HCV isolates, partial 5'UTR sequences were obtained for 36 isolates representative of genotypes most commonly isolated in the Wessex region (1a, 1b, 2a, 2b, 3a) and of the rarer genotypes 4 and 5. More strains of genotypes 1a and 3a were analysed because these are the most commonly detected UK (Harris *et al.*, 1999) and Wessex genotypes (S. Green, personal communication).

A 305nt region (nt -297 to 8) of the 5'UTR of each of the 36 selected HCV isolates was amplified by PCR with Bio-X-act DNA polymerase (Bioline) using primers HCV939 and HCV209 (Fig.3.7). The PCR products were then purified and sequenced on both strands. A truncated version of primer HCV209, HCV209T (Fig.3.7) was used due to poor quality sequence data obtained from primer HCV209. The sequence data were deposited in the GenBank database (accession numbers: AF217293 - AF217315). The multiple alignment of the 36 5'UTR sequences is shown in Appendix III (Fig.10.1) and nucleotide variation is shown in Fig.3.8.

The Wessex HCV sequences were compared with HCV 5'UTR sequences reported on the GenBank computer database using FASTA. Reference sequences were chosen which represented the range of genotypes analysed in this study (Table 3.1).

The percentage identities between each of the Wessex sequences with the GenBank sequence of the same genotype were determined (Appendix III, Table 10.3) and represented as a phylogenetic tree (Fig.3.9). The Wessex sequences showed 94.1 - 100% identity with GenBank sequences of the same genotype. This verified that the correct region had been amplified and sequenced. The highest degree of variation between genotypes was found to occur between Wessex type 3a sequence and GenBank type 2b sequences (87.9 - 94.9% identity).

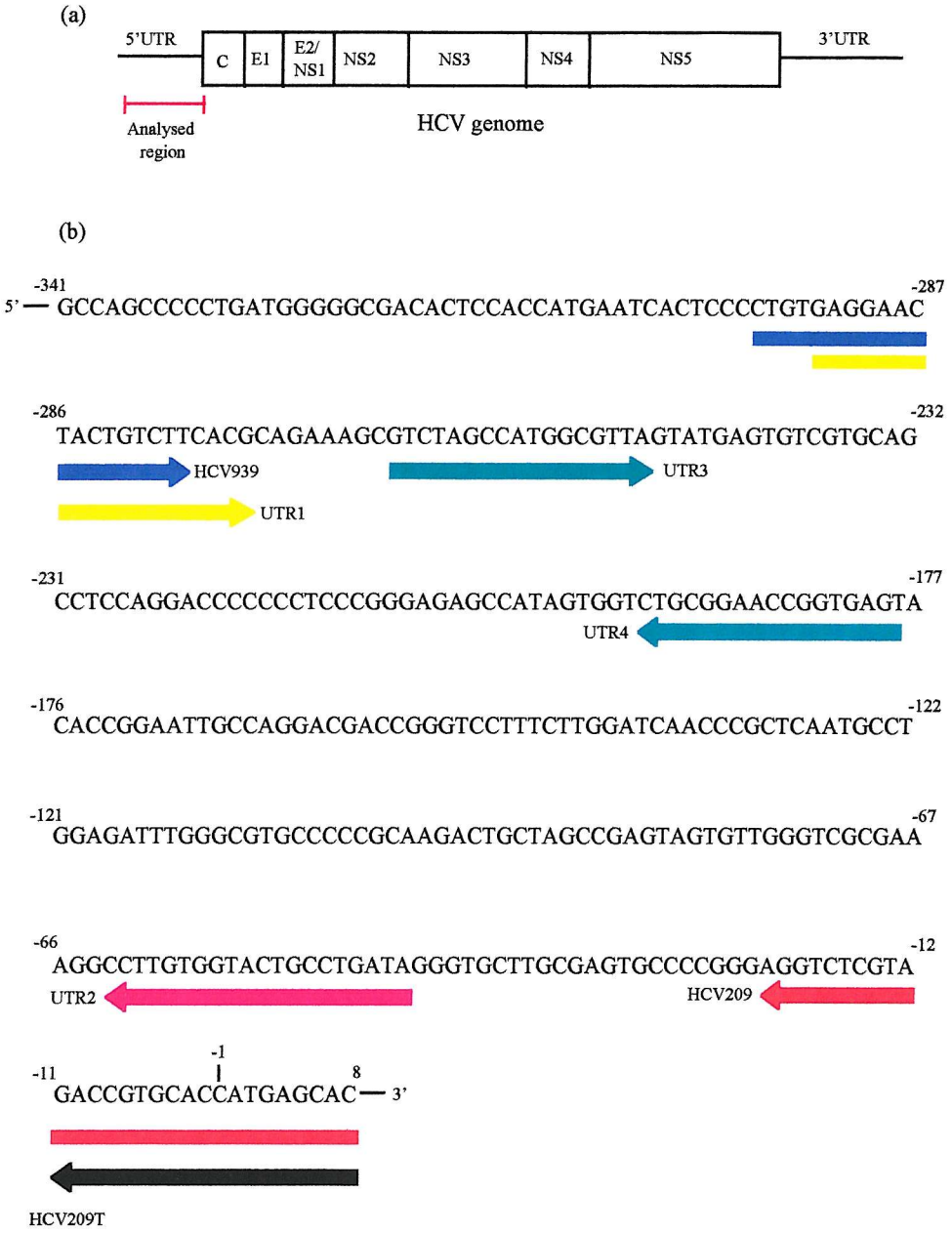


Fig.3.7 Locations of primers used for amplification and sequence analysis of Wessex HCV 5'UTRs.
(a) The region of the HCV genome (nt -297 to 8) amplified by PCR. Boxes represent RNA coding regions. (b) The primer positions (arrows) are shown against the 5'UTR sequence of HCV-1, the prototype strain, (Choo *et al.*, 1991) and are numbered relative to the polyprotein translation start site. Nucleotide positions are shown as superscript.

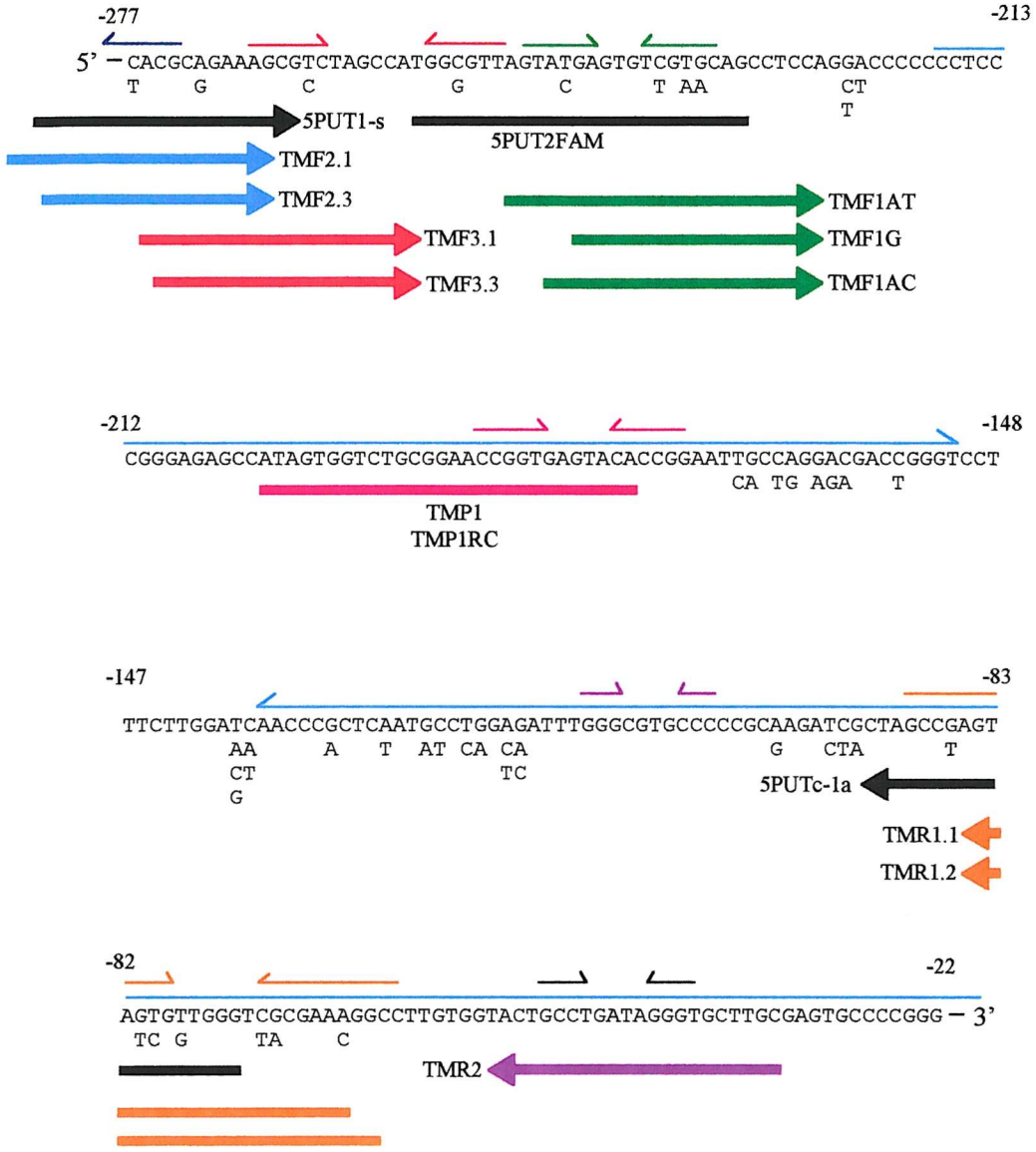


Fig.3.8 Nucleotide variation in the 5'UTR of Wessex HCV strains.
Nucleotide variations are shown (subscript) relative to strain Sot1-1a. Nucleotide co-ordinates (superscript) correspond to those of the prototype strain, HCV-1 (Choo *et al.*, 1991). The positions of the primers (arrows) and probes (lines) used for the published TaqMan PCR assay (Morris *et al.*, 1996) are illustrated in black. The primers (arrows) and probes (lines) used for the in-house TaqMan PCR are shown with primer mixtures represented in the same colour. Positions of predicted secondary structure (Smith *et al.*, 1995) are shown. Pairs of thin semi-arrows representing complementary sequences (based on HCV-1 sequence) are shown in the same colour, with the arrow heads pointing towards the loop region.

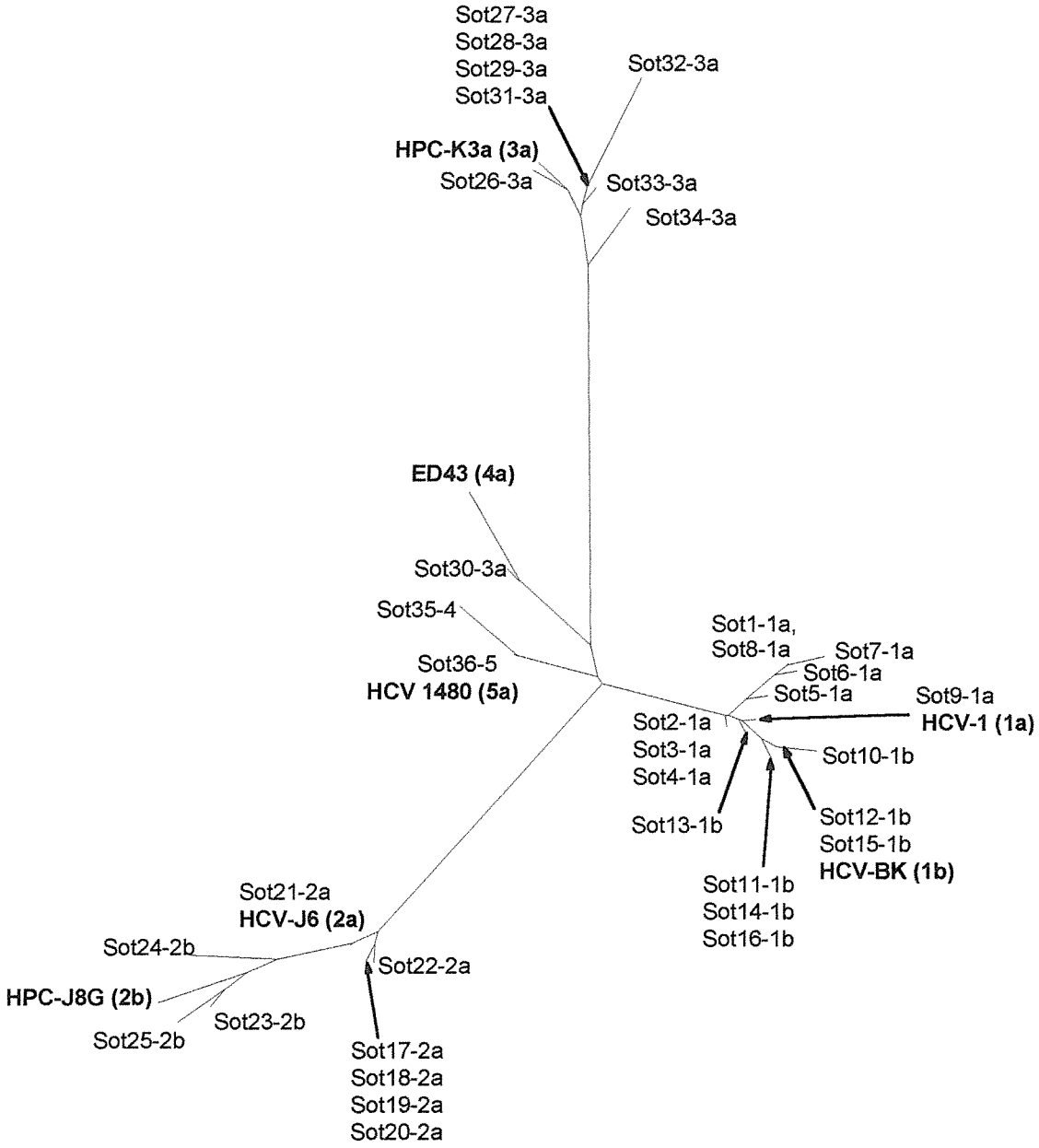


Fig.3.9 Unrooted phylogenetic analysis of a 255nt section (nt -277 to -22) of the 5'UTRs of Wessex and published HCV strains created using PHYLIP.
Reference strains are in bold type with genotypes in brackets.

Table 3.1 Published HCV sequences from GenBank database used for comparison with Wessex 5'UTR sequences.

Sequence	Genotype	Country of origin	GenBank accession number	Reference
HCV-1	1a	USA	M62321	Choo *et al.*, 1991
HCV-BK	1b	Japan	M58335	Takamizawa *et al.*, 1991
HCV-J6	2a	Japan	D00944	Okamoto *et al.*, 1991
HPC-J8G	2b	Indonesia	D10988	Okamoto *et al.*, 1992
HPC-K3a	3a	Japan	D28917	Yamada *et al.*, 1994
ED43	4a	Middle East	Y11604	Chamberlain *et al.*, 1997(a)
HCV 1480	5a	South Africa	Y13184.1	Chamberlain *et al.*, 1997(b)

The range of sequence identity between Wessex isolates was 87.9 - 100% (Appendix III, Table 10.4). Sequence identity was 87.9 - 99.6% between Wessex isolates of different genotype and 93 - 100% between isolates of the same genotype.

3.2.2. HCV TaqMan PCR primer and probe design

The alignment of Wessex HCV 5'UTR sequences was used to identify conserved areas of the 5'UTR to which TaqMan PCR primers and probes could be targeted. Primers were designed to amplify target sequences from all of the panel of HCV isolates and to have annealing temperatures of approximately 60°C. They were designed according to the rules described in Section 2.17. The primers shown in Table 3.2 and Fig.3.8 were designed by analysing the Sot1-1a sequence of the HCV panel with Primer Express, Gene Runner and manually to assess whether the method of primer design used affected TaqMan PCR efficiency. The chosen TMF1, TMF2, TMF3 and TMR1 primer sites included some sequence variation. Therefore, the primers were designed to include degenerate nucleotides at these positions (Table 3.2).

The variations at nt-238 of Sot30-3a and at nt-236 of Sot35-4 and Sot36-5 were not accounted for when designing degenerate primers. This was so as not to compromise detection of the most common sequences. In these cases, the mismatches occurred away from the critical 3′ primer positions and would theoretically affect amplification only slightly.

Table 3.2 Primers designed for amplification of the HCV 5'UTR by TaqMan PCR.

Primer set	Primer name	Primer mix	Design method*	Position on HCV genome (nt)	Degeneracy[+]	Amplicon length (nt)
1	TMF1	TMF1G	PE	-244 to -226	-	179 to 186
		TMF1AT		-249 to -226	A(-235)	
		TMF1AC		-246 to -226	C(-245)	
	TMR1	TMR1.1	PE	-85 to -65	-	
		TMR1.2		-85 to -63	C(-80), T(-72)	
2	TMF2	TMF2.1	GR	-291 to -267	-	254 to 257
		TMF2.3		-288 to -267	G(-272)	
	TMR2	TMR2	GR	-55 to -34	-	
3	TMF3	TMF3.1	M	-276 to -256	-	241 to 242
		TMF3.3		-275 to -256	C(-264),G(-272)	
	TMR2	TMR2	GR	-55 to -34	-	

Nucleotide co-ordinates correspond to those of HCV-1 (Choo *et al.*, 1991).
*PE = Primer Express. GR = Gene Runner. M = Manual.
[+]Primer mixes consisting of equimolar amounts of different oligonucleotides, each designed to anneal to a different variation of the primer site identified in the Wessex strains.

The TaqMan probes TMP1 and TMP1RC (Fig.3.8) were designed using Primer Express according to the rules described in Section 2.18. The target region was conserved throughout the Wessex isolates. TMP1 was designed to anneal to the sense template strand as this meant the probe had more C than G residues. However, this resulted in the probe being 89 - 120nt away from the primer on the same strand. In case distance from the primer had an effect on probe cleavage efficiency, TMP1RC was designed as the reverse complement to anneal to the anti-sense template strand. This probe was only 23 - 64nt away from the primer on the same strand. Probes were labelled with FAM at the 5' end as reporter dye and TAMRA at the 3' end as quencher.

3.3. Evaluation of primers and probes

The following experiments were carried out to identify the most efficient combination of HCV TaqMan primers and probes to use in the assay. For the evaluation, cDNA from HCV isolates Sot2-1a, Sot8-1a, Sot29-3a and Sot30-3a was used, representing the most commonly identified genotypes of the Wessex region. These were also representative of the most common sequence variants within their respective genotypes. The performance of the in-house

primer-probe sets was compared with that of the primer-probe set described by Morris *et al.* (1996) (Fig.3.8).

3.3.1. Primer evaluation

Different combinations of primers were used to amplify the above cDNA sequences using TaqMan PCR conditions but excluding the TaqMan probes. The amplification products were analysed by agarose gel electrophoresis (Fig.3.10). The products from amplifications carried out with primer pairs TMF1/TMR1 and TMF2/TMR1 included significant levels of primer-dimer structures. The most specific PCRs, giving products of only the expected size, used the primer pairs 5PUTc-1a/5PUT1-s and TMF3/TMR2.

3.3.2. Probe evaluation

All primer-probe sets were evaluated for detection efficiency of HCV cDNA in complete TaqMan PCRs (Fig.3.11). Reaction C_T was used as a measure of the amplification and detection efficiency. The primer-probe combination TMF1/TMR2/TMP1 gave the lowest mean C_T values, indicating that the most efficient amplification, probe binding and cleavage, and reporter fluorescence detection was occurring for this set. The primer-probe set 5PUTc-1a/5PUT1-s/5PUT2FAM gave the highest mean C_T value, indicating the lowest detection efficiency. This set also amplified genotype 3a sequence less efficiently than genotype 1a sequence. Amplifications including probe TMP1 had consistently lower C_T values than amplifications including TMP1RC and 5PUT2FAM, indicating more efficient probe annealing, cleavage or fluorescence detection.

The primer-probe set TMF1/TMR2/TMP1 was identified as the most efficient for the amplification and detection of HCV 5'UTR for genotypes 1a and 3a. The set was therefore chosen for use in the complete assay.

3.4. Optimisation of TaqMan PCR conditions

To obtain the maximum efficiency for amplification of HCV 5'UTR cDNA, the concentration of primers, probe and Mg^{2+} ions were optimised. The C_T of the TaqMan PCR was again used as a measure of reaction efficiency.

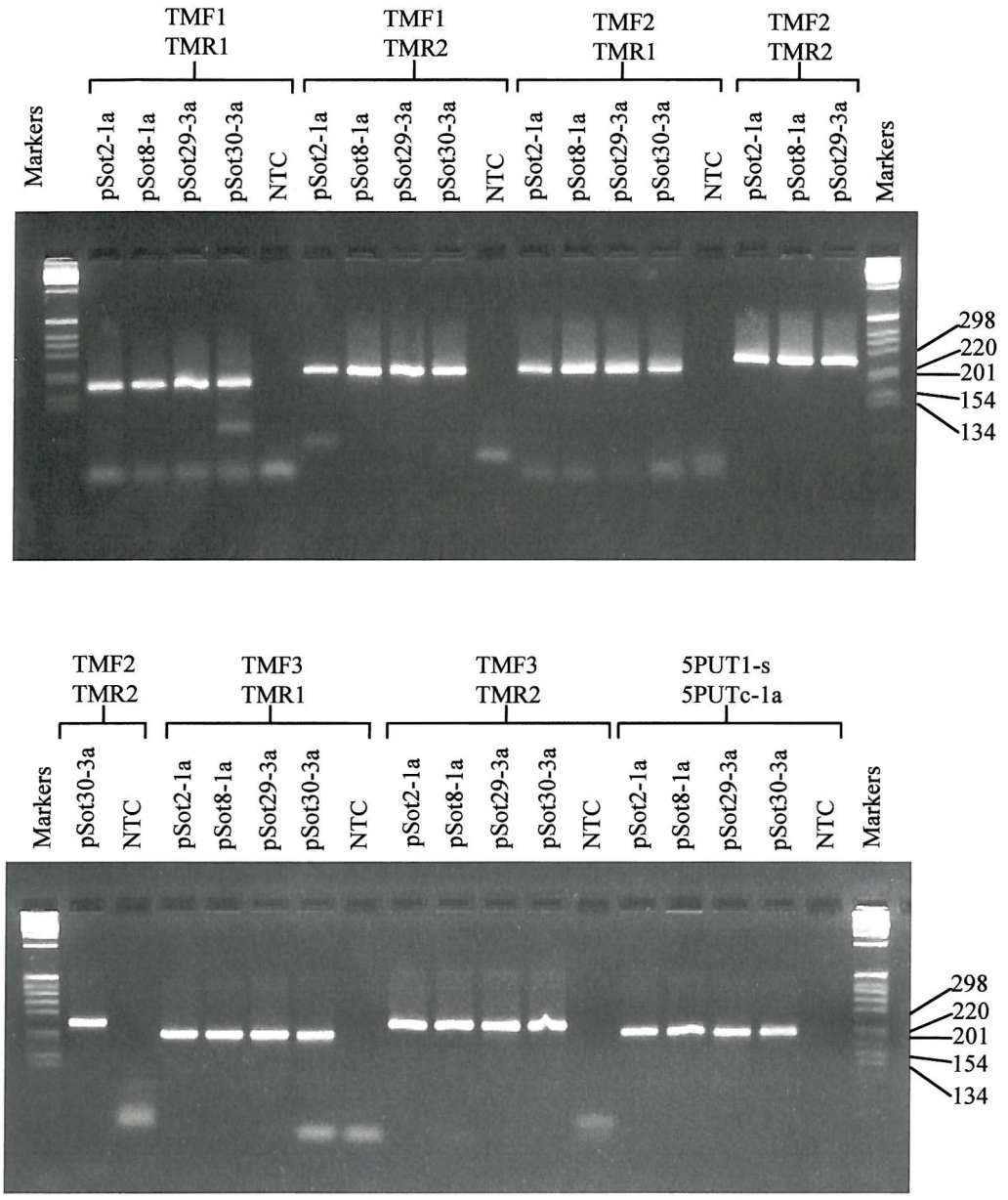


Fig.3.10 Agarose gels of amplification products produced by PCR with in-house and published primers.

Sot2-1a, Sot8-1a, Sot29-3a and Sot30-3a cDNAs were amplified using the following primer combinations (amplicon sizes): TMF1/TMR1 (186bp), TMF1/TMR2 (215bp), TMF2/TMR1 (226bp), TMF2/TMR2 (257bp), TMF3/TMR1 (211bp), TMF3/TMR2 (242bp), and 5PUT1-s/5PUTc-1a (215bp). Each cDNA sample (1µl) was amplified using the default β-actin TaqMan PCR conditions, but excluding TaqMan probes. Primers were at a final concentration of 0.15µM as described by Morris *et al.* (1996). PCRs were carried out with each primer combination for each cDNA. A 10µl sample of each product was analysed on a 4% NuSieve agarose gel with 1kb ladder markers (150ng; selected band sizes are shown (bp)). NTC = no template control.

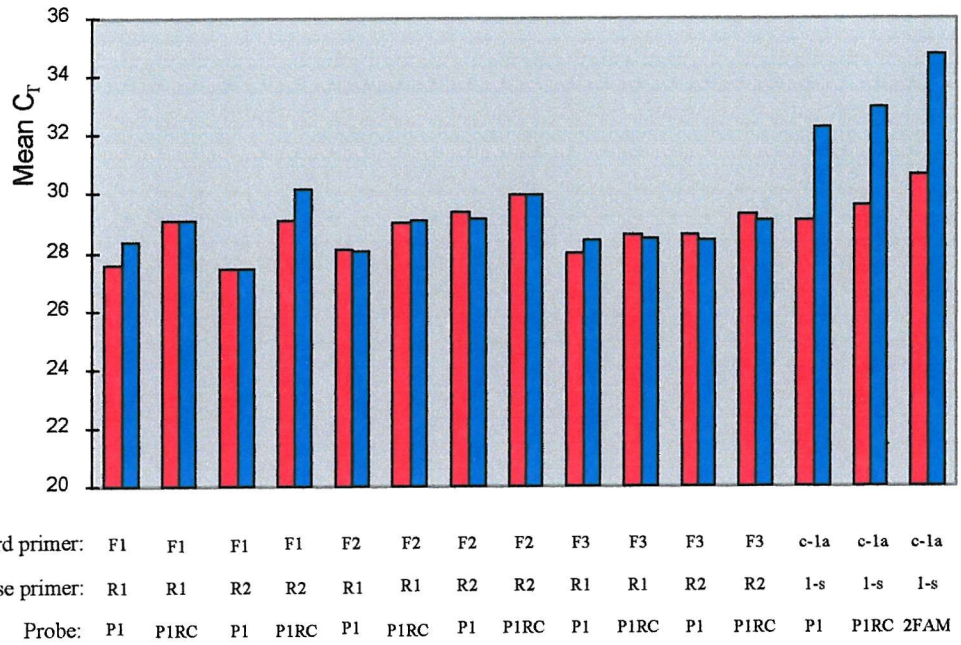


Fig.3.11 Chart of mean reaction C_Ts for different primer/probe combinations.
A 1µl sample of Sot8-1a (red) or Sot29-3a (blue) cDNA was included in 25µl PCRs. Each cDNA sample was amplified using the β-actin TaqMan PCR conditions and the primer/probe combinations shown. Primers and probes were at a final concentration of 0.15µM and 0.04µM, respectively, as described by Morris *et al.* (1996). Duplicate PCRs were carried out with each primer combination for each cDNA. The prefixes, TM and 5PUT have been omitted from the primer and probe names for clarity.

3.4.1. Optimisation of primer concentration

To determine the optimal concentration of primers for the reaction, TaqMan PCRs were performed in which TMF1 and TMR2 concentrations were varied independently between 0.1μM and 0.4μM (Fig.3.12). TMF1 and TMR2 concentrations of 0.4μM and 0.2μM, respectively resulted in the lowest mean C_T values for both genotype 1a and 3a amplification.

The concentration of TMF1 that gave the lowest C_T was at the top of the concentration range tested (0.4μM). Therefore, a further experiment (Fig.3.13) was carried out to more accurately determine the optimum concentration of the forward primer. The lowest mean C_Ts for amplification of both cDNAs was given at a TMF1 concentration of 0.3μM. When the results of this and the previous experiment were combined, the lowest mean C_T was given by a TMF1 concentration of 0.4μM and this was used in subsequent experiments.

3.4.2. Optimisation of probe concentration

TaqMan PCRs were carried out to determine the optimum concentration of TMP1 for the reaction using primers, TMF1 and TMR2 at the optimum concentrations previously determined (Fig.3.14). A TMP1 concentration of 200nM gave the lowest mean C_T value for the genotype 1a cDNA, whereas a concentration of 175nM gave the lowest mean C_T value for the genotype 3a cDNA. The results for both genotypes were combined and the lowest mean C_T was given at the 175nM concentration of TMP1. This concentration was used in subsequent experiments.

3.4.3. Optimisation of $MgCl_2$ concentration

$MgCl_2$ concentration for the TaqMan PCR was optimised by testing in the range 0.8 - 9mM (Fig.3.15) The lowest mean C_T for detection of the genotype 1a and 3a cDNAs was given by $MgCl_2$ concentrations of 4 and 5mM, respectively. When the C_Ts obtained for detection of 1a and 3a cDNAs were combined, the lowest value was given by a $MgCl_2$ concentration of 4mM. This was therefore identified as the optimum $MgCl_2$ concentration.

3.5. Evaluation of optimised HCV TaqMan PCR

Cloned 5'UTR sequences were used as template to assess the sensitivity of the in-house TaqMan PCR and the effect of HCV genotype. Using cloned sequences enabled known amounts of template to be added to PCRs. The performance of the in-house assay was compared with that of the published assay (Morris *et al.*, 1996).

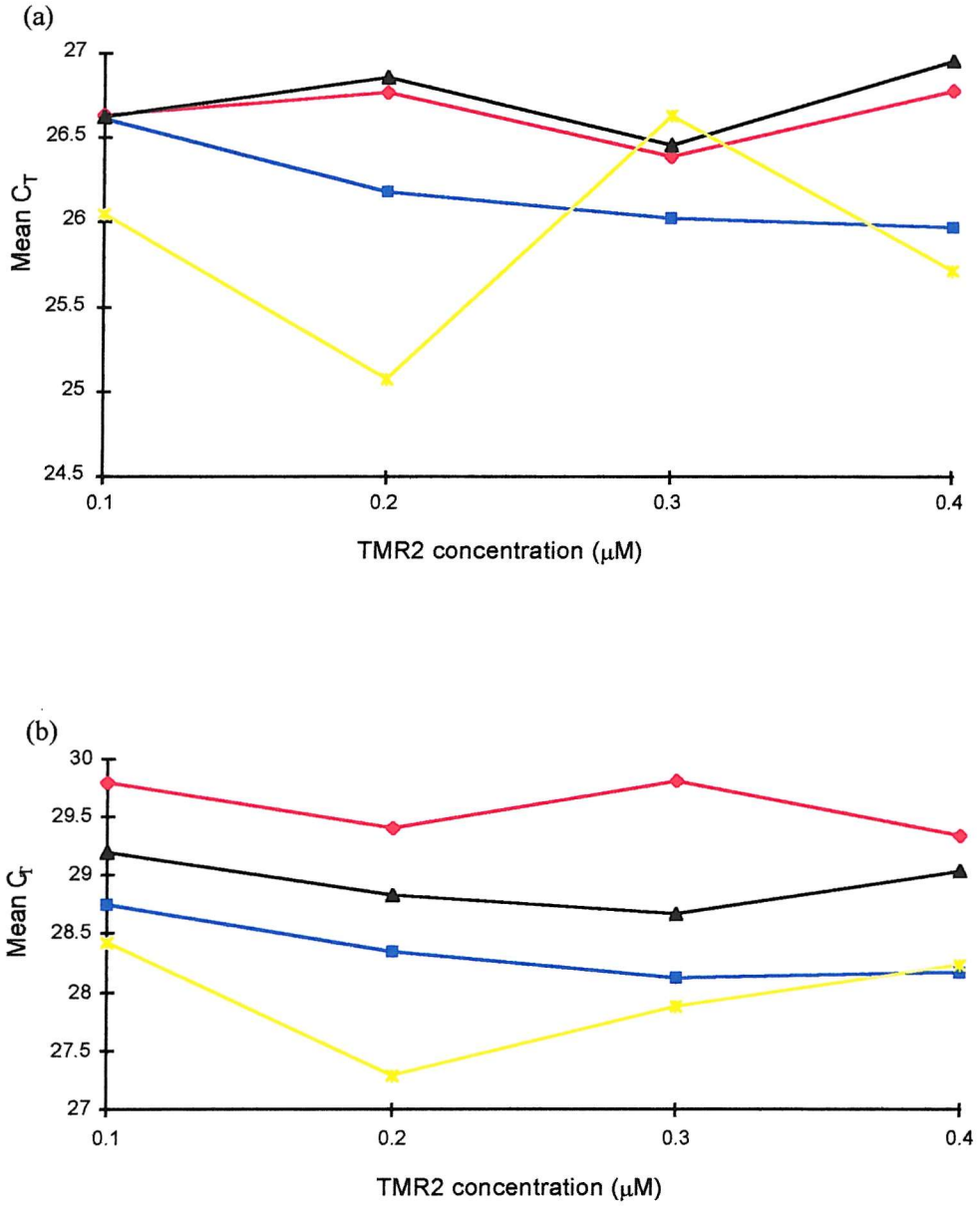


Fig.3.12 Plots of C_T against TMR2 concentration for TaqMan PCRs using different TMF1 concentrations.

A constant volume (1μl) of the Sot8-1a (a) and Sot29-3a (b) cDNA samples was amplified in duplicate TaqMan PCRs. Each primer was included at final concentrations of 0.1μM (red), 0.2μM (blue), 0.3μM (black), and 0.4μM (yellow) in all possible combinations. The concentration of the probe was kept at 40nM. The concentration of the other reaction components and conditions were as for the β-actin control TaqMan PCR reaction.

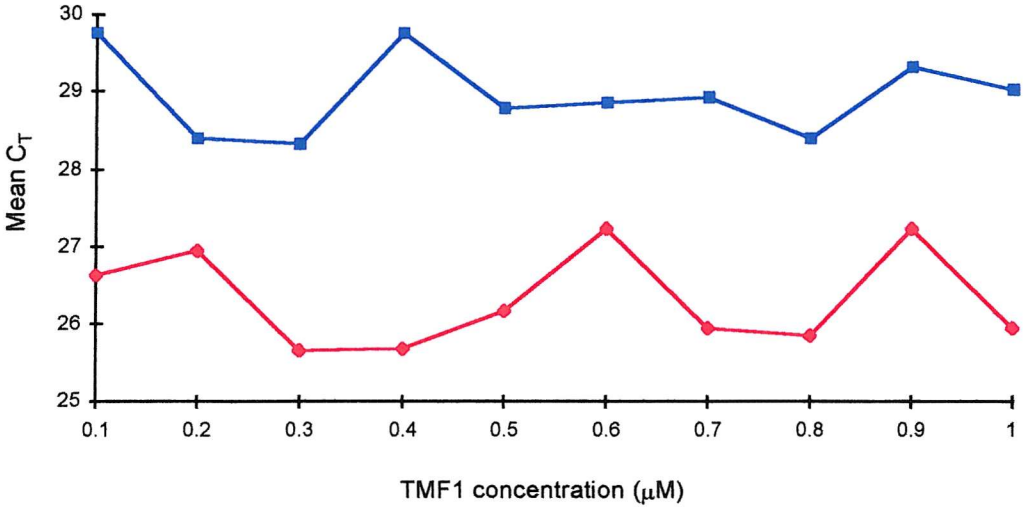


Fig.3.13 Plots of mean C_T against TMF1 concentration for amplification of 1a and 3a cDNAs.
Samples (1μl) of Sot8-1a (red) and Sot29-3a (blue) cDNA were amplified in duplicate 25μl TaqMan PCRs. Primer TMR2 was included at a final concentration of 0.2μM. TMF1 was at final concentrations of 0.1 - 1.0 μM. Other reagents and reaction conditions were as for the β-actin control reaction.

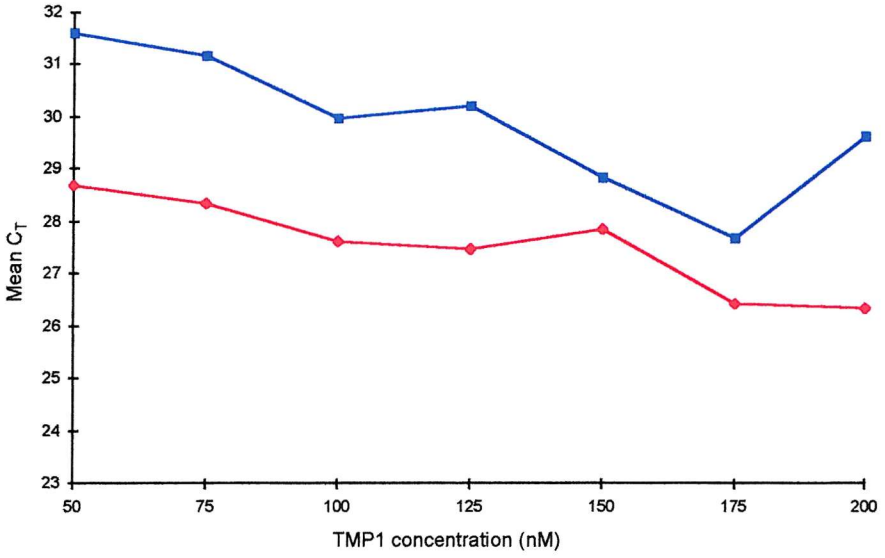


Fig.3.14 Plots of C_T against probe concentration for genotype 1a and 3a cDNA samples.
Sot8-1a (red) and Sot29-3a (blue) cDNA samples (1μl) were included in duplicate 25μl TaqMan PCRs. TMF1 and TMR2 concentrations were 0.4μM and 0.2μM, respectively. TMP1 was included at final concentrations of 50 - 200nM. Other reagents and conditions were as for the β-actin control reaction.

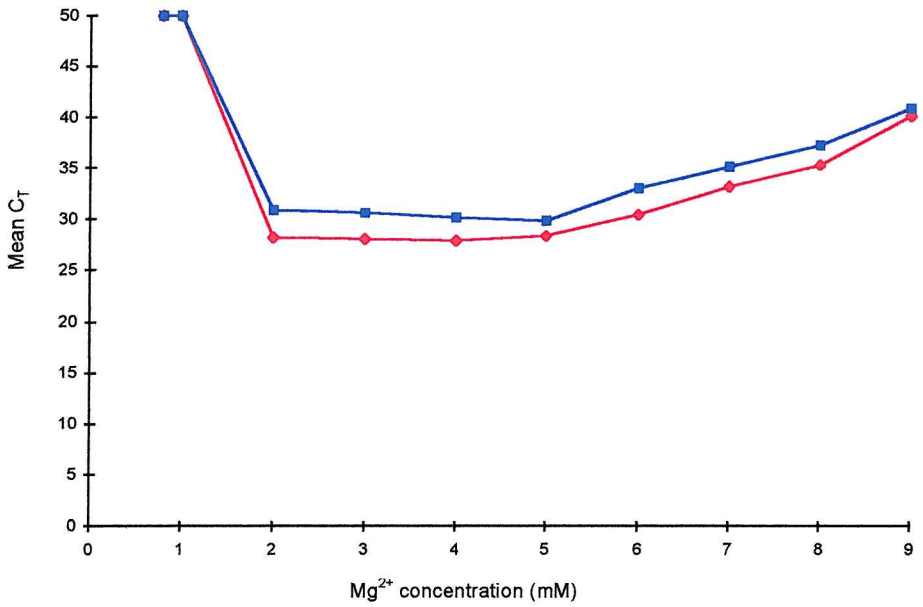


Fig.3.15 Plot of C_T against $MgCl_2$ concentration for genotype 1a and 3a cDNA samples. Sot8-1a (red) and Sot29-3a (blue) cDNA samples (1µl) were included in duplicate 25µl TaqMan PCRs. TMF1, TMR2 and TMP1 concentrations were 0.4µM, 0.2µM and 0.175µM, respectively. $MgCl_2$ was included at final concentrations of 0.8 - 9mM. Other reagents and conditions were as for the β-actin control reaction.

3.5.1. Cloning of 5'UTR sequences

Of the 36 sequenced Wessex HCV 5'UTR sections (305nt), cDNA from the following was cloned: Sot6-1a, Sot7-1a, Sot10-1b, Sot15-1b, Sot17-2a, Sot21-2a, Sot23-2b, Sot24-2b, Sot26-3a, Sot34-3a, Sot35-4, and Sot36-5. These were chosen to represent HCV genotypes 1a, 1b, 2a, 2b, 3a, 4, and 5. Plasmid clones of the 5'UTR sequences were constructed by ligating 28ng of *EcoRI*/*Hind*III-digested HCV939E/HCV209TH amplicons with 100ng of *Eco*RI/*Hind*III-digested pGEM-3Z and transforming competent *E. coli* Top10 cells. For each transformation, plasmid DNA from three white colonies was screened for 5'UTR insert DNA by PCR with the primer pair, HCV 939E/HCV209TH. PCR with the primer pair, pGEM3Z-SP6/pGEM3Z-T7 (annealed to the pGEM3Z SP6 and T7 promoter sequences, respectively) was used to determine the length of DNA inserted into the MCS. PCRs with the primer pairs, pGEM3Z-SP6/HCV939E and pGEM3Z-T7/HCV209TH were used to check the presence and orientation of insert DNA. Plasmid DNA was then analysed by cycle sequencing with primers pGEM3Z-SP6 and pGEM3Z-T7. The sequence of each of the 5'UTR clones was compared with the original sequence data for each cDNA. No variations from the original sequence was found for any of the 12 clones selected for the study.

3.5.2. Efficiency of detection of HCV genotypes

An experiment was performed to evaluate the effect of genotype on PCR efficiency (Fig.3.16). Each 5'UTR clone was linearised by digestion with *Hind*III. Complete digestion was confirmed by agarose gel electrophoresis (not shown). The DNA concentration was determined by A_{260} measurement and used to calculate the number of template copies per µl. The HCV 5'UTR plasmids were diluted in UHQ H_2O to a concentration of 100 copies/µl. Optimised TaqMan PCRs were then performed including constant numbers of template copies and C_T values obtained with the in-house and published (Morris *et al.*, 1996) assay confirmed.

The C_Ts for the in-house TaqMan PCR (mean = 29.73) were significantly lower ($P = 0.02$) than those for the published assay (mean = 35.23), indicating a more efficient reaction. The mean C_Ts for the in-house assay were also more consistent across the range of genotypes tested than those of the published assay (SDs of 0.43 and 4.79, respectively), indicating that detection was less affected by genotype.

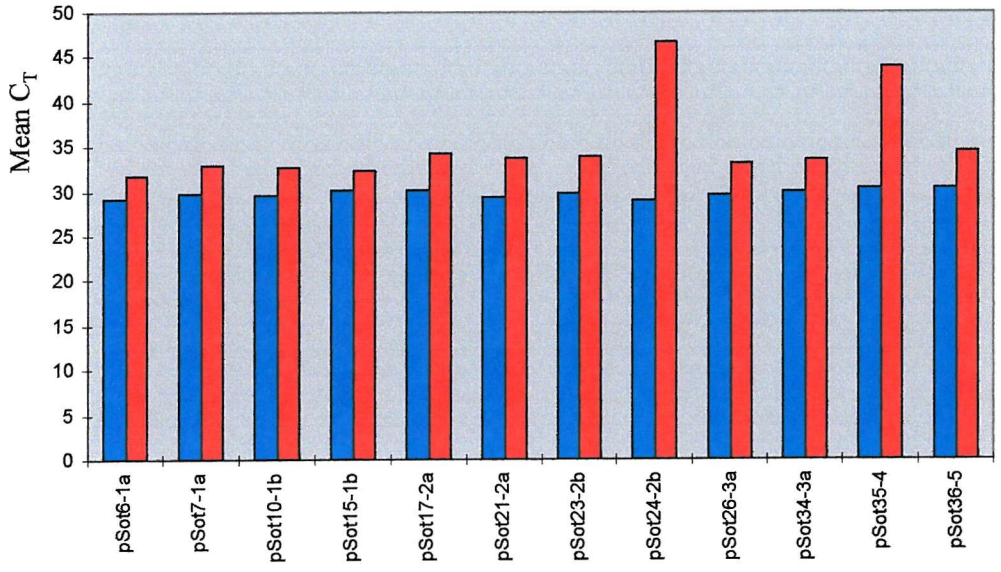


Fig.3.16 Chart showing mean C_T values from detection of the HCV 5'UTR plasmids with in-house and published HCV TaqMan PCRs.
Triplicate in-house (blue) and published (Morris *et al.*, 1996) (red) TaqMan PCRs were performed including 1000 copies of linearised, cloned Sot6-1a, Sot7-1a, Sot10-1b, Sot15-1b, Sot17-2a, Sot21-2a, Sot23-2b, Sot24-2b, Sot26-3a, Sot34-3a, Sot35-4 and Sot36-5 5'UTR sequences.

3.5.3. Determination of TaqMan PCR assay sensitivity

The sensitivity of the in-house TaqMan PCR was assessed and compared with that of the published TaqMan PCR and an in-house nested 5'UTR PCR using cloned 5'UTR sequences (Table 3.3). Half-log dilution series of linearised pSot7-1a and pSot26-3a were made and tested with the in-house TaqMan, published TaqMan and in-house nested PCRs. The results indicated a detection limit of approximately ten template copies for the in-house TaqMan assay (6/8 reactions positive), the nested PCR (7/8 reactions positive) and the published TaqMan PCR (8/8 reactions positive).

Table 3.3 Comparison of sensitivities of in-house and published TaqMan PCRs and in-house nested PCR for the detection of cloned 5'UTR sequences.

PCR method	DNA	100 copies	30 copies	10 copies	3 copies	1 copy
In-house TaqMan	pSot7-1a	4/4	4/4	3/4	1/4	0/4
	pSot26-3a	4/4	4/4	3/4	3/4	1/4
Morris' TaqMan	pSot7-1a	4/4	4/4	4/4	1/4	1/4
	pSot26-3a	4/4	4/4	4/4	2/4	2/4
In-house Nested	pSot7-1a	4/4	4/4	3/4	3/4	0/4
	pSot26-3a	4/4	4/4	4/4	1/4	0/4

Duplicate dilution series of linearised pSot7-1a and pSot26-3a were made in UHQ H_2O. Duplicate in-house and published TaqMan PCRs and in-house nested PCRs were performed for each dilution series including one to 100 template copies of each plasmid. A C_T of less than 50 was indicative of a positive TaqMan PCR. Nested PCR was performed with primers, UTR1, UTR2, UTR3 and UTR4. Products were analysed on a 4% NuSieve agarose gel.

3.6. Discussion

Closed-tube detection of HCV RT-PCR products using TaqMan probes offers several advantages over conventional methods of PCR product detection. No post-amplification manipulation of products is required, resulting in reduced contamination risk, shorter processing time, and increased throughput. Morris *et al.* (1996) developed TaqMan primers and a probe for detection of HCV, based on sequence data from HCV detected in patients from the USA, Japan and Italy (Cha *et al.*, 1991). Since the start of the project, several closed-tube TaqMan PCR-based assays for detection of HCV RNA have been developed (Petrik *et al.*, 1997; Mercier *et al.*, 1999; Martell *et al.*, 1999; Takeuchi *et al.*, 1999; Kawai *et al.*, 1999). However, none have been designed to detect HCV genotypes found in the UK. Hence, there

remains a need for a closed-tube TaqMan PCR-based assay for detection of HCV genotypes commonly identified in the UK.

The 5'UTR is one of the most conserved regions of the HCV genome (Bukh *et al.*, 1992), with greater than 90% identity existing between different isolates, compared to as little as 66% identity between complete genomes (Simmonds, 1995). To design primers and probes for maximum efficiency of amplification and detection of UK HCV sequences, the 5'UTR of 36 local strains, representing the most commonly identified genotypes in the Wessex region, were determined. The distribution of genotypes identified in Wessex correlated with that reported across the UK (Harris *et al.*, 1999). Bio-X-act polymerase was used to amplify the sequences from cDNA because of it's proof-reading capability and high fidelity (greater than seven-fold higher than *Taq*) (Bioline product data). The poor sequence data derived when using primer HCV209 was probably due, either to secondary structures in the 5'UTR, or to mis-priming at the annealing temperature used.

The sequence data generally agreed with that of 5'UTR sequences reported on the GenBank database and showed the relative conservation of 5'UTR sequence within genotypes from different geographical regions (93.8 - 100 % identity). The Sot30-3a Wessex strain showed greatest sequence identity with the type 4 reference strain, suggesting that the Sot30-3a strain may have been mis-typed. Alternatively, the patient may have been infected with more than one genotype, with a different genotype being dominant in the genotyping sample from the sequencing sample. Another possibility is that either the genotyping PCR or the sequencing PCR was contaminated with nucleic acid from another genotype.

The sequence data was used to identify relatively conserved regions within the 5'UTR, to which primers and probes were designed. Three different methods were used to design the prospective primers and probes, the Primer Express and Gene Runner computer programs, and a manual method. Each identified a different primer set, but the reverse primers designed using Gene Runner and manually were identical (TMR2). When used in a complete TaqMan PCR there was no significant difference in efficiency between forward primers designed using the three methods (Fig.3.11).

Due to the limited availability of totally conserved sequence, several of the primers had to be designed to bind sites with a small degree of sequence variation. This is not the ideal situation, as there should be no mismatch between the primer and template for efficient annealing, especially in the in the 3′ five to six bases of the primer (Dieffenbach and Dreksler, 1993). Different strategies can been used for overcoming template sequence variation (Sambrook *et al.*, 1989): Primers can be designed with degenerate bases to allow primer-binding to all possible template sequence variations; Primers can be designed to account for

particular sequence variations; Or a "neutral" base such as inosine, which base pairs with A, C, G and T with approximately equal efficiency (Lin and Brown, 1992), can be included to bind at the variable position. The second method was employed to design primers to anneal to regions of variable nucleotide sequence because the limited degeneracy identified could be accounted for by a small number of primers.

The efficiency of the designed primers was assessed and compared with those designed by Morris *et al.* (1996) using clinical cDNA samples. Patient cDNA was used as template to simulate the conditions of a detection run as closely as possible. Also, the absolute starting template copy number did not need to be known, as the relative amplification efficiencies of each primer-probe set was assessed. All primer combinations gave good amplification of the clinical cDNA samples, as assessed by agarose gel electrophoresis. The Morris primers and the in-house primer pair, TMF3/TMR2 produced the lowest levels of primer-dimer structures. These primers might, therefore, be expected to give the most efficient reactions in a full TaqMan PCR, but this needed to be tested.

Different probe preparations and variations in probe flexibility and nucleotide composition may produce different levels of background reporter dye fluorescence. The purity of the probe preparation can also affect background fluorescence level due to the presence of unbound reporter or quencher dye molecules. The background fluorescence level can affect the differentiation of amplification-specific fluorescence and hence detection efficiency. To fully assess the efficiency of the designed primers and probes, reactions were carried out with all combinations of primers and probes. C_T was used as a measure of reaction efficiency as it was assumed that the more efficient a reaction, the earlier its amplification product will be detected. The primer-probe set, TMF1/TMR2/TMP1 was identified as the most efficient. This confirmed that full TaqMan PCRs needed to be performed to choose the most efficient primer pair.

Amplifications including TMP1 gave consistently lower C_T values than those including TMP1RC. This indicated more efficient probe annealing, cleavage or fluorescence detection. This agrees with the rules for TaqMan probe design described by Livak *et al.* (1995) because TMP1 contained more C than G residues. The distance between the probe and the primer on the same strand was shorter for TMP1RC (23 - 64nt) than for TMP1 (89 - 120nt). This might be expected to result in greater cleavage efficiency for TMP1RC, but this did not produce lower C_Ts. Therefore, the distance from probe to the primer on the same strand had less of an effect on probe efficiency than probe G:C ratio.

Optimisation of the primer, probe and $MgCl_2$ concentrations in the TaqMan PCR was performed by testing concentrations over ranges recommended by the TaqMan core reagent

kit manufacturers (Perkin Elmer, 1996). Primers should be at a high enough concentration to be in excess for a PCR, but should not be at too high a concentration as this can lead to formation of non-specific amplification products (Cha and Thilly, 1993). The optimum concentration of probe to use in a TaqMan PCR is affected by the amount of background fluorescence and the concentration of primers (Perkin Elmer, 1996). Therefore, the concentration of probe was optimised after that of the primer.

Mg^{2+} ions are essential for *Taq* polymerase activity (Chien *et al.*, 1976). Too low a concentration can result in low amplification efficiency, whereas too high a concentration can result in amplification of non-target sequences (Williams, 1989). The concentration of Mg^{2+} should therefore be optimised for a particular target-primer combination for maximum PCR efficiency and specificity. The optimum Mg^{2+} concentration (4μM) for the PCR was within the range suggested by the TaqMan kit manufacturers (Perkin Elmer, 1996).

Mg^{2+} concentration had a much greater effect on PCR efficiency than primer or probe concentration. Variation of primer and probe concentrations resulted in C_T only deviating by approximately two and three C_Ts, respectively. Titration of Mg^{2+} concentration, however, resulted in C_T varying by approximately 22 C_Ts. This suggests that optimisation of Mg^{2+} concentration is considerably more important.

To assess the effect of genotype on the optimised TaqMan PCR, 5'UTR sequences from representatives of the genotypes of the Wessex HCV panel were cloned and validated by sequence analysis. Amplification of the cloned cDNAs was consistent across the range of genotypes. The inclusion of degenerate positions in the forward primer thus did not significantly affect the efficiency of the PCR and allowed efficient detection of all genotypes tested. The mismatches between TMF1 and genotype 4 and 5 sequences at nt-236 did not significantly affect amplification efficiency. The published assay (Morris *et al.*, 1996) gave higher mean C_T values for all genotypes tested. This was probably due to mismatches between this primer-probe set and several target sequences identified from the sequence analysis data. The forward primer, 5PUT1-s contained a single mismatched base for 8/9 of the genotype 3a sequences at nt-72, and for the genotype 4 sequence at nt-277. The probe, 5PUT2FAM contained a single mismatch at nt-235 for 1/9 1a sequences (pSot7-1a), 6/6 of the genotype 2a sequences, and 2/3 of the genotype 2b sequences (pSot23-2b, pSot24-2b). The probe also mismatched pSot30-3a at nt-235 and nt-238, and mismatched pSot35-4 and pSot36-5 at nt-235 and nt-236. The reverse primer, 5PUTc-1a contained a mismatch at nt-80 for all of the genotype 2a and 2b Wessex sequences, and a second mismatch at nt-86 for pSot24-2b, and at nt-81 for pSot34-3a.

The published TaqMan PCR (Morris *et al.*, 1996) amplified pSot24-2b and pSot35-4 with much reduced efficiency compared with the other plasmids (Fig.3.16). This can probably be attributed to several mismatches; one nt in the centre of the forward primer and two in the centre of the probe for pSot35-4; and one nt in the centre of the probe and two in the centre of reverse primer for pSot24-2b. It has been reported that mismatches in the centre of the probe have the greatest effect on probe-template hybrid stability (Livak *et al.*, 1995). However, certain mismatches seem to be tolerated, such as that between 5PUT2FAM and genotype 2a sequences. Also, the mismatched pair of nucleotides between the genotype 4 sequence and the probe did not result in a significant loss in PCR efficiency. The effect of probe mismatches may be cumulative with the effect of primer mismatches, such as in the case of pSot24-2b and pSot35-4. The difference in detection efficiencies between the in-house and published assays illustrate the effect that mismatches can have on amplification and detection efficiency.

The HCV 5'UTR is predicted to form extensive secondary structures, thought to be involved in viral replication (Smith *et al.*, 1995). It is possible that secondary structure may have an effect on PCR efficiency by inhibiting primer-binding and polymerase processivity. TaqMan PCR primers and probes investigated in this study all annealed at positions of predicted stem-loop structures (Fig.3.8). All of the TaqMan PCR forward primer sites and the 5PUT2FAM site were situated in the region: nt-226 to nt-291. The region is predicted to form three minor stem-loop structures. The reverse primers and the probes, TMP1 and TMP1RC annealed within the region: nt-34 to nt-202, predicted to include a major stem-loop structure (nt-58 to nt-208). Binding of all of the TaqMan PCR primers and probes studied may, therefore, have been affected by secondary structure. As most of the 5'UTR is predicted to form secondary structures, primers and probes could not be designed to avoid these regions. The HCV 3'UTR is also predicted to form extensive secondary structures (Kolykhalov *et al.*, 1996). Therefore, this region was rejected as a possible alternative target for TaqMan primers and probes. Secondary structures within the cDNA might be expected to be broken down during the denaturing step of PCR. The primer annealing temperature (60°C) would be expected to be high enough to prevent major secondary structures from reforming. Secondary structures may have had more of an effect during the RT reaction which was incubated at a lower temperature (42°C). However, as the stem-loop structures have been predicted to be conserved across genotypes (Smith *et al.*, 1995), all RT-PCRs are probably equally affected.

The sensitivity of the in-house TaqMan PCR was compared with that of the published TaqMan HCV PCR and an in-house HCV nested PCR for the detection of cloned HCV 5'UTR template. The limit of detection for all of the PCRs was determined to be approximately ten input template copies. Template was not detected in all of the PCRs at this

dilution, probably due to sampling variation at the low copy number. The number of template copies in a given sample corresponds to a Poisson distribution about a mean equal to the expected copy number in that sample (Simmonds *et al.*, 1990a). This, coupled with the fact that ten input copies is near the actual limit of detection for PCR, would result in some negative reactions.

The TaqMan PCR developed has been shown to be sensitive and to detect the most prevalent UK genotypes with equal efficiency. Thorough optimisation of the TaqMan PCR was performed to maximise sensitivity and broad reactivity of the quantitative assay that it will form the basis of.

3.7. Summary

1. An efficient TaqMan primer-probe set (TMF1/TMR2/TMP1) for detection of HCV 5'UTR cDNA was identified.
2. The concentrations of TMF1, TMR2, TMP1 and Mg^{2+} in the TaqMan PCR were optimised (0.4μM, 0.2μM, 0.175μM and 4μM, respectively).
3. The designed primer-probe set was shown to be more efficient at detecting a panel of cloned HCV 5'UTR sequences and relatively genotype-independent compared to a published assay from the USA.
4. The sensitivity of the PCR was shown to be ten input copies of HCV 5'UTR DNA which was comparable to that of a published TaqMan PCR and an in-house nested PCR.

4. Preparation of HCV standard and internal control RNAs

4.1. Introduction

4.1.1. RNA controls for monitoring RT-PCR efficiency

PCR efficiency can be affected by substances present in clinical samples or introduced during sample preparation, such as phenol, ethanol, isopropanol, SDS, sodium citrate, haemoglobin, heparin and potassium-EDTA (Löffert *et al.*, 1997). To distinguish between genuinely negative PCRs and false negatives caused by the presence of inhibitors, PCR efficiency can be monitored by co-amplification of an internal control (IC). DNA sequences have been used as ICs, but for RNA detection and quantitation by RT-PCR, the efficiency of the RT step also has to be considered. The efficiency of RT has been reported as being between 5 and 90% (Noonan *et al.*, 1990; Henrard *et al.*, 1992; Simmonds *et al.*, 1990b).

Exogenous and endogenous ICs have been utilised. An endogenous control is a cellular nucleic acid sequence, known to be present in the sample at relatively invariant levels, such as the glyceraldehyde-3-phosphate dehydrogenase, cyclophilin, β-actin, human leukocyte antigen, or β_2-microglobin messenger RNAs (Zhong and Simons, 1999; Noonan *et al.*, 1990; Kim *et al.*, 1996). The use of an endogenous RNA control has the advantage of monitoring the integrity of the nucleic acid target, allowing improperly collected, stored or processed specimens to be identified. However, amplification of the endogenous control may not accurately represent that of the target due to differences in the primer sequences, amplicon size and relative amounts of the two targets (Rosenstraus *et al.*, 1998). The use of a cellular endogenous control is generally not applicable to detection of nucleic acids in cell free samples such as serum.

An exogenous control is a nucleic acid template that is added to a sample prior to RNA extraction. A known, constant number of IC molecules can be added, allowing more precise monitoring of relative detection efficiencies. However, degradation of nucleic acid during sample collection or storage can not be controlled for using exogenous controls.

Exogenous controls may be competitive or non-competitive, depending on whether the IC molecule has binding sites for the primers used for PCR amplification of the test template and significant internal sequence similarity. A competitive control is a synthetic template with primer sites identical to those of the test target. These usually consist of cloned target template engineered to contain a marker such as a deletion or insertion, restriction site, or probe-binding site to allow distinction between the IC and target PCR products (Wang *et al.*, 1989;

Hahn *et al.*, 1995; Rosenstraus *et al.*, 1998). Competitive controls have the advantage of having similar primer-binding efficiencies as the test template. However, competition between primers, and annealing between IC products and target products may compromise amplification of the primary target, especially if the target is present at a low copy numbers or the IC is added at a high copy number (Cleland *et al.*, 1999).

There are two main types of non-competitive IC. The first type consist of a nucleic acid sequence which does not contain binding sites for the test PCR primers. This has the advantage of not compromising detection of the target sequence through competition for primers, but may be less representative due to differences in amplification efficiency. Cleland *et al.* (1999) employed a non-competitive IC for detection of HCV, consisting of cultured bovine viral diarrhoeal virus. This virus has similar physicochemical properties as the target virus, but no sequence similarity. This has the advantage of controlling for the extraction off viral RNA from clinical samples.

The second form of non-competitive control includes the same primer-binding sites as the target template, but has a different internal sequence. This reduces the risk of annealing between IC amplification products and target amplification products (Young, 1996; Miyachi *et al.*, 1998). Although termed non-competitive, there is competition with the test template for primer.

4.1.2. RNA standards for quantitative RT-PCR

Molecular standards are used for quantitation of nucleic acids by RT-PCR. The standard may be a DNA or RNA template, but for RNA quantitation, an RNA standard is more representative of target amplification as RT is accounted for. Several strategies that have been employed which either use the level of an IC RNA to quantify target template (and hence can also be used to monitor assay efficiency as described above), or an external standard curve which is used solely for quantitation.

(i) Endogenous ICs

RT-PCR of the control and target are performed simultaneously and the product level of the endogenous control is used to normalise the product level of the target RT-PCR. This strategy is not fully quantitative as it only gives the number of target copies relative to the level of IC. This is because the number of control molecules in a reaction is unknown and the amplification efficiency of the IC and target may differ (Ferre, 1992).

(ii) Exogenous ICs

There are two possible formats for competitive RT-PCR using exogenous ICs. Either the analyte is diluted, and a range of template amounts co-reverse transcribed and amplified with a constant amount of IC (Roth *et al.*, 1996), or *vice versa* (Manzin *et al.*, 1994). The number of initial target templates can then be calculated from the initial number of IC molecules in the reaction which gives equal amounts of IC and target products.

Alternatively, an external standard curve can be produced by amplifying serial dilutions of cloned standard template with constant copy numbers of the IC. The ratio of target product to IC product in sample PCRs can then be compared with the standard curve product ratios (Ravaggi *et al.*, 1995).

Quantitation by competitive RT-PCR with an IC has the advantage of simultaneously monitoring RT and PCR efficiencies and that the amplification efficiency of the IC closely resembles that of the target. However, competition may affect target amplification efficiency.

(iii) External standard curves

The use of external standard curves involves RT and PCR of serial dilutions of template in a separate series of reactions from the sample PCRs. The standard may consist of purified target RNA, or RNA *in vitro* transcribed from cloned DNA template. The level of PCR product resulting from amplification of the diluted external standard is plotted against input copy number to create a standard curve. *In vitro* transcribed cloned HCV 5'UTR sequence has been used as an external standard for quantitation of HCV (Haberhausen *et al.*, 1998). An external quantitation standard has the advantage of having the same sequence as the target molecule, but not affecting the amplification efficiency of the target. It has the disadvantage of not controlling for RT and PCR efficiency in individual tests.

4.1.3. Standard and control RNAs for the HCV TaqMan PCR assay

For this project two types of RNA were developed:

(a) An IC for monitoring extraction, RT and PCR efficiency of HCV quantitation reactions.

(b) HCV RNA standards for production of external quantitation standard curves.

This involved three important steps: *in vitro* transcription of cloned template DNA, purification of RNA transcripts, and quantitation of the RNA transcripts.

(i) In vitro transcription

In vitro transcription involves the use of bacteriophage RNA polymerases to transcribe RNA from template DNA cloned downstream of the polymerase promoter sequence. The T7, T3

and SP6 polymerases have been used to produce RNA for a range of applications (Gurevich, 1996). These have included synthesis of full length HCV RNA (Kolykhalov *et al.*, 1997). This technique has also been widely used to produce control RNAs for use in RT-PCR assays for detection of viruses such as HIV (Mulder *et al.*, 1994) and HCV (Collins *et al.*, 1995; Miyachi *et al.*, 1998; Rosenstraus *et al.*, 1998; Haberhausen *et al.*, 1998).

(ii) Purification of RNA

Before *in vitro* transcribed RNA can be quantified and used in RT-PCRs, proteins, buffers, salts and unincorporated nucleotides must be removed. The two most commonly employed strategies for RNA purification are those based on organic extraction, and those based on binding of RNA to a solid matrix. Examples of both strategies were used in this project to purify IC and HCV standard RNAs.

The most commonly used organic extraction methods involve phenol/chloroform mixtures (Sambrook *et al.*, 1989). Phenol is a potent protein denaturant, and most proteins are more soluble in phenol than in the aqueous phase. Conversely, nucleic acids are more soluble in the aqueous phase. Below pH 7.0, DNA is denatured and partitions into the organic phase. Chloroform is included for its ability to denature proteins and to enhance phase separation. Isoamyl alcohol is often added to prevent foaming. RNA is precipitated from the aqueous phase by addition of isopropanol. Precipitation with isopropanol, rather than ethanol, reduces the amount of free nucleotides precipitated.

Purification methods based on binding to a solid phase usually use silica-gel-based membranes. The RNeasy mini kit (Qiagen) protocol involves addition of a GITC-containing buffer which denatures proteins and provides a high salt environment. This promotes binding of RNA to a spin-column-mounted silica membrane after addition of ethanol. The solution is passed through the membrane by centrifugation and RNA species >200nt in length bind to the matrix. After washing with high salt buffers, the RNA is eluted in nfH_2O. This method has the advantage over precipitation methods of removing truncated transcripts (<200nt long) and free nucleotides.

(iii) Quantitation of RNA transcripts

RNA to be used to generate standard curves must be precisely and accurately quantified. The quantitation method also has to be reproducible, as RNA standards must be remade periodically. Various methods are available including A_{260} measurement (Sambrook *et al.*, 1989), hyperchromicity analysis (Warsaw and Tinocco, 1966), multiple wavelength hyperchromicity analysis (Henderson *et al.*, 1992), phosphate determination (Webb, 1992),

and isotopic tracer methods (Kinloch *et al.*, 1993). Hybridisation methods such as ribonuclease protection assays have also been used (Xia *et al.*, 1992) but these can not be used to quantify RNA standards because these assays require RNA standards themselves. Radioactively labelled nucleotide incorporation, followed by measurement using a scintillation counter has been used for quantitation of HCV RNA standards (Martell *et al.*, 1999). Quantitation of RNA standards should not be based on a single method, as each method has a different set of interfering impurities (Collins *et al.*, 1995). For this project, quantitation of HCV standards and IC RNA was carried out by two separate methods, measurement of A_{260} and RiboGreen (Molecular Probes) fluorimetry.

4.2. Selection of IC template

The IC target sequence was chosen according to the following criteria:

1. The sequence should show no significant sequence similarity to known human or pathogen sequences.
2. There should be no potential binding sites for the HCV TaqMan primers and probe contained within the sequence.
3. The sequence should not be one that is commonly handled in diagnostic virology laboratories. This excluded the use of plasmid vector sequences.
4. Commercial availability of target DNA.

A 350bp section (nt1801 - 2150) of the *Drosophila melanogaster tailless* (*tll*) gene (Pignoni *et al.*, 1990) was chosen as the target sequence. The *tll* sequence was scanned for similarities with known human and pathogen sequences on the GenBank database. No similarities were found. The IC RNA was designed to include the HCV TMR2 primer-binding site at it's 3' end to allow the option of co-priming RT of the IC and HCV RNA with the HCV TaqMan PCR reverse primer.

4.3. Selection of HCV standard templates

The HCV 5'UTR plasmids, pSot6-1a, pSot7-1a, pSot26-3a and pSot34-3a were chosen to produce standard RNA templates for use in the quantitative assay as they were representative

of the most common UK genotypes (Harris *et al.*, 1999). They were also selected to represent the most common sequence variations identified within their respective genotypes.

4.4. Production of the IC plasmid

The Primer Express program was used to design a primer-probe set for the IC TaqMan PCR. The *tll* segment sequence was input with the HCV TMR2 site sequence added downstream. The TMR2 sequence was fixed as the reverse primer site and a forward primer and TaqMan probe were designed. The identified forward primer (2DTMF1) and probe (2DTMP1) were scanned against the GenBank database (release 109.0). They showed no similarity with known human or pathogen sequences.

The IC plasmid, pSotIC was created by firstly cloning the 350nt region of *tll* using the strategy described in Fig.4.1. The *tll* sequence was amplified with Bio-X-act polymerase and ligated with a TMR2 site cassette. The cassette was created by incubation of an equimolar mixture of TMR2 and the 5' phosphorylated oligonucleotide HF at 90°C for 5min, followed by annealing by cooling to room temperature. The ligation product was then amplified by PCR with Bio-X-act polymerase using primers, HR and 2DF. The products were resolved on an agarose gel and DNA from the 394nt band was re-amplified. The products from this second PCR were analysed on an agarose gel and the 394nt band purified. The product was cut with *Eco*RI and *Pst*I, and ligated with similarly cut pGEM3Z. Competent *E. coli* Top10 cells were transformed and resultant white clones screened for recombinant plasmids by PCR using primer-pairs 2DF/HR, pGEM3Z-T7/pGEM3Z-SP6, and pGEM3Z-T7/HR. The clones were sequence verified (Fig.4.2) using the primers pGEM3Z-T7 and pGEM3Z-SP6. These produced read lengths of only 256nt and 227nt, respectively. Therefore, the primers pGEM3Z-T72, 2DR2A and 2DR2B were used to complete the double-stranded sequence data.

4.5. *In vitro* transcription of IC and HCV standard RNAs

The IC and HCV standard RNAs were produced by *in vitro* transcription with T7 RNA polymerase using the T7 promoter in pGEM-3Z. To allow run-off transcription, the HCV standard and IC plasmids were first linearised with *Hind*III.

Prior to full-scale *in vitro* transcription of the IC and HCV plasmids, a time-course experiment was performed to determine the optimum incubation time. Small-scale (20µl) *in*

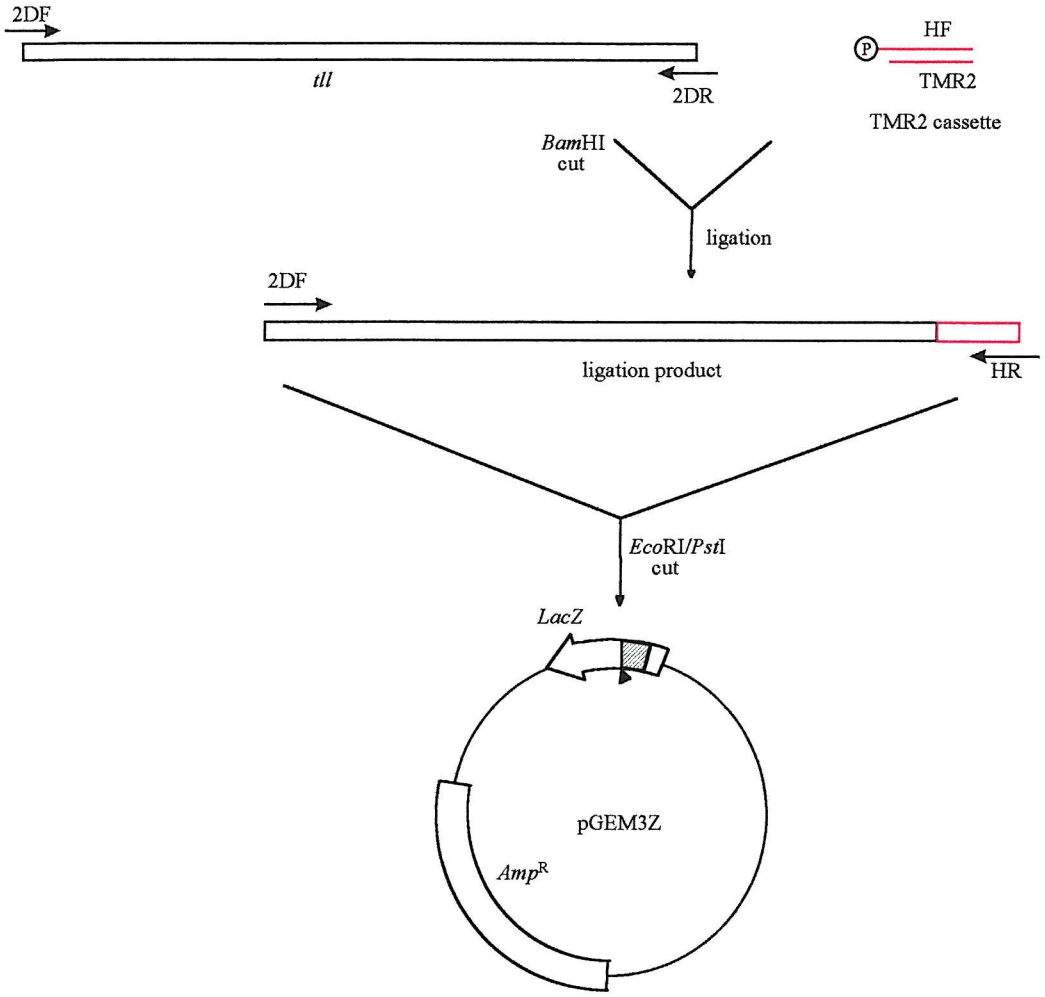


Fig.4.1 Diagrammatic representation of the construction of pSotIC.
The 350nt region (nt 1801 - 2150) of the *D. melanogaster tll* gene was amplified using the primers 2DF and 2DR (tailed with cutting sites for *Eco*RI and *Bam*HI, respectively). The product was then cut with *Bam*HI and ligated with the TMR2 cassette using a 3:1 molar ratio. The ligation product was then amplified using primers HR and 2DF and cloned into the MCS (▨) of pGEM3Z in the correct orientation for transcription from the T7 polymerase promoter (▶). Ⓟ = Phosphorylation site.

Fig.4.2 Positions of oligonucleotides used for sequencing of pSot IC and TaqMan PCR of rSotIC cDNA.

The cloned *D. melanogaster tll* region (nt1801 - 2150) is shown with flanking pGEM-3Z vector sequences (underlined). TaqMan PCR primers and probe binding sites are represented by blue arrows or bar, respectively. Sequencing primer binding sites are represented by black arrows.

vitro transcription reactions of the linearised pIC and the control plasmid pTRI-xef (Ambion) were performed. Samples were taken after 2, 4, 6 and 21hr (Fig.4.3).

The maximum yield of RNA was achieved after 21hr incubation. The yields calculated from RiboGreen values were 15.0 - 37.7% (mean = 24.3%) lower than those from A_{260} values. This may have been due to incomplete removal of nucleotides from the RNA during the purification process, resulting in inaccurate A_{260} readings. It was therefore decided to remove free nucleotides, short transcripts (<200nt), reaction buffers and enzymes from products of further *in vitro* transcription reactions using the RNeasy method (Qiagen). The A_{260} readings may also have been affected by the pH of the samples after dilution of the RNA in RNA storage solution (pH6.4). Therefore, purified RNA from subsequent reactions was eluted in nfH_2O.

The products were also analysed by formaldehyde-agarose gel electrophoresis (Fig.4.4). The RNA was estimated to be only 350nt, 24% shorter than the expected length (459nt). The transcripts from overnight incubation included an extra RNA species, approximately 700nt in length (not visible in Fig.4.4). Therefore, 6hr was used as the optimum incubation time for maximum RNA synthesis without non-specific product formation.

A pilot experiment (data not shown) was performed where pSotIC, pSot6-1a, pSot7-1a, pSot26-3a and pSot34-3a were *in vitro* transcribed in 10µl reactions. The products were purified using the RNeasy kit and eluted in nfH_2O. The RNAs were analysed by formaldehyde-agarose gel electrophoresis, spectroscopy, fluorimetry, and RNA from all of the *in vitro* transcription reactions was detected. The RNA was also analysed by RT followed by PCR using the primer pairs TMF1/TMR2, HCV939E/TMR2, TMF1/5PUTc-1a, HCV939E/UTR4 and 2DTMF/TMR2. All of the RNAs were detected except rSot7-1a. Therefore, pSot7-1a was not included in the scaled-up RNA production.

Scaled-up (60µl) *in vitro* transcription reactions of linearised pSotIC, pSot6-1a, pSot26-3a, pSot34-3a and pTRI-xef plasmids were performed. The products were treated with DNaseI, purified using the RNeasy kit and eluted in nfH_2O. The products were then quantified by measurement of A_{260} and by RiboGreen fluorimetry (Table 4.1). The yields deduced by the RiboGreen method were 16.7 - 26.4% (mean = 21.7%) lower than those from the A_{260}. The RiboGreen values were used to calculate the number of copies in each sample, as quantitation by this method is not affected by residual free nucleotides that might be present after the purification process. The A_{260}/A_{280} ratios varied from 1.59 - 1.75 (mean = 1.69), indicating acceptable purity. GITC was shown not to have been carried through the purification step, as A_{260}/A_{230} ratios varied from 2.27 - 2.57 (mean = 2.44). The RNA templates were diluted in

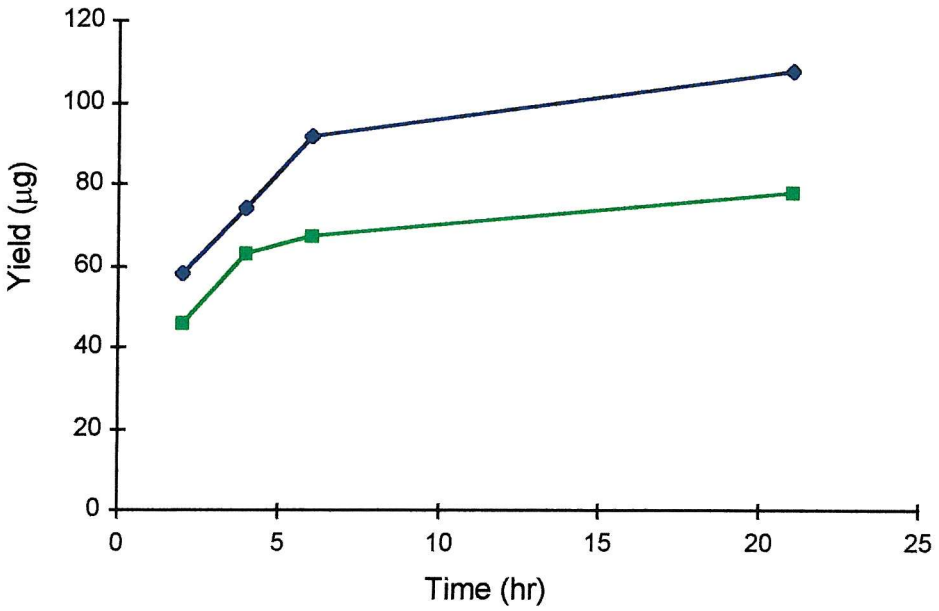


Fig.4.3 Plots of pSot6-1a *in vitro* transcription reaction yields for different incubation times.

Single A_{260} (blue) and triplicate RiboGreen (green) measurements (mean shown) were performed on *in vitro* transcription reaction samples (2µl) taken after 2, 4, 6 and 21hr incubation. The RNA products were purified using phenol/chloroform/isoamyl alcohol extraction followed by isopropanol precipitation and re-suspended in RNA storage solution (Ambion) for quantitation.

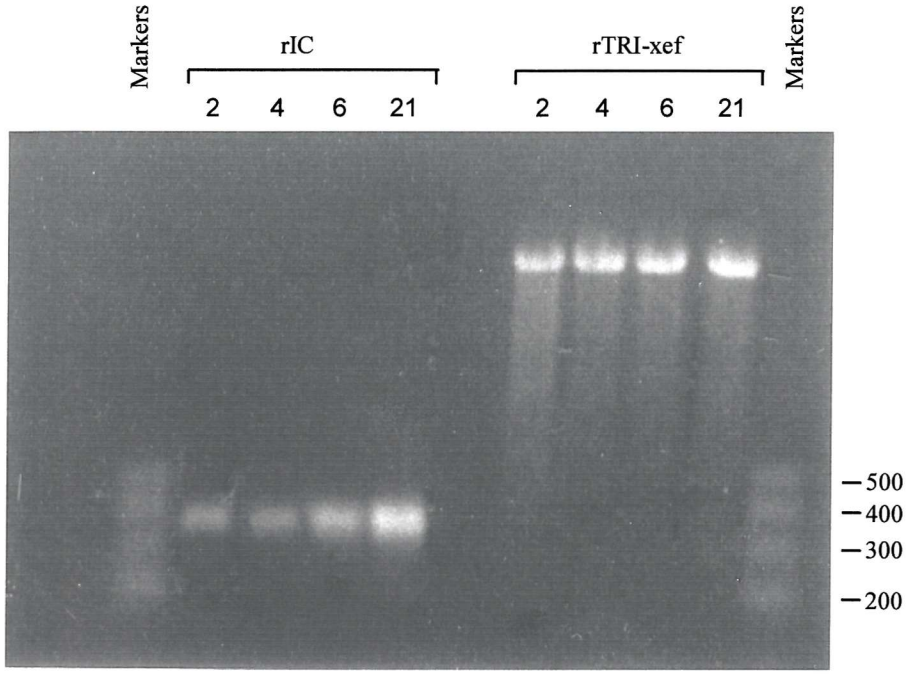


Fig.4.4 Formaldehyde-agarose gel of RNA from a time-course study of *in vitro* transcription using pSotIC and pTRI-xef.
Purified pSotIC and pTRI-xef *in vitro* transcription reaction products sampled (5µl) at 2 - 21hr (shown above each lane) were resolved on a 2% denaturing agarose gel. Century marker RNA (5µg) was also resolved and band sizes (nt) are shown.

Table 4.1 Yields of scaled-up *in vitro* transcription reactions.

Method	Yield (µg/60µl reaction)				
	pSot6-1a	pSot26-3a	pSot34-3a	pSotIC	pTRI-xef
A_{260}	372.00	388.80	381.60	417.60	357.60
RiboGreen	309.88	319.25	300.89	307.27	263.62

Mean yields derived from triplicate A_{260} measurements and triplicate RiboGreen measurements of three different sample dilutions are shown.

RNA storage solution with *S. cerevisiae* tRNA, RNasin and DTT. The solutions were stored in siliconised tubes as single-use aliquots at -70°C.

4.6. Evaluation of IC and HCV standard RNAs

The integrity and size of the transcripts were assessed by resolving samples on a formaldehyde-agarose gel (Fig.4.5). Discrete single bands were observed for each product, indicating that no significant RNA degradation had occurred, and that plasmid linearisation resulted in successful termination by polymerase run-off. However, the sizes of the HCV 5'UTR transcripts were deduced to be approximately 250nt in length, 25% shorter than the expected length (333nt). The IC RNA was estimated to be approximately 350nt in length, 24% shorter than the expected length (459nt).

The size and integrity of the transcripts were investigated further by performing random primed RT on $1x10^6$ RNA copies, followed by PCR (Fig.4.6). When resolved on an agarose gel, the PCR products were of the expected sizes, corresponding to the full-length RNAs. The RNA transcripts were therefore deduced to be of the correct length and suitable for use as TaqMan PCR controls and standard RNAs. No template DNA was detected at the input RNA copy number used. All positive controls were detected and no cross-reactivity was observed for the IC primers with HCV template or for HCV primers with IC template.

The IC and HCV standard RNAs were assessed for their use in the HCV TaqMan assay. TMR2-primed RT was performed with $1x10^6$ RNA copies. Duplicate TaqMan PCRs were then carried out using the HCV primers and probe and the IC primer-probe set, 2DTMF/TMR2/2DTMP for amplification of the HCV standards and IC cDNA, respectively (data not shown). TaqMan PCRs containing only HCV or IC RNA were also conducted to detect DNA contamination. All RNA transcripts were detected and no DNA contamination was found at this input RNA copy number.

The optimal Mg^{2+} concentration for the IC TaqMan PCR was determined by performing reactions with varying Mg^{2+} concentrations with a constant input RNA copy number (Fig.4.7). C_T was used as a measure of reaction efficiency. The lowest mean C_T, hence the optimal Mg^{2+} concentration for the RT reaction, was found to be from 2 - 4μM. The 4μM concentration was used for further experiments because of the sharp increase in C_T for Mg^{2+} concentrations below 2μM.

An experiment was performed to establish the limit of detection of the IC RNA when extracted from sera using the QIAamp kit, and to investigate whether the presence of HCV RNA affected IC amplification, or *vice verse*. Nucleic acid from HCV-negative, and HCV

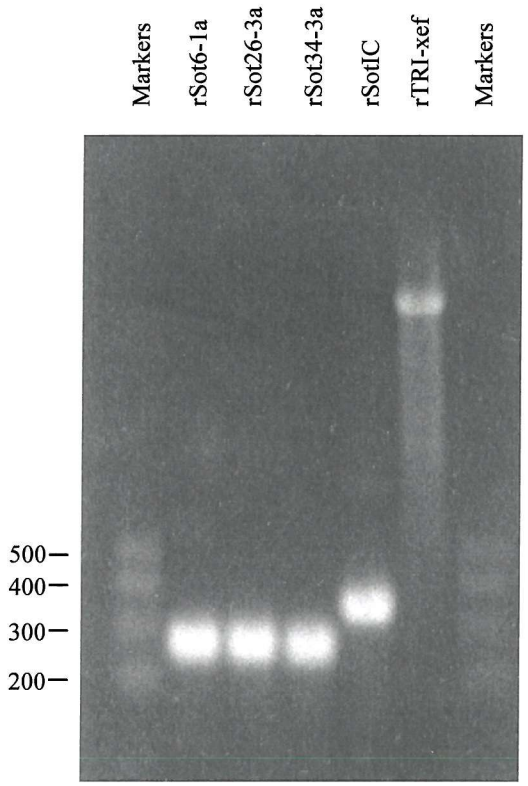
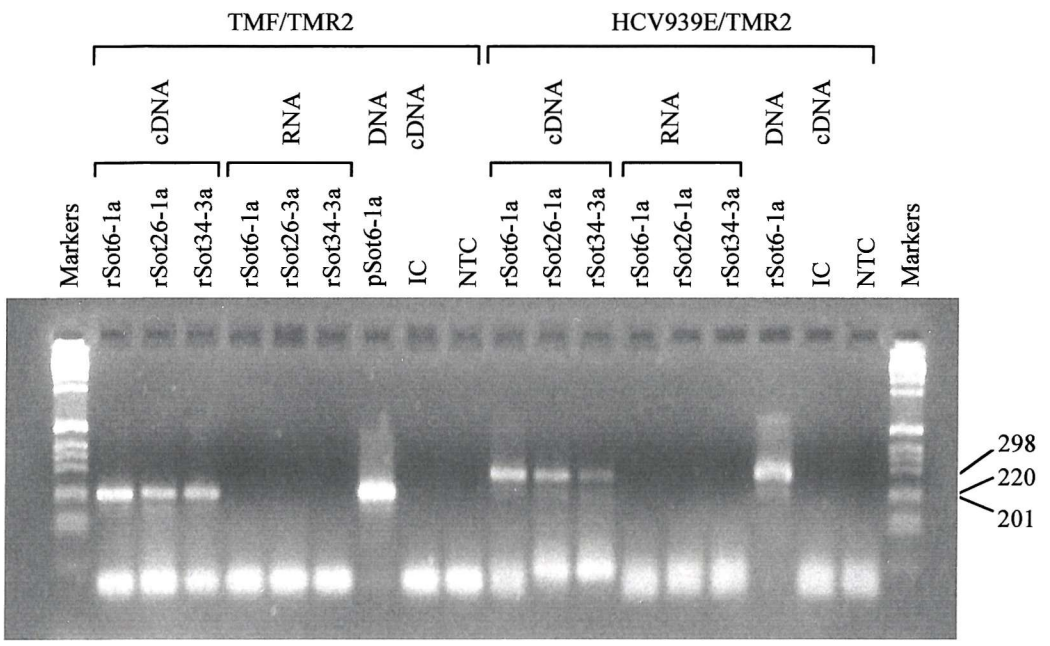


Fig.4.5 Formaldehyde-agarose gel of *in vitro* transcription products.
Undiluted samples (5µl) of purified pSotIC, pSot6-1a, pSot26-3a, pSot34-3a and pTRI-xef *in vitro* transcription reaction products were resolved on a 2% formaldehyde-agarose gel. Century marker RNA (5µg) was also resolved and band sizes (nt) are shown.

(a)


TMF/TMR2
HCV939E/TMR2
cDNA
RNA
DNA
cDNA
cDNA
RNA
DNA
cDNA
Markers
rSot6-1a
rSot26-1a
rSot34-3a
rSot6-1a
rSot26-3a
rSot34-3a
pSot6-1a
IC
NTC
rSot6-1a
rSot26-1a
rSot34-3a
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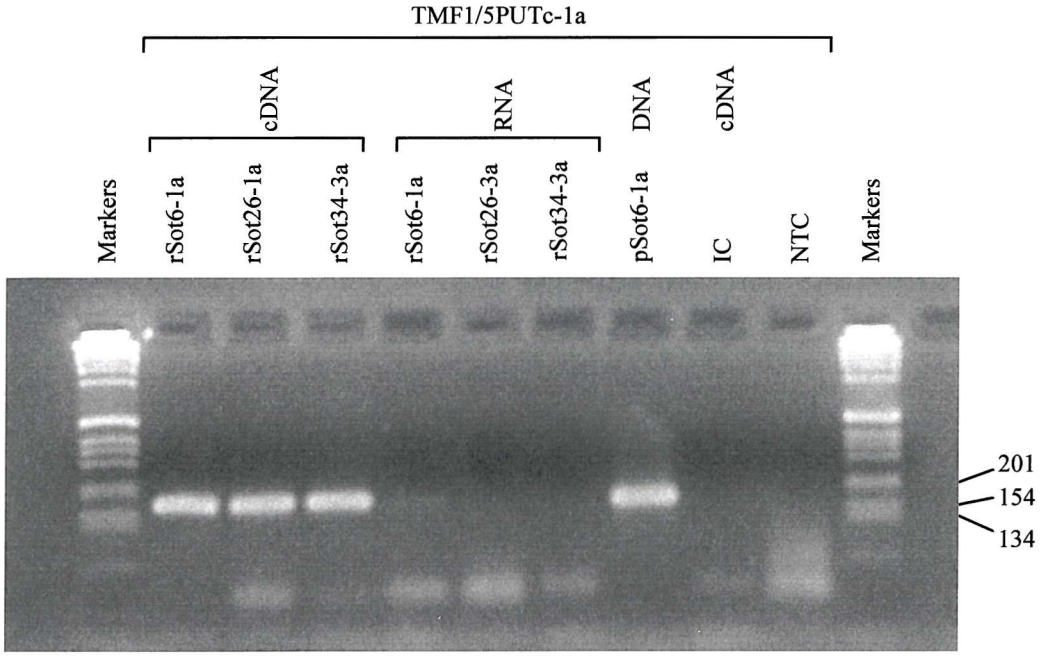
rSot26-1a
rSot34-3a
rSot6-1a
IC
NTC
Markers
298
220
201


TMF1/5PUTc-1a
cDNA
RNA
DNA
cDNA
Markers
rSot6-1a
rSot26-1a
rSot34-3a
rSot6-1a
rSot26-3a
rSot34-3a
pSot6-1a
IC
NTC
Markers
201
154
134

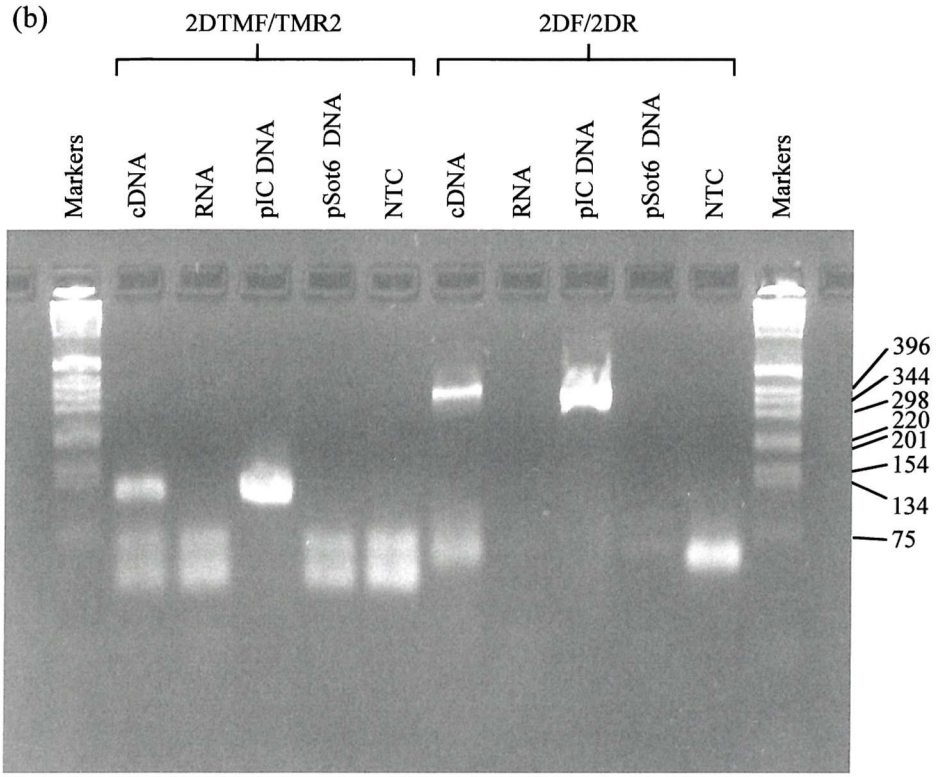


Fig.4.6 NuSieve agarose gels (4%) of products from PCR of (a) HCV standard and (b) IC cDNA and RNA.
For the HCV cDNA PCRs, the primer pairs TMF1/TMR2, HCV939E/TMR2 and TMF1/5PUTc-1a were used (expected product sizes: 215bp, 272bp and 175bp, respectively). The primer pairs 2DTMF/TMR2 and 2DF/2DR were used for the IC cDNA PCR (expected product sizes: 141bp and 396bp, respectively). To control for contamination by undegraded template DNA, PCRs were performed on RNA without RT. Plasmids pSot6-1a and pSotIC were included as positive controls and to control for primer cross-reactivity in the HCV standard and IC PCRs. 1kb ladder size marker (150ng) was resolved in the outer lanes and selected band sizes (bp) are shown. NTC = no template control.

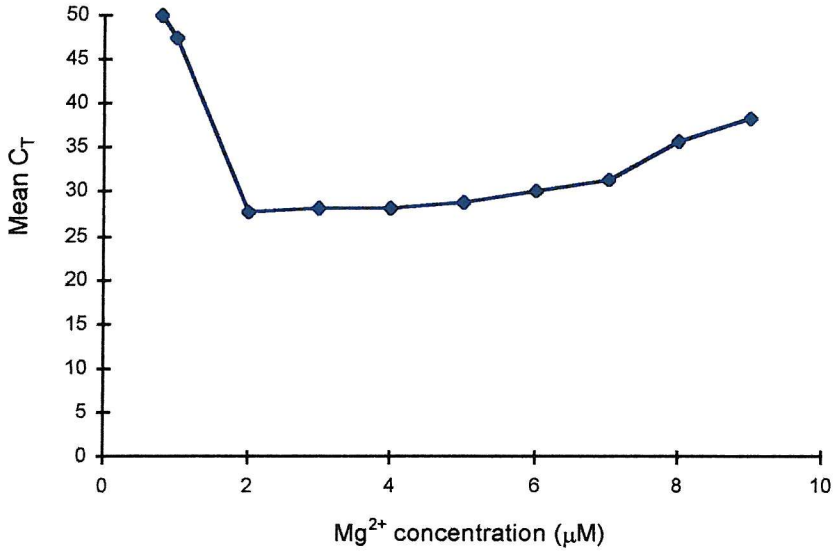


Fig.4.7 Plot of mean C_T against Mg^{2+} concentration for the IC TaqMan PCR.
Triplicate TaqMan PCRs were conducted on cDNA produced by TMR2-primed RT with $1x10^6$ IC RNA copies/RT. Mg^{2+} was included at concentrations of 0.8 - 9mM. Concentrations of other reagents were as for the β-actin control reaction.

genotype 1a and 3a-positive sera was extracted with varying copy numbers of IC added to the lysis buffer. Extraction of nucleic acid from the patient serum in the absence of IC RNA was performed as a control. Extracted RNA was reverse transcribed, and amplified by HCV and IC TaqMan PCR in separate tubes.

The mean C_T for the HCV PCRs did not vary significantly across the range of IC input copies (Fig.4.8) and no trend indicating competition was observed. There was also no significant difference between mean C_Ts for the IC-negative and IC-positive HCV PCRs (P = 0.5). Therefore, the presence of IC RNA had no significant effect on HCV detection. All HCV PCRs on cDNA from HCV-negative serum gave negative results.

IC PCRs were negative for all tests containing 0, 10^1, 10^2 and 10^3 IC copies/extraction. The limit of detection of the IC RNA was found to be at $1x10^4$ copies/extraction (4/4 PCRs positive) in the presence and absence of HCV genotype 1a and 3a RNA. This indicated that the presence and genotype of HCV RNA did not affect the detection limit of the IC RNA. Mean IC PCR C_Ts at the 10^4 IC input copy number for the HCV genotype 1a and 3a cDNAs did not differ significantly from the mean C_Ts for the HCV-negative cDNA (mean C_Ts of 37.39, 40.15 and 40.34, respectively; P = 0.2). This showed that HCV RNA had no significant effect on detection of the IC RNA.

The HCV standard RNAs were analysed for the presence of undegraded template DNA by TaqMan PCR. Varying copy numbers of RNA template were included in PCRs without prior RT (Table 4.2). The lowest input copy number was tested with the RT and PCR steps as a positive control. Template DNA was detected at input RNA copies above $1x10^6$ for rSot6-1a, above $1x10^5$ copies for rSot26-1a, and above $1x10^8$ for rSot34-3a. The rSot34-3a preparation was selected as the standard curve template because it appeared to contain the lowest level of contaminating DNA. The IC RNA was not tested again for DNA contamination as none had been detected previously by nested PCR at $1x10^6$ input copies (Fig.4.6) which is a higher input copy number than was to be used in the assay.

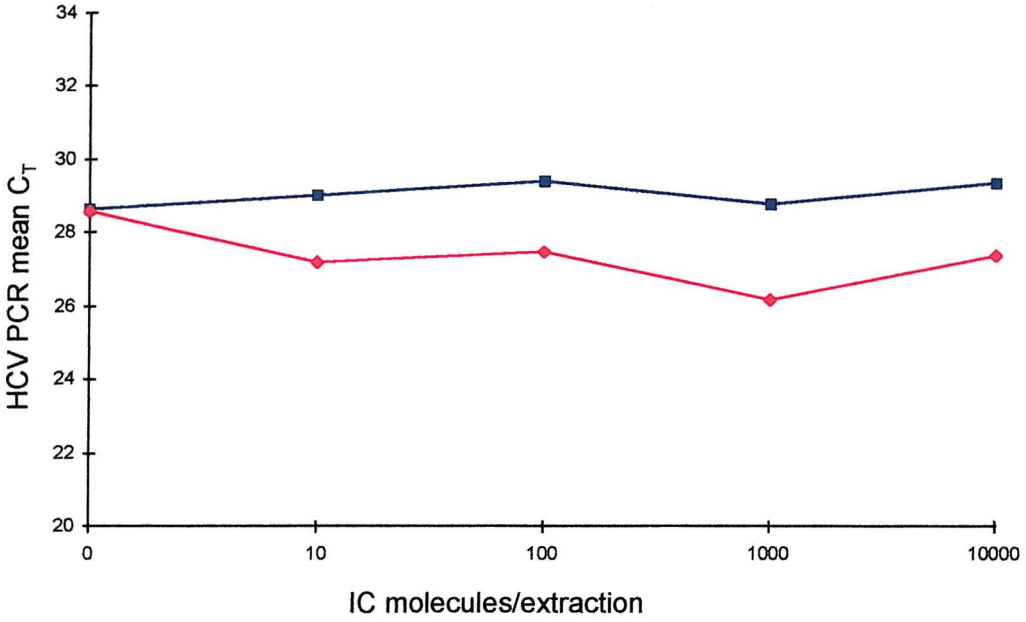


Fig.4.8 Plots of mean HCV PCR C_T for assays performed in the presence of IC RNA. RNA was extracted from duplicate HCV genotype 1a-positive (Sot37-1a, red) and genotype 3a-positive (Sot38-3a, blue) sera with 0, 10^1 - 10^4 copies of IC added to buffer AVL of the QIAamp viral RNA extraction kit. RNA was reverse transcribed after priming with TMR2, and duplicate HCV and IC TaqMan PCRs were performed.

Table 4.2 Analysis of *in vitro* transcribed HCV RNA standards for the presence of plasmid template DNA contamination.

	RNA				cDNA
Input copies	10^6	10^7	10^8	10^9	10^6
rSot6-1a	0/4	2/4	4/4	3/4	2/2
rSot26-3a	2/4	0/4	3/4	4/4	2/2
rSot34-3a	0/4	0/4	3/4	4/4	2/2

Quadruplicate HCV TaqMan PCRs were performed with 10^6 - 10^9 input copies of RNA. Duplicate PCRs were carried out on cDNA from RT of 10^6 RNA copies as a positive control. The number of positive reactions is shown as a fraction of the number of PCRs performed.

4.7. Discussion

PCRs have been shown to be inhibited by a range of substances found in clinical samples. Of relevance to HCV RNA detection from serum, the blood components, haemoglobin and heparin have been shown to be potent PCR inhibitors. Nucleic acid purification methods may not remove all such inhibitory substances from a clinical sample (Miyachi *et al.*, 1998). Co-amplification of IC molecules has been shown to be useful for detection of the inhibitory effects of these substances on PCR. Schwab *et al.* (1997) showed that an exogenous IC can be used to identify Norwalk virus RT-PCR false negatives caused by inhibitors carried over from stool samples. Miyachi *et al.* (1998) showed that an exogenous IC can be used to identify HCV RT-PCR false negatives caused by inhibitors found in blood.

A section of the *D. melanogaster tll* gene was chosen as an IC template sequence for the HCV TaqMan PCR assay. No significant risk of IC PCR false positivity due to environmental contamination was posed as the *D. melanogaster* genome is not handled routinely in the laboratory. The *tll* sequence was found to have no significant sequence similarity with known human or pathogen sequences and no binding sites for the HCV assay primers and probe. Therefore, it should have no direct effect on the HCV PCR. The TMR2 primer site was incorporated into the IC template to give the option of using TMR2 to prime RT.

In the complete quantitative assay, HCV and IC RNA will be co-extracted, and reverse transcribed in the same tube. This will allow monitoring of extraction and RT efficiency. HCV and IC TaqMan PCR will then be performed in separate tubes, eliminating competition for TMR2 that could result if PCR was carried out in the same tube. Carrying out HCV and IC TaqMan PCRs in separate tubes will negate the need to distinguish between IC and HCV PCR products. Multiplex TaqMan PCR, using IC and HCV probes labelled with different reporter dyes, was therefore not required.

To allow quantitation of HCV RNA by TaqMan PCR, standard HCV template RNAs were created by *in vitro* transcription of a cloned 305nt section of the HCV 5'UTR sequences from representatives of the most common UK genotypes, 1a and 3a (Harris *et al.*, 1999). The accuracy with which extraction, RT and amplification of *in vitro* transcribed standard RNA templates represents that of viral RNA is limited by several factors. The standard RNAs are not contained within viral particles, which may affect the extraction efficiency compared with natural virus. Viral RNA may remain bound to viral proteins during extraction of whole virus. The 333nt standard RNAs are much shorter than the complete viral genome (approximately 9.6kb) which may also affect extraction efficiency due to potentially different silica matrix binding properties. The 5'UTR sequence of the standard RNAs would differ from that of

many clinical HCV samples due to the high sequence variability of HCV. This may result in different primer or TaqMan probe binding efficiencies. The secondary structure of full length viral RNA would also differ from that of the standard, affecting RT and PCR efficiency. However, using whole virus for generating standard curves is not practical. Production of large quantities of viral particles is not possible with current cell culture techniques. The use of artificial template RNA allowed production of high copy numbers by *in vitro* transcription and quantitation by physical methods.

The IC and HCV standard templates were created by firstly cloning their respective sequences. Problems were encountered during sequence analysis of the IC plasmid. The short length of sequence data produced using primers pGEM3Z-T7 and pGEM-SP6 may have been due to secondary structure in the IC sequence resulting in poor polymerase processivity. However, such difficulties were not encountered when sequencing the HCV clones, even though the 5'UTR is known to have extensive secondary structure (Smith *et al.*, 1995).

In vitro transcription was performed on linearised plasmids in order that transcription of the template sequence terminated at the cut end, producing transcripts with discrete 3' termini. The IC and 5'UTR plasmids were cut so as to produce protruding 5' ends. This was done to avoid non-specific transcripts being produced as can occur from templates with protruding 3' ends (Schenborn and Mierendorf, 1985).

The length of the *in vitro* transcription incubation was optimised by performing a time-course experiment. Too long an incubation period resulted in formation of a second product of approximately 700nt in length. This may be due to accumulation of products of non-specifically primed transcription.

In vitro transcription products were analysed by formaldehyde-agarose gel electrophoresis. However, RNA did not migrate at the expected rate, compared with the marker bands. The length of the product RNA was confirmed as being of the expected length by RT-PCR. The discrepancy between the mobilities of the marker and sample RNA may have been due to transcript RNA secondary structures being incompletely disrupted.

The *in vitro* transcribed RNA was quantified by both the RiboGreen fluorimetry method and by spectroscopy. Slightly greater discrepancy between the two concentration measurements was observed for the time-scale *in vitro* transcription experiment than for the full-scale production of IC and HCV standard RNAs. This was probably due to two differences in methodology:

(i) RNA from the time-course experiment was purified by phenol/chloroform extraction followed by precipitation with isopropanol, whereas the scaled-up production transcripts

were purified by the RNeasy kit. The RNeasy kit probably resulted in more efficient removal of free nucleotides which could have affected the A_{260} measurements.

(ii) RNA from the time-course experiment was diluted in RNA storage solution prior to quantitation. The A_{260} measurement was therefore probably affected by sample pH.

The yields given by RiboGreen fluorimetry were still an average of 21.7% lower than those given by spectroscopy for the full-scale RNA production. The RiboGreen measurements were used when calculating the number of transcript RNA molecules in a sample because the RiboGreen method is not affected by free nucleotides, which may have been present in the sample having come through the purification procedure or after degradation of RNA.

The stability of the RNA transcripts is an important issue affecting the reproducibility and accuracy of the assay. Therefore, RNA was stored in RNA storage solution which is at a low pH and contains sodium citrate, an efficient Mg^{2+} chelator, to reduce potential RNA hydrolysis. Yeast tRNA was included as a carrier molecule. RNasin was included to inhibit RNase activity and DTT was included to stabilise the RNasin structure. RNA from scaled-up transcription reactions were quantified before being diluted in RNA storage solution with tRNA, RNasin and DTT to prevent the tRNA and pH from affecting the readings.

Freeze-thawing has been shown to affect HCV RNA stability (Halfon *et al.*, 1996). Therefore, the number of freeze-thaw cycles that HCV standard and IC RNAs were subjected to was kept constant and to a minimum. *In vitro* transcription, quantitation, dilution in storage solution and storage at -70°C were performed on the same day. Samples were stored in single-use aliquots to avoid multiple freeze-thaws.

Precise, consistent dilution of IC and HCV standard RNAs is important for assay reproducibility and accuracy. Siliconised tubes were used for storage and dilution of RNA to minimise adherence to tube surfaces. Yeast tRNA was included as a carrier molecule when performing serial dilutions of IC and standard RNAs to reduce RNA aggregation which might lead to sampling variation.

Assessment of IC and HCV standard RNAs by PCR revealed detectable DNA contamination when high template copy numbers were included. This supports findings by Schwab *et al.* (1997) who found that DNA contamination was not fully eliminated unless high concentrations of DNaseI were used. Detection of DNA in *in vitro* transcribed RNA samples put an upper limit on the copy number that could be included in TaqMan PCR. C_T values for detection of the HCV RNA standards at high concentrations could be biased by contaminating template DNA molecules. The concentration of standard RNA that could be used was sufficient to produce a standard curve with an upper limit of 10^7 copies/RT from which it would be possible to quantify the normal range of HCV viraemia levels, which have

an upper level of approximately 10^8 genome equivalents/ml (Lau *et al.*, 1993). Samples with virus concentrations above the standard curve range could be diluted to allow quantitation. Therefore, the RNA was not treated with higher concentrations of DNaseI.

In vitro transcription of pSot7-1a was shown to produce RNA detectable by formaldehyde-agarose gel electrophoresis, spectroscopy and fluorimetry, but undetectable by RT followed by PCR. Failure of PCR with the primer-pair, HCV939E/UTR4 suggested that PCR failure was not due to RNA product truncation. PCR failure may have been due to inhibitory RNA secondary structure, or to inefficient removal of inhibitory substances from the RNA preparation.

Extraction, RT and TaqMan PCR of the IC in the presence of HCV RNA showed that there was no significant competition between the two templates. This may be because the IC shares only the TMR2 primer-binding site with HCV. Amplification of IC cDNA, therefore, cannot occur in the HCV TaqMan PCR. There may be competition for TMR2 binding during the RT step, but this should be insignificant due to the excess concentration of primer used. The IC RNA was accepted for use in the HCV assay because of its efficient detection by RT followed by IC TaqMan PCR and the lack of detectable competition with HCV RNA.

Preparation of HCV standard RNAs will allow the development of a TaqMan PCR-based assay for quantitation of HCV RNA. A dilution series of an HCV standard RNA will be made and each dilution reverse transcribed and amplified to produce a standard curve. The successful development of an IC RNA for monitoring of extraction, RT and TaqMan PCR efficiency will allow identification of false negative results from the quantitative and qualitative HCV assays. It will also be used to identify inaccurate quantitation results caused by inefficiencies in extraction and RT or by PCR inhibitors carried over from clinical samples.

4.8. Summary

1. An IC RNA for monitoring extraction, RT and PCR efficiency for the HCV assay was designed, produced by *in vitro* transcription and quantified by measurement of A_{260} and RiboGreen fluorimetry.
2. Inclusion of the IC in clinical samples was shown not to be deleterious to the detection of HCV RNA.
3. HCV RNA standards were also *in vitro* transcribed and quantified for generation of standard curves for TaqMan PCR-based HCV quantitation.

5. Development and evaluation of a real-time quantitative assay for hepatitis C virus

5.1. Introduction

5.1.1. Hepatitis C viral load measurement

Quantitation of serum viral load is important for patient management, assessment of treatment efficacy, and for understanding the natural history of chronic viral infections such as HIV, HBV and HCV. For HCV infections, serum viral load is used as a surrogate marker for virus replication in the liver. Serum viral loads range from up to 10^8 genome equivalents/ml in untreated patients down to levels undetectable by currently available assays (<10 copies/ml by TMA) in patients successfully treated with antiviral therapy (Lau *et al.*, 1993; Weiland, 1998). The main applications of HCV viral load data have been prediction of treatment outcome, monitoring of treatment responses and study of viral kinetics during therapy (reviewed in Chapter 7). Assays for quantitation of HCV viral load have also been used to develop models of how chronic and acute hepatitis C virus infections progress (Alter, 1995).

5.1.2. Methods used for quantitation of HCV RNA

HCV quantitation is not possible using conventional methods such as plaque assays due to the lack of an efficient cell culture system and detectable cytopathic effects. Therefore, HCV load determination is performed mainly by quantitation of RNA using molecular techniques. Methods that have been used to quantify HCV RNA can be classified as either signal amplification, or template amplification techniques. Limits of quantitation of these methods are summarised in Table 5.1.

(a) Signal amplification: Quantiplex bDNA assay

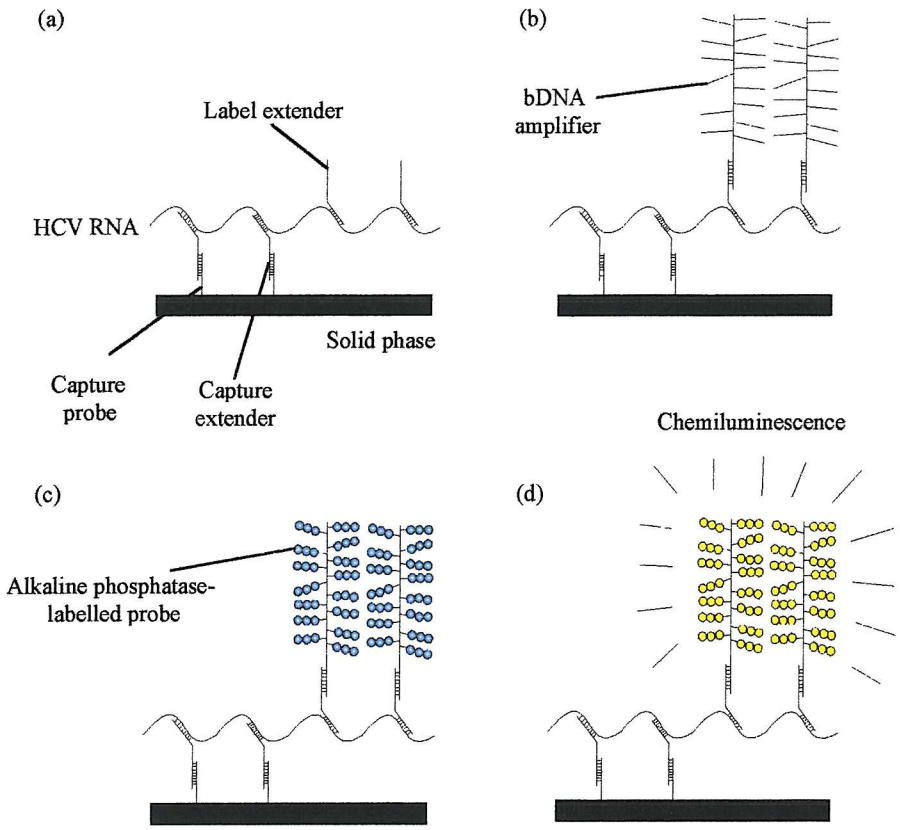
Quantitation by signal amplification involves detection of nucleic acid by probe hybridisation, without amplification of the nucleic acid (reviewed by Nolte, 1998). The Quantiplex branched DNA (bDNA) assay from Bayer has been used to quantify HCV in peripheral blood (Davis *et al.*, 1994) and liver tissue (Idrovo *et al.*, 1996). In the first generation assay (Quantiplex HCV RNA 1.0) patient serum is added to microplate wells coated with oligonucleotide capture probes designed to anneal within the HCV 5'UTR and core region (Fig.5.1A). Virus particles are lysed with proteinase K and HCV RNA is immobilised to the solid phase via hybridisation

Table 5.1 Upper and lower quantitation limits of published and commercially available assays for HCV RNA quantitation.

Assay	Lower quantitation limit	Upper quantitation limit	Reference
Amplicor Monitor 2.0 PCR	$1x10^3$ copies/ml 500 IU/ml	$5x10^5$ copies/ml $5x10^5$ IU/ml	Mellor *et al.*, 1999 Lee *et al.*, 2000
SuperQuant PCR	$1x10^2$ copies/ml	$5x10^6$ copies/ml	Blatt *et al.*, 1997
Quantiplex HCV RNA 2.0 bDNA	$2x10^5$ copies/ml $3.2x10^4$ IU/ml	$5x10^7$ copies/ml $1.9x10^7$ IU/ml	Mellor *et al.*, 1999 Germer and Zein, 2001*
Versant HCV RNA 3.0 bDNA	$2.5x10^3$ copies/ml 521 IU/ml	$4x10^7$ copies/ml $8.3x10^6$ IU/ml	Germer and Zein, 2001*
Competitive RT-PCR	$5x10^3$ copies/ml $1x10^4$ copies/ml	$5x10^6$ copies/ml $10^{9.5}$ copies/ml	Roth *et al.*, 1996 Hagiwara *et al.*, 1993
Limiting dilution RT-PCR	$4x10^3$ copies/ml	ND	Simmonds *et al.*, 1990b
NASBA	$3x10^4$ copies/ml	ND	Lunel *et al.*, 1999
Real-time PCR	$2x10^3$ copies/ml $3.6x10^4$ copies/ml $4.0x10^1$ copies/ml $1x10^3$ IU/ml $1x10^3$ copies/ml	$2x10^8$ copies/ml $3.6x10^8$ copies/ml $4x10^8$ copies/ml $1x10^7$ IU/ml $1x10^7$ copies/ml	Kawai *et al.*, 1999 Martell *et al.*, 1999 Takeuchi *et al.*, 1999 Kleiber *et al.*, 2000 Schroter *et al.*, 2001

ND = not determined. *Based on unpublished manufacturer's data.

A.



B.

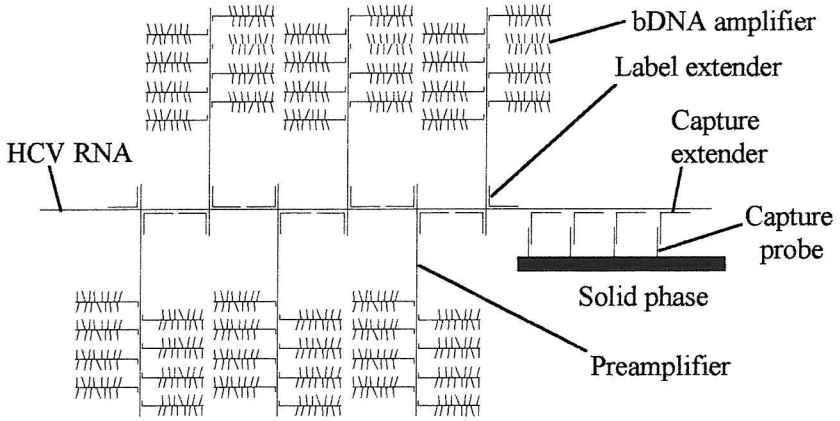


Fig.5.1 Diagrammatic representation of the bDNA assay.
A. Quantiplex HCV RNA 1.0 assay.
(a) HCV RNA immobilisation and binding of label extenders. (b) bDNA amplifier molecule binding. (c) Alkaline phosphatase-labelled probe binding.
(d) Chemiluminescence detection after addition of dioxetane substrate.
B. Probe strategy for the Quantiplex HCV RNA 2.0 assay.
For clarity, alkaline phosphatase-labelled probes have not been shown.
Adapted from Nolte (1998).

to capture extender oligonucleotides. A second set of HCV 5'UTR/core-specific oligonucleotides, known as label extenders also hybridise to the target RNA molecule. Branched DNA amplifier oligonucleotides are added and bind to complementary sequences on the label extenders. Amplifier oligonucleotides have 15 identical arms, each of which can bind three alkaline phosphatase-labelled probes. The second version of the assay (Quantiplex HCV RNA 2.0) includes a pre-amplifier molecule to increase the number of labelled probes that can bind to the target (Kern *et al.*, 1996) (Fig.5.1B). Each pre-amplifier can bind up to eight bDNA amplifier molecules. Dioxetane substrate is then added and chemiluminescence is measured using a luminometer. The concentration of HCV RNA is determined by comparing the chemiluminescence level with a standard curve produced by signal amplification of dilutions of a recombinant ssDNA standard.

Version 1.0 of the test was shown to underestimate genotype 2 virus level by a factor of three and genotype 3 virus by a factor of two (Lau *et al.*, 1995). The assay had a lower detection limit of $3.5x10^5$ copies/ml. Version 2.0 has refined oligonucleotide probes, designed to reduce discrepancy of quantitation between genotypes (Detmer *et al.*, 1996). The test has been shown to give equal measurement of genotypes 1 to 5, but underestimates type 6 by a factor of two to four. The bDNA version 2.0 assay is reported to have a dynamic range of $2x10^5$ to $5x10^7$ copies/ml (Mellor *et al.*, 1999), an intra-assay coefficient of variation (CV) of 7 - 18% and an inter-assay CV of 5 - 13% (Bayer product information, 1998). The failure of the assay to detect low levels of viraemia may limit its use for monitoring patient response to therapy. A third version of the HCV bDNA assay (Versant HCV RNA 3.0) has recently been released by Bayer which is claimed to have improved sensitivity (Table 5.1).

(b) Template amplification methods

Template amplification methods involve enzymatic nucleic acid amplification prior to detection.

(i) Limiting dilution polymerase chain reaction (PCR)

The first PCR-based assay for HCV quantitation used a limiting dilution method (Simmonds *et al.*, 1990b). This involved reverse transcription, limiting serial dilution of cDNA, followed by PCR with two nested pairs of primers. PCR products were then detected by agarose gel electrophoresis. Addition of a specific volume of cDNA to replicate PCRs gave a Poisson distribution of positive and negative results. This allowed the concentration of HCV cDNA to

be determined using the equation:

Mean number of template molecules per reaction = -ln[F]

(where F is the fraction of negative reactions).

The method required good initial recovery of target nucleic acids from the specimen, large numbers of replicate PCRs being performed and a PCR capable of detecting a single copy of cDNA. The initial number of RNA copies was then calculated using an estimated RT efficiency of 5%. This approach allowed quantitation to within one $\log_{10}$ and had a lower limit of $4x10^3$ RNA copies/ml.

(ii) Competitive RT-PCR

Quantitative competitive RT-PCR assays use an exogenous IC RNA molecule with the same primer sequences as the test target (as described in Section 4.1.2(ii)). The control is also similar to the target in length and base composition, but is distinguishable by the presence or absence of a restriction site, a deletion or insertion, or inclusion of a different probe site. Any variation in the efficiency of RT and PCR between reactions theoretically affects the yield of IC and target amplification product equally (Ferre, 1992). There have been numerous in-house competitive RT-PCR assays developed for quantitation of HCV. These have used several different strategies for achieving quantitation:

(i) Extraction and RT-PCR of a constant amount of sample with serial dilutions of IC (Manzin *et al.*, 1994) or *vice versa* (Roth *at al.*, 1996). Roth *et al.* (1996) quantified reaction products by Southern blotting followed by chemiluminescence and laser densitometry. Manzin *et al.* (1994) quantified products by gel electrophoresis and video densitometry. The initial amount of template was determined from the number of IC molecules in the reaction yielding equal amounts of IC and target amplicons.

(ii) Extraction and RT-PCR of a constant amount of sample with a constant amount of IC (Haberhausen *et al.*, 1998). An external standard curve was generated by amplification of increasing amounts of HCV RNA with constant amounts of IC RNA. The products were quantified by detection of electrochemiluminescence (ECL) using luminometry. The ratios of target to IC PCR product in the sample were then compared with those of the standard curve to determine HCV RNA starting copy number.

(iii) Extraction and RT-PCR of a constant amount of sample with a constant amount of IC without generation of a standard curve (Haberhausen *et al.*, 1998). PCR products were quantified by ECL detection. Quantitation was achieved by applying the formula:
Initial template concentration = (signal from sample / signal from IC) x initial IC amount.

As competitive RT-PCR involves amplification of two molecules with very similar sequences can result in hybridisation between the amplified product strands of IC and target. This may reduce amplification efficiency, and if IC and target amplification are not equally affected, quantitation accuracy may be compromised (Young, 1996).

(iii) Amplicor Monitor

The Amplicor Monitor assay (Roche) is based upon RT-PCR of HCV RNA in the presence of an internal non-competitive quantitation control (QC) RNA. The QC shares primer sequences with HCV but has a different internal sequence. Sample preparation involves lysis of virus from plasma or serum with GITC and RNA precipitation with isopropanol. RT-PCR is performed by *Tth* polymerase in one tube using a single set of biotinylated primers targeted to the HCV 5'UTR. Serial dilutions of the amplified sample are then added to microtitre plate wells coated with QC-specific or target-specific capture probes. After hybridisation, avidin-horseradish peroxidase conjugate and tetramethylbenzidine substrate are added and the colour change detected by spectrophotometry. The number of target RNA copies in the sample is determined using the dilutions that give optical densities (ODs) within the assay's linear range. The OD in each well is proportional to the initial amount of target RNA which is determined by the equation:

$$\text{Target RNA copies/ml} = \frac{\text{Target OD x dilution factor}}{\text{QC OD x dilution factor}} \times \text{Input QC copies/reaction}$$

The sensitivity of the version 1.0 assay was determined to be approximately $1x10^3$ copies/ml but showed significant differences in the efficiency of detection for genotypes 1, 2 and 3, with genotype 2 virus level being underestimated by a factor of nine, and genotype 3 virus level being underestimated by a factor of 12 relative to genotype 1 (Hawkins *et al.*, 1997). The version 2.0 assay has been shown to give equal quantitation of genotypes 1 to 6 within the range, 10^3 to $5x10^5$ copies/ml. The assay significantly underestimates RNA concentration above this range (Mellor *et al.*, 1999).

(iv) SuperQuant

The SuperQuant assay (National Genetics Institute, NGI) is an RT-multiple cycle PCR test (Conrad *et al.*, 1997). The assay is not commercially available but is performed as a service at the NGI laboratories. RNA is extracted by viral lysis with GITC/phenol/chloroform and precipitated with ethanol/ammonium. RT with MMLV reverse transcriptase is performed and the cDNA products split into four identical PCR reactions. These are incubated for 25, 30, 35, or 40 cycles. The products are analysed by gel electrophoresis followed by automated

Southern blotting with a digoxygenin-labelled probe. The Southern blots are scanned and quantified by densitometry and compared to an external standard curve. The dynamic range of the test has been reported as $1x10^2$ to $5x10^6$ copies/ml (Blatt *et al.*, 1997). The SuperQuant assay has been used to assess patient treatment with IFN (Tong *et al.*, 1997). No IC is employed by this assay, so inter-assay and intra-assay variability is not controlled for.

(v) Nucleic acid sequence based amplification (NASBA)

The NASBA assay (Organon Teknika) involves viral lysis with GITC, nucleic acid purification using silica particles, followed by isothermal amplification of RNA using three enzymes: AMV reverse transcriptase, RNase H, and T7 RNA polymerase (Fig.5.2). Prior to extraction, three competitor RNAs are added to each sample at different concentrations and co-amplified with the sample RNA. Amplified RNA is captured, by hybridisation to oligonucleotide probes on paramagnetic beads. Amplified HCV and competitor RNAs are distinguished by electrochemiluminescent probes labelled with a ruthenium molecule and detected by a semi-automated ECL detection system (NucliSens reader; Organon Teknika). The initial amount of HCV RNA is determined by comparing the HCV RNA product signal with a standard curve generated from the signals produced by the competitor RNAs. The assay was found to have a detection limit of $3x10^4$ RNA copies/ml (Lunel *et al.*, 1999). The assay is no longer commercially available.

(vi) Real-time PCR

The PCR amplification process can be described by the equation:

$$N = N_0(1+eff)^n$$

where N is the amount of PCR product, N_0 is the initial amount of nucleic acid, eff is the amplification efficiency and n is the cycle number (Wang *et al.*, 1989). Theoretically, PCR products will double at each cycle. However, in reality PCR efficiency is less than 100%. Also, the increase in PCR product is exponential only for a limited number of cycles, after which it reaches a plateau. This occurs due to substrate saturation of the enzyme, product strand re-annealing, and incomplete strand separation. In the post-exponential phase the amount of amplified product is no longer proportional to the starting copy number. Therefore, quantitation is more accurate if based on measurement of PCR products during the exponential phase. This is difficult to achieve with conventional RT-PCRs as the range of cycles over which product increase is exponential depends on the number of starting template molecules (Ferre, 1992).

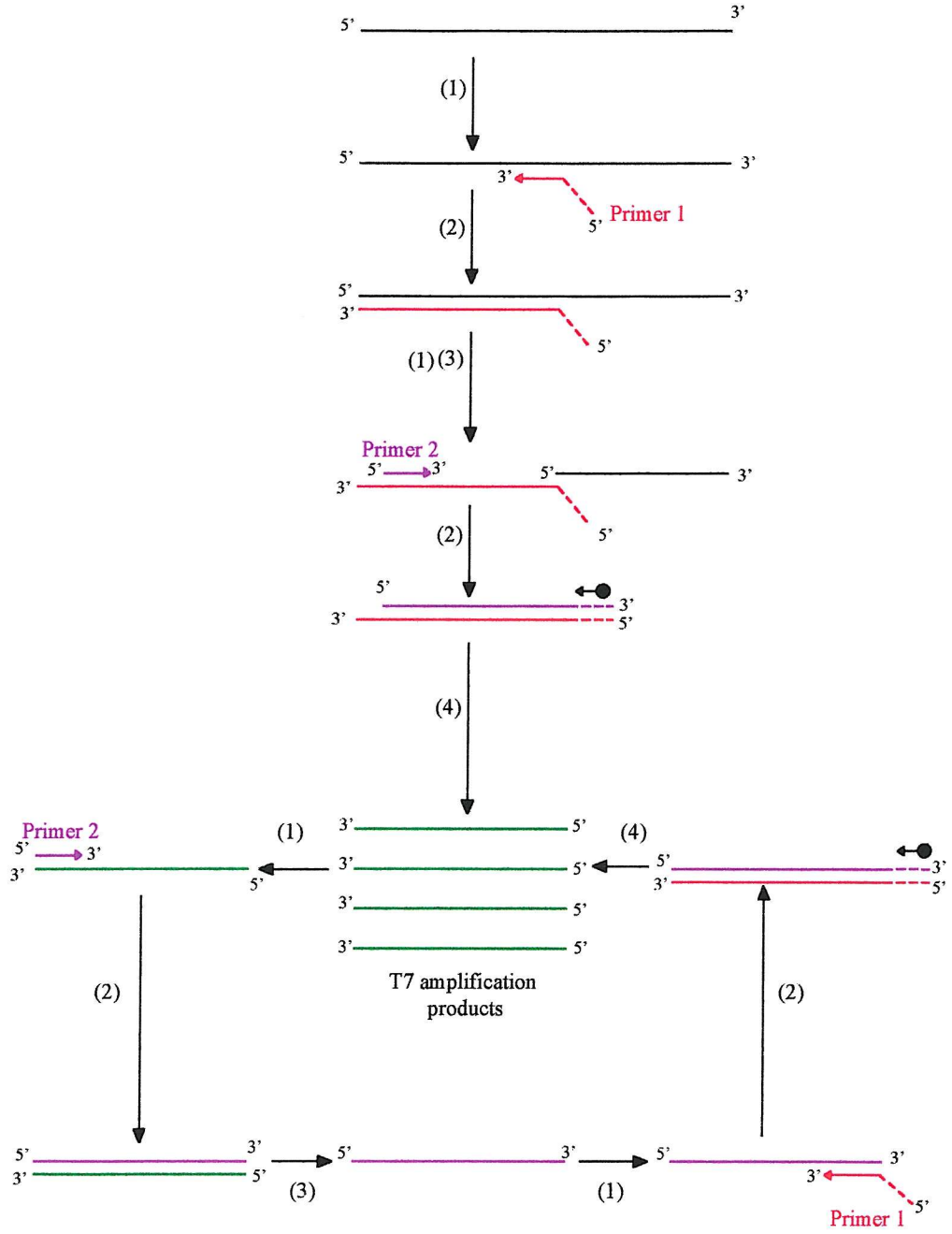


Fig.5.2 Diagrammatic representation of the NASBA process.
(1) Primer annealing. (2) Reverse transcription. (3) RNase H activity. (4) T7 polymerase activity. Black line = template RNA. Red line = anti-sense DNA. Purple line = sense DNA. Green line = anti-sense RNA. Dashed lines = T7 RNA polymerase promoter site. ⊣● = T7 polymerase.

Real-time PCR methods measure the level of reaction products at every cycle of the PCR as described in Section 3.1.5. This ensures that quantitation of the initial target concentration is based on the level of product formation during the exponential phase of PCR, rather than the plateau phase.

Prior to the start of this project there were no real-time PCR assays available for HCV quantitation. Several have since been reported (Martell *et al.*, 1999; Takeuchi *et al.*, 1999; Kawai *et al.*, 1999; Kleiber *et al.*, 2000; Schroter *et al.*, 2001). All but one of these have used the TaqMan PCR system (Applied Biosystems) in conjunction with the PRISM 7700 Sequence Detection System (Applied Biosystems). TaqMan PCR is performed on HCV cDNA samples and the threshold cycle (C_T) is determined (as described in Section 3.1.5(iii)). An external standard curve of C_T against starting copy number is created using cDNA produced by RT of serial dilutions of synthetic HCV RNA standards (Fig.5.3). The 7700 software plots the standard curve and determines the amount of starting template in the sample PCR from the C_T. A real-time PCR for quantitation of HCV using the Light Cycler (Roche) has also been developed recently (Schroter *et al.*, 2001).

The reported HCV TaqMan PCR assays have all employed a single tube RT-PCR strategy. Takeuchi *et al.* (1999) and Kawai *et al.* (1999) used *Tth* polymerase and Kleiber *et al.* (2000) used ZO5 DNA polymerase for RT and PCR, while Martell *et al.* (1999) utilised MMLV reverse transcriptase and *Taq* DNA polymerase. Takeuchi *et al.* (1999) showed that this assay format has the potential to be sensitive and have up to an eight $\log_{10}$ dynamic range (defined by the upper and lower quantitation limits) (Table 5.1). Only one of these assays (Kleiber *et al.*, 2000) employed an IC to monitor extraction, RT and PCR efficiency. This assay was developed by Roche and the TaqMan probe sequence was not disclosed.

5.1.3. Performance of current assays

An assay for quantitation of HCV RNA would ideally be sensitive, specific, accurate, reproducible, inexpensive, easy to perform, have a large dynamic range and quantify all major genotypes equally. To date, there is no commercially available assay which fulfils all of these criteria. The two most widely used assays, the Quantiplex bDNA assay and the Amplicor Monitor assay both lack sensitivity and have limited dynamic ranges (Mellor *et al.*, 1999).

As the currently available assays have been developed and evaluated using a range of standard sera or RNA transcripts, comparison and standardisation of different assays has not been possible. The first World Health Organisation (WHO) International Standard for HCV RNA (NIBSC; Saldanha *et al.*, 1999) was recently characterised and released and will now

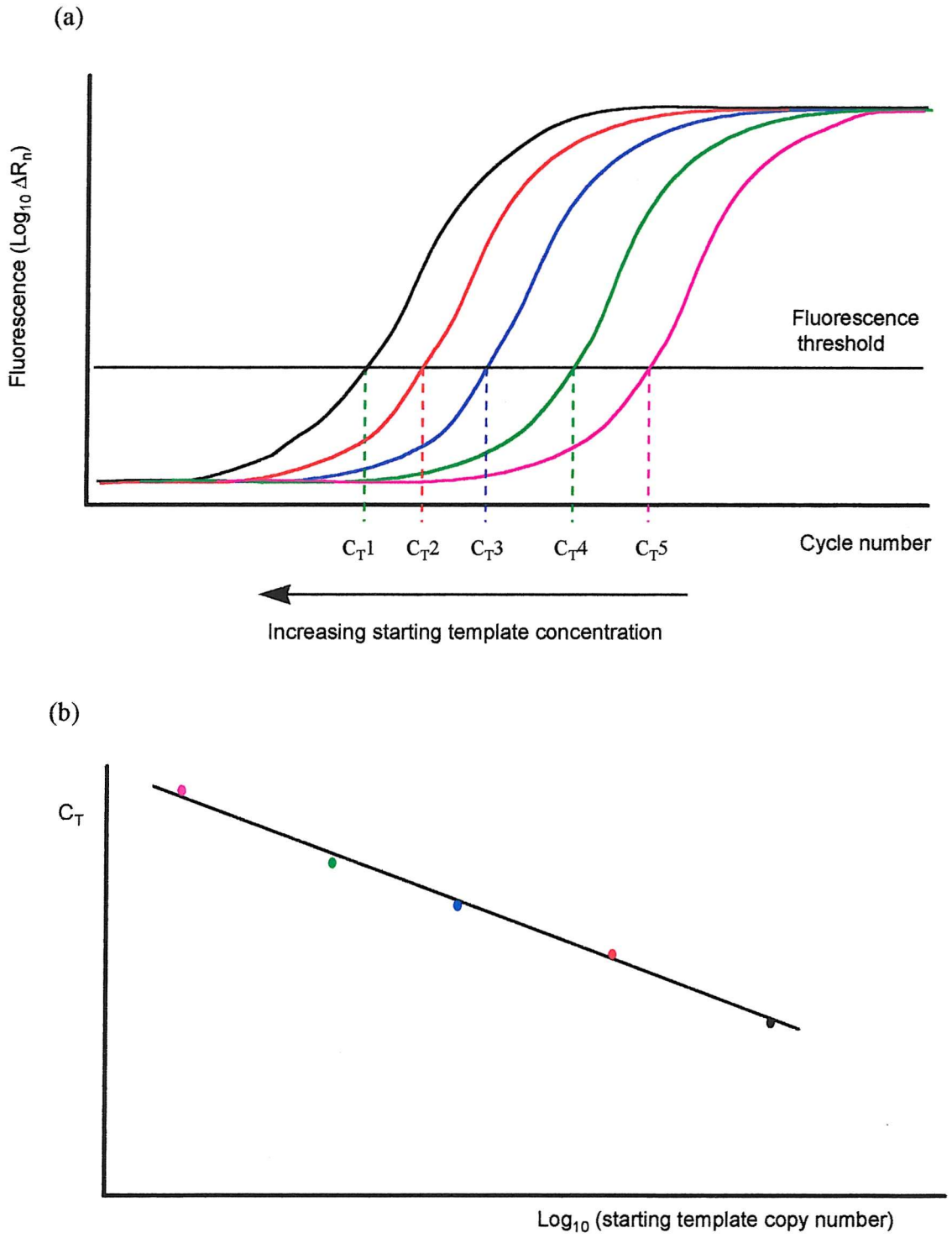


Fig.5.3 Diagrammatic representation of TaqMan PCR quantitation.
(a) Amplification plots produced by TaqMan PCR with different amounts of starting template. The threshold cycle (C_T) of each plot is illustrated by a dashed line of the same colour. A standard curve (b) is generated by plotting log_{10} starting template copy number against C_T.

allow standardisation of HCV quantitative assays. Several assays have recently been calibrated in IU (Table 5.1).

5.2. Development of the quantitative HCV TaqMan PCR assay

To convert the HCV TaqMan PCR assay described in Chapter 3 into a quantitative assay, the RNA extraction, RT and PCR stages were integrated in such a way as to maximise the sensitivity and linear dynamic range of the assay. The HCV standard and IC RNAs described in Chapter 4 were incorporated to allow HCV RNA quantitation and monitoring of assay efficiency, respectively.

5.2.1. Optimisation of RT sensitivity

To maximise the sensitivity of the assay, attempts were made to increase the proportion of cDNA used in the PCR. Preliminary studies (data not shown) showed that cDNA from Superscript and AMV RT reactions was inhibitory to TaqMan PCR when present at >8% v/v. cDNA from *Tth* RT reactions had a less marked effect on the PCR but the assay lacked sufficient sensitivity. The effects of including increasing volumes of cDNA in the PCR were therefore studied further.

The effect of increasing proportions of cDNA produced by three different reverse transcriptases in the PCR was assessed (Fig.5.4). C_T was used as a measure of PCR efficiency. All of the RT reactions were primed with TMR2 to allow direct comparison. Mg^{2+} content was adjusted in the Superscript PCRs to compensate for the addition of Mg^{2+} from the RT reaction and keep the concentration at 4μM. The other RT reactions did not contain Mg^{2+}. Reactions were incubated at the temperatures recommended by the respective manufacturers. Sensiscript reactions were incubated at 37°C or 42°C as recommended by Qiagen. Increasing proportions of cDNA resulted in increasing C_Ts for the Sensiscript reactions and Superscript reactions in Superscript buffer. Sensiscript reactions performed at 42°C and 37°C showed similar rates of C_T increase with increasing cDNA proportion. C_Ts decreased when the volume of cDNA from *Tth* RT was increased from 2 - 4μl per PCR, but remained relatively constant at higher cDNA contents indicating some inhibition. RT reaction performed with Superscript in PCR buffer II gave lower C_Ts at 8μl per PCR than at other proportions. Subsequent Superscript RT reactions were therefore performed in PCR buffer II.

As the *Tth* and Superscript enzymes allowed higher proportions of cDNA to be included in the PCR, their sensitivities for HCV detection were compared (Fig.5.5). RT reactions carried

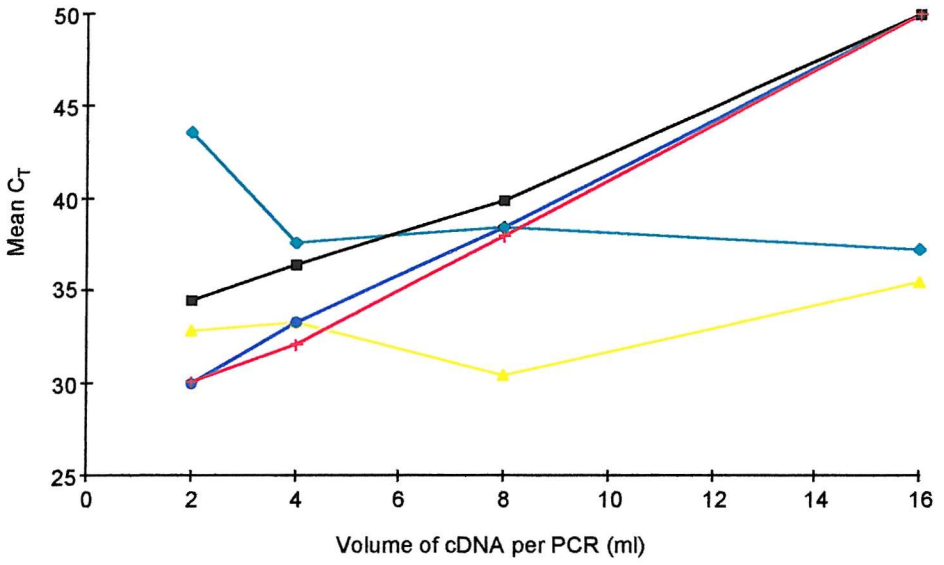


Fig.5.4 Plot showing the effect of cDNA proportion on TaqMan PCR.
RT reactions were performed using Superscript (in Superscript buffer (black) and PCR buffer II (yellow)) with Mg^{2+} concentration at 4μM in PCRs, Sensiscript (incubated at 37°C (red) and 42°C (blue)) and *Tth* (green) reverse transcriptases. Each reaction included 10^5 copies of rSot34-3a RNA. Duplicate 50μl TaqMan PCRs were performed including 2 - 16μl of cDNA per reaction.

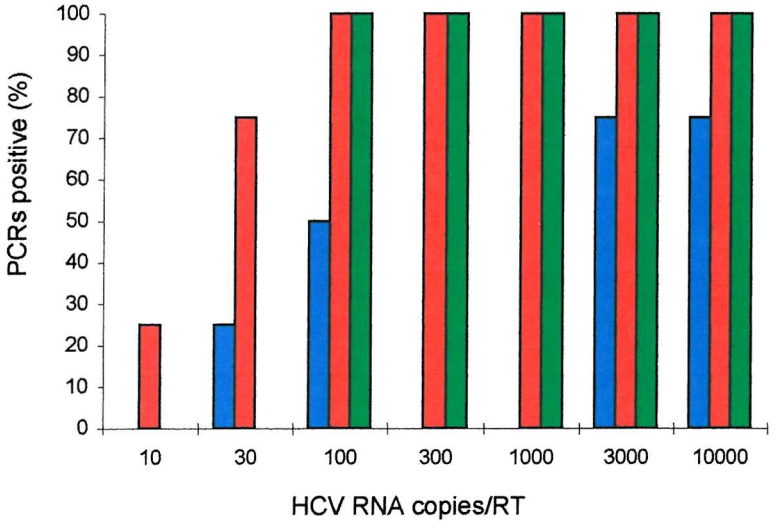


Fig.5.5 Chart showing the sensitivity of RT reactions performed with different enzymes.
RT reactions (25μl volume) were carried out with 10 - 10^4 copies of rSot34-3a using Superscript enzyme and random (red) or TMR2 priming (green), or *Tth* polymerase and TMR2 priming (blue). Quadruplicate 50μl TaqMan PCRs were performed including 8μl of each cDNA preparation. Mg^{2+} concentration was adjusted as described previously.

out using *Tth* showed inconsistent RNA detection (negative PCRs at 300 and 1000 HCV RNA copies/RT) down to 30 HCV RNA copies (25% PCRs positive). TMR2-primed Superscript reactions detected HCV RNA in 100% PCRs down to 100 copies/RT but did not detect RNA at lower copy numbers. The highest sensitivity was achieved with random-primed Superscript RT reactions (25% detection at 10 RNA copies/RT, 75% at 30 RNA copies/ml and 100% at 10^2 - 10^4 copies/RT). Therefore, for subsequent assays, random-primed Superscript RT and compensation of PCR Mg^{2+} concentration were performed.

The effect of different RT incubation temperatures on assay sensitivity was assessed (Table 5.2). Highest sensitivity (100% PCRs positive at 50 HCV RNA copies/RT) was attained by incubation at 50°C and therefore subsequent reactions were incubated at this temperature.

Table 5.2 Results of a comparison of the effect of incubation temperature on assay sensitivity.

	HCV RNA copies/RT				
Temperature (°C)	10	30	50	100	300
50	1	1	2	2	2
42	0	1	0	2	2

Random-primed Superscript RT reactions (25µl) were performed with 10 - 300 copies of rSot34-3a and incubated at 42°C or 50°C. Duplicate PCRs were carried out including 8µl of each cDNA sample and Mg^{2+} compensation as described previously. Results show number of positive TaqMan PCRs.

An attempt was made to enhance the sensitivity of the assay by further increasing the proportion of cDNA in TaqMan PCRs to 50% of the reaction volume. It was hoped that use of PCR buffer II instead of the manufacturer's buffer in Superscript RT reactions would lead to less PCR inhibition without loss of RT efficiency. To assess the effect of the buffer composition on the PCR, the sensitivity of RT reactions performed in TaqMan PCR buffer A and PCR buffer II were compared (Table 5.3). A higher sensitivity was attained when RT was carried out in PCR buffer II (100% detection at 10 HCV RNA copies/RT) than in TaqMan PCR buffer A (100% detection at 50 HCV RNA copies/RT). Subsequent reactions were therefore performed in PCR buffer II.

In summary, the highest sensitivity was achieved using random-primed MMLV RT reactions in PCR buffer II, with compensation of Mg^{2+} content (4µM in the PCR) and 50°C incubation.

Table 5.3 Results of a comparison of assay sensitivity when using different buffers for the RT reaction.

	HCV RNA copies/RT					
Buffer	10	30	50	100	300	1000
A	0	1	2	2	2	2
II	2	2	2	2	2	2

Duplicate 25µl Superscript RT reactions were performed including 10 - 1000 copies of rSot34-3a. The entire volume of each reaction product was then included in a 50µl TaqMan PCR. Results show number of positive TaqMan PCRs. Mg^{2+} concentration was adjusted as described previously.

5.2.2. Evaluation of the quantitative assay

The complete HCV quantitative assay format is described in Section 2.23.2. Briefly, RNA was extracted from 280µl of serum in the presence of 10^5 copies of IC, the HCV and IC RNAs were co-reverse transcribed in a single tube and equal volumes of the cDNA used in duplicate HCV PCRs and a single IC PCR. Inclusion of 10^5 IC copies/extraction was based on preliminary results (data not shown) using *Tth* RT reactions which detected the IC at 10^4 copies but with considerable variation in C_T. Duplicate HCV PCRs were performed to improve the precision of results. Assays were repeated if the results of the individual HCV PCRs were >50% from the mean of the duplicates (consistent with Suryanarayana *et al.*, 1998). The fluorescence threshold was set at 0.05 $\log_{10} \Delta R_n$ for all runs as this was found to be consistently above the background fluorescence level and within the log-linear phase of all amplification plots analysed (data not shown).

An external standard curve was produced by performing RT and duplicate HCV PCRs on tenfold dilutions (10^1 - 10^7 copies/PCR) of the HCV standard RNA. Sample HCV C_Ts were compared with the standard curve by the ABI 7700 SDS software to calculate the HCV load and the IC C_T was used to monitor assay efficiency (as described in Section 2.23.2). The mean of the results of the duplicate HCV PCRs was used to calculate the serum HCV RNA concentration.

(i) Assessment of standard curve linearity

The linearity of the standard curve and the effect of the IC on detection of the HCV standard RNA were studied (Fig.5.6) by producing standard curves in the presence and absence of 10^5 IC RNA copies. The HCV standard RNA was detected in 100% of PCRs over the range 10^1 - 10^7 copies/PCR and the standard curve produced was linear with ($r = 0.904$, $P = 0.000$) and

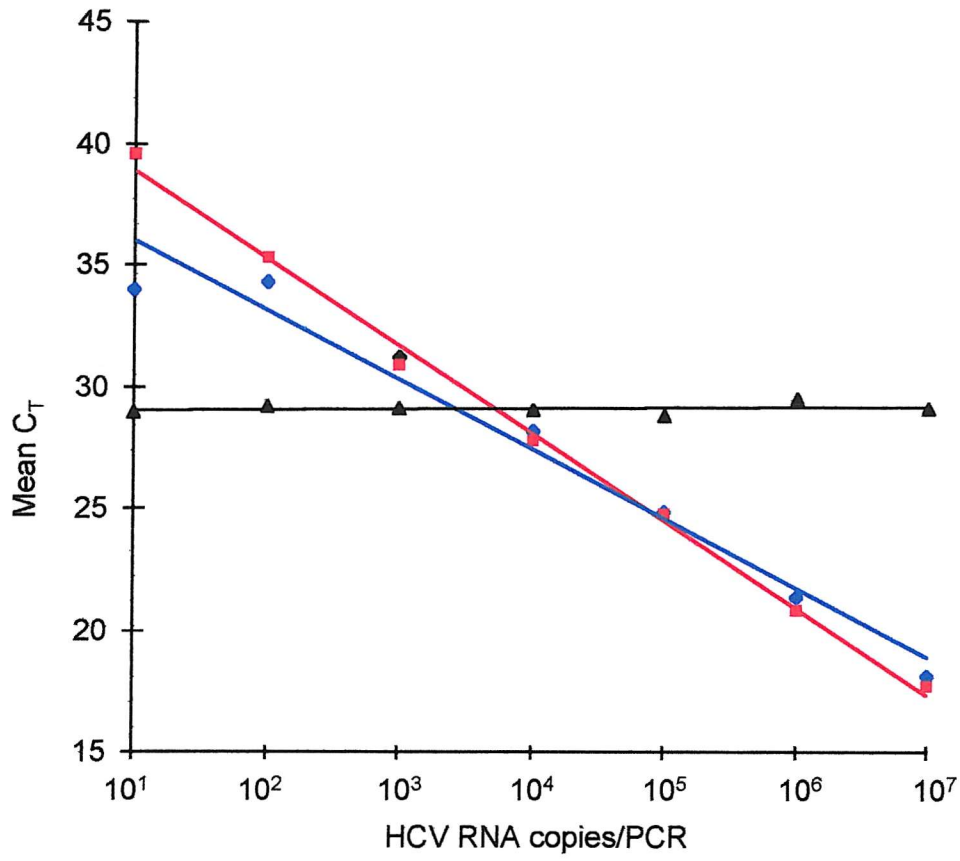


Fig.5.6 Plots showing mean C_Ts for standard curves produced in the presence and absence of IC RNA, and mean C_Ts for the corresponding IC PCRs.
RT reactions containing 10^1 - 10^7 copies of HCV RNA standard were performed in the presence (blue) and absence (red) of 10^5 copies of IC RNA. Mean C_T values from triplicate HCV and IC (black) PCRs and least-squares regression lines are shown.

without (r = 1.000, $P = 0.000$) IC RNA. There was no significant difference between mean C_T values produced in the presence or absence of IC ($P = 0.910$).

HCV concentration had no detectable effect on IC amplification (Fig.5.6). There was no detectable trend that would indicate inhibition of IC detection and IC C_Ts varied by <1.03-fold across the range of HCV standard RNA copy numbers.

The reproducibility of individual standard curve points was assessed by determination of the CV across the range of HCV RNA input copies (Table 5.4). The presence of IC had no significant effect on the CVs of the standard curve points from 10^2-10^7 copies/PCR. There was, however, an increased (7.3-fold higher) CV for the 10^1 copies/PCR point produced in the presence of IC compared with CVs for the 10^2-10^7 copies/PCR points (mean CV = 2.14). This was due to an outlying C_T from a single PCR, probably the result of sampling variation at the low copy number.

Table 5.4 CVs for standard curves produced in the presence and absence of IC RNA.

HCV RNA copies/PCR	10^1	10^2	10^3	10^4	10^5	10^6	10^7
CV with IC (%)	15.60	2.68	4.23	0.78	1.37	2.44	1.33
CV without IC (%)	1.47	3.37	4.08	1.19	2.14	1.49	1.13

CVs for triplicate HCV PCR C_Ts at each standard curve point performed in the presence and absence of 10^5 IC RNA copies (as shown in Fig.5.6).

(ii) Determination of assay sensitivity, accuracy and reproducibility

The limit of detection of the assay was determined using the first WHO International Standard for HCV RNA (96/790, NIBSC; Saldanha *et al.*, 1999) (Table 5.5). To maximise sensitivity, RNA was extracted from 280µl of serum with the QIAamp extraction kit. This was twice the volume described by the standard kit method (Qiagen, 1999) and required two more centrifugation steps. The volume was the maximum volume which could be processed without making the method impractical. HCV was detected in 100% of assays down to 200 IU/ml, 80% at 100 IU/ml, 70% at 50 IU/ml and 60% at 25 IU/ml. At least one of the duplicate HCV PCRs were positive for each of the five replicate assays at all dilutions except 25 IU/ml (3/5 assays positive). As duplicate HCV PCRs would be performed for each assay, the limit of detection was estimated to be 50 IU/ml.

The accuracy of the assay was determined by testing the undiluted WHO International Standard. The standard was tested twice and gave values of 1.29×10^5 and 1.70×10^4 copies/ml (mean = 7.3×10^4 copies/ml, SD = 7.9×10^4 copies/ml). These values were 5.88-fold lower and

Table 5.5 Detection of WHO HCV International Standard.

HCV IU/ml	25	50	100	200	400	600	800
PCRs positive (%)	60	70	80	100	100	100	100

The WHO HCV international standard was diluted in HCV-negative serum to give 25 - 800 IU/ml of serum. RNA extraction, RT and duplicate HCV TaqMan PCR were performed on five replicate 280µl samples at each dilution. Percentage detection is shown for the ten PCRs at each concentration.

1.29-fold higher than the published titre ($1x10^5$ IU/ml), respectively. From the mean TaqMan assay value, a conversion factor of 1.37 was derived that could be applied to TaqMan assay results in RNA copies/ml to give values in IU/ml. Taking into account the variability of quantitation near the detection limit, the limit of detection was approximated to 50 HCV RNA copies/ml.

Inter-assay and intra-assay variation were determined by testing multiple aliquots of a genotype 1a HCV-positive serum sample (Table 5.6). The intra-assay CV was found to be 23.8% and the inter-assay CV 29.4%. Overall there was a 3.36-fold variation in load measurement. The SD of the combined values from the intra- and inter-assay variability samples was 0.18 $\log_{10}$ copies/ml. Therefore, changes in viral load beyond two SDs (0.36 $\log^{10}$ copies/ml) were considered as true fluctuations and not due to assay variation ($P<0.05$). This was rounded to 0.5 $\log_{10}$ copies/ml (in agreement with Pontisso *et al.*, 1999).

Table 5.6 Variation in viral loads obtained for replicate measurements made intra-assay and inter-assay.

	Viral load (copies/ml)				CV (%)
Intra-assay	2,494,084	4,420,121	3,901,248	4,392,404	23.8
Inter-assay	2,275,997	1,315,566	2,851,023	2,494,084	29.4

Assay reproducibility was assessed using multiple aliquots of a single serum sample from a known HCV-positive patient. Inter-assay variation was assessed by quantitation of HCV load in four consecutive assays. Intra-assay variation was assessed by performing four replicate assays in the same run.

(iii) Assessment of IC function

The efficacy of the IC for detecting the presence of PCR inhibitors in clinical samples was assessed by performing assays in the presence of varying amounts of sodium heparin (Fig.5.7).

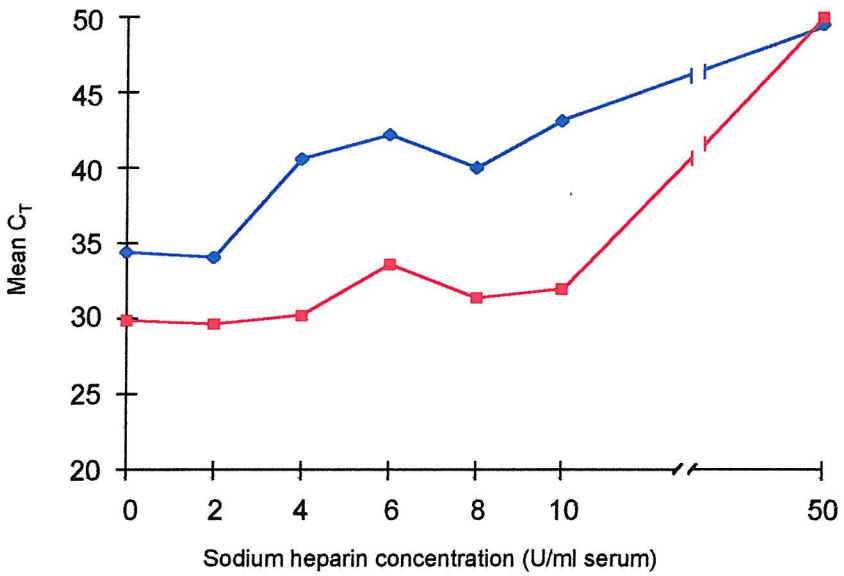


Fig.5.7 Plots showing the effect of sodium heparin on HCV and IC RNA C_Ts.
Serum from a chronically infected HCV-positive patient (load, 2.5 x 10^5 copies/ml) was spiked with 0 - 50U/ml sodium heparin. RNA was extracted, reverse transcribed and duplicate HCV (red, mean C_Ts shown) and single IC (blue) TaqMan PCRs performed.

Sodium heparin concentrations of 4U/ml and above produced a significant increase in IC C_Ts (19.1% higher C_T at 4U/ml than at 2U/ml). The effect of sodium heparin on HCV C_Ts was less pronounced. Both HCV and IC PCRs were completely inhibited at a sodium heparin concentration of 50U/ml.

For HCV PCRs that were significantly inhibited by inclusion of sodium heparin, all of the corresponding IC C_Ts were more than three SDs from the mean IC C_T of uninhibited HCV PCRs. The results were used to formulate the assay acceptance criteria detailed in Section 2.23.2.

(iv) Comparison of TaqMan and Quantiplex HCV RNA 2.0 assays

The TaqMan assay was compared with the commercially available Quantiplex HCV RNA 2.0 bDNA assay from Bayer (Fig.5.8). Of 36 samples tested 17 contained HCV genotype 1, four genotype 2, eight genotype 3, one genotype 4 and one genotype 5. All were quantified by the TaqMan assay but four (11.1%) were below and one (2.8%) was above the range of the bDNA assay ($2.0x10^5$ - $1.20x10^8$ genome equivalents (GEq)/ml). There was a significant correlation ($r = 0.902$, $P = 0.000$) between the results from the two assays. Overall, results from the bDNA assay were a mean of 19.67-fold higher than those given by the TaqMan assay. There was no detectable trend in fold difference with load. From the least-squares regression line the equation for conversion of quantities given by the TaqMan PCR-based assay into bDNA GEq/ml is:

$$B = 0.8141T + 2.2143$$

(where B = bDNA result ($\log_{10}$ GEq/ml) and T = TaqMan result ($\log_{10}$ copies/ml)).

5.3. Discussion

Quantitation of HCV load in patient serum is important for management of disease, evaluation of new treatments and for the study of virus biology. The most important properties of a useful quantitative assay are sensitivity, specificity, accuracy, reproducibility and dynamic range. Real-time PCR has the potential to accurately quantify template over a wide dynamic range with excellent sensitivity (Heid *et al.*, 1996).

The sensitivity of the TaqMan PCR-based assay was maximised by optimisation of the RNA extraction and RT steps and by increasing the proportion of cDNA in the PCR. RNA extraction was optimised by addition of the highest volume of patient serum that was practical to the extraction column. This strategy increases the number of RNA molecules per RT and

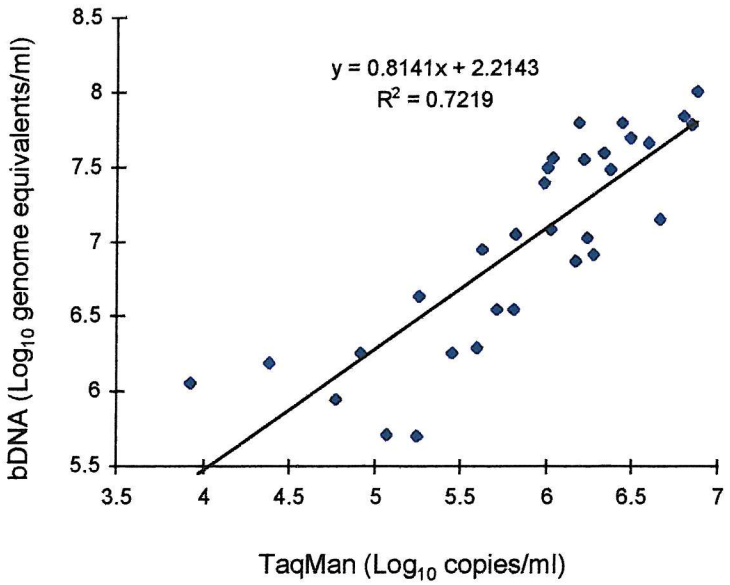


Fig.5.8 Plot showing comparison of HCV quantitation between the Quantiplex HCV RNA 2.0 bDNA assay and TaqMan PCR.

The TaqMan and bDNA assays were used to quantitate 36 HCV positive patient serum samples. The plot shows results of the 31 samples which gave viral loads within the acceptable limits of both assays. The least-squares regression line, equation and R^2 value are shown.

has been used successfully to improve the sensitivity of the Amplicor Monitor assay (Lee *et al.*, 2000).

The RT step was optimised by incubating at 50°C and priming with random instead of sequence-specific primers. Higher incubation temperatures probably allowed increased enzyme processivity through regions of secondary RNA structure as described by Gerard *et al.* (1997). Random-priming is thought to increase primer-binding efficiency in regions of secondary structure (Radhakrishnan *et al.*, 1999). The use of random-priming made the TMR2-binding site in the IC redundant but this did not impair the performance of the assay. Significant competition between the IC and HCV RNA for random primer was not expected due to the excess concentration of primers used. This was supported by the similarity of standard curves produced in the presence and absence of the IC.

Increasing the volume of cDNA in the PCR led to inhibition of amplification, especially when using Sensiscript and Superscript enzymes, suggesting that the RT buffer was incompatible with the PCR. Using PCR buffer II for the RT step and compensating for the Mg^{2+} being introduced into the PCR from the RT reaction allowed higher proportions of cDNA to be included in the PCR without affecting amplification efficiency. This strategy was similar to that employed by Suryanarayana *et al.* (1998) for quantitation of SIV by TaqMan PCR. PCR buffer II is the same as TaqMan buffer but without ROX dye. The use of a different reaction buffer seemed to have no detrimental effect on RT step and halving the level of ROX dye in the PCR did not prevent R_n measurement.

The sensitivity and accuracy of the TaqMan assay were determined by testing multiple dilutions of the WHO International Standard (Saldanha *et al.*, 1999). The HCV RNA concentration in the standard has been quantified by limiting dilution analysis using a range of different methods (Roche Amplicor, TMA and in-house PCR assays) at 20 different laboratories and is the best characterised HCV standard available. The TaqMan assay gave quantitation readings within the 3.4 $\log_{10}$ copies/ml distribution of values reported by Saldanha *et al.* (1999) and the mean result was only 0.33 $\log_{10}$ copies/ml from the mean for the standard (5 $\log_{10}$ copies/ml). This compares with a difference from the mean of 0.27 - 1.03 $\log_{10}$ copies/ml by the Amplicor Monitor, 0.77 - 0.91 $\log_{10}$ copies/ml by the Quantiplex HCV assay, and 1.02 $\log_{10}$ copies/ml by the NASBA assay (Saldanha *et al.*, 1999). The results suggest the accuracy of the TaqMan assay is at least comparable with those of the commercially available assays. More replicates would need to be performed to confirm the finding, however, this was not possible due to the limited availability of the WHO standard.

Quantitation by the TaqMan assay was based on comparison of sample C_Ts with an external standard curve derived by RT-PCR of a 333nt *in vitro* transcribed RNA. Use of an

external standard curve assumes that RT and amplification of the standard and sample RNAs occurred with equal efficiency. Furthermore, unlike RNA from patient samples, the standard RNAs did not undergo the extraction process, a possible source of RNA loss. However, these factors and the fact that the standard RNA was not full-genome length did not appear to significantly affect accuracy as assessed using the WHO International Standard.

Based on the standard curve range (10^1 - 10^7 copies/PCR), the quantitative range of the TaqMan PCR assay was $1x10^2$ - $1x10^8$ copies/ml. However, as HCV RNA is detectable down to 50 copies/ml, the standard curve can be extrapolated to quantify to this level if both of the duplicate HCV PCRs of an assay are positive and meet the acceptance criteria (described in Section 2.23.2). The theoretical quantitation range was therefore 50 - $1x10^8$ copies/ml (or 50 - $1.37x10^8$ IU/ml).

It is hoped that in future all HCV quantitation standards will be calibrated in IUs using the WHO International Standard, allowing comparison of the different assays formats available (Saldanha *et al.*, 2001). Of the TaqMan PCR-based assays described for quantitation of HCV, only that of Kleiber *et al.* (2000) has been calibrated in IUs. This is the first commercial TaqMan HCV quantitative assay to be described (Roche). The quantitative assay developed for the current report has a broader dynamic range than that of the Roche assay (50 - $1.37x10^8$ IU/ml versus 10^3 - 10^7 IU/ml, respectively). However, the Roche TaqMan assay was more sensitive, detecting the WHO International Standard in 95% of assays at 28 IU/ml. The dynamic ranges and sensitivities of the other published quantitative HCV TaqMan assays have not been reported in IUs (Takeuchi *et al.*, 1999; Kawai *et al.*, 1999; Martell *et al.*, 1999). However, assuming one IU to be approximately equal to one copy/ml, the dynamic range of the TaqMan PCR assay described in the current study surpasses that of all but the assay described by Takeuchi *et al.* (1999). The TaqMan PCR also has a larger dynamic range than the Light Cycler real-time quantitative assay described by Schroter *et al.* (2001).

The TaqMan PCR assay described in this report also has a larger dynamic range and better sensitivity than the most commonly used commercial assays, the Amplicor Monitor 2.0 assay (500 - $5x10^5$ IU/ml) and the Quantiplex HCV RNA 2.0 assay ($3.2x10^4$ - $1.9x10^7$ IU/ml), and the recently released Versant HCV RNA 3.0 assay (521 - $8.3x10^6$ IU/ml).

The TaqMan assay performed well when compared directly with the Quantiplex HCV RNA 2.0 assay, showing superior dynamic range and sensitivity. The overall run-time was shorter for the TaqMan assay (ca. 8hr) than the bDNA assay (21hr), but 'hands-on' time was similar for the two assays (ca. 5hr). The TaqMan assay was cheaper to perform than the bDNA assay (£20 - £24 versus £35 per sample). However, the TaqMan PCR assay is more technically demanding than the bDNA assay.

The intra- and inter-assay CVs of the TaqMan assay (23.8% and 29.4%, respectively) were higher than those reported for the Quantiplex HCV RNA 2.0 assay (12% and 11%, respectively; Bayer, 1998) and for the Roche TaqMan assay (17.0% and 21.6%, respectively; Kleiber *et al.*, 2000) for samples of similar load (mean = $2.8x10^6$ copies/ml for present study). This suggests that the reproducibility of the current assay could be improved. The RNA extraction stage could possibly be made more reproducible by use of an automation system such as the NucliSens Extractor (Organon Teknika).

Extraction, RT and PCR efficiencies were monitored using the IC RNA. Sodium heparin was used to evaluate the effect of inhibitory substances on IC detection. Heparin compounds are polyanions known to inhibit PCR by competing with DNA for protein binding (Beutler *et al.*, 1990) and are not removed from serum samples by the QIAamp viral RNA mini kit (Qiagen, 1999). The strategy of using 10^5 IC copies/extraction differs from that used some researchers (Arnauld *et al.*, 1998; Rosenstraus *et al.*, 1998) who have used ICs at low copy numbers, near the assay's limit of detection. The use of IC at low copy number allows detection of low level inhibition by complete elimination of IC amplification, but is susceptible to sampling variation. Including higher numbers of template copies was possible because IC amplification efficiency was monitored using the C_T. Inclusion of the IC at the lysis stage of RNA extraction allowed the efficiency of all stages of the assay to be monitored and showed that amplification of HCV and IC templates were inhibited in a dose-dependent manner. This supports results by Miyachi *et al.* (1998) who also demonstrated a dose-dependant relationship between PCR inhibition and heparin concentration. Sodium heparin had a greater effect on IC amplification than on HCV amplification, probably because the IC was present at lower copy numbers than HCV RNA in the patient sample used.

The TaqMan assay developed by Kleiber *et al.* (2000) is the only other TaqMan PCR assay to incorporate an IC. The IC RNA described by the group had primer-sites identical to those of the HCV PCR and a unique probe sequence. The authors did not reveal the IC copy number added to each sample, its performance for detecting reaction inhibition or the IC probe sequence used. Kleiber *et al.* (2000) performed the IC and HCV PCRs in a single tube and distinguished their amplification by using different reporter dyes for each probe. This made the assay more simple to perform than the test described in the current report but has increased potential to compromise amplification efficiency of both templates.

In conclusion, the TaqMan PCR assay described here offers the potential to quantitate HCV RNA over a wide range of viral loads with good sensitivity and reproducibility. The assay is not as sensitive as the TMA qualitative assay (detection limit of 10 IU/ml, Sarrazin *et al.*, 2001) and therefore may not detect very low level viraemia. However, the

assay is suitable for use in studies of viral load in chronically infected patients and for monitoring viral load changes in response to treatment. Modification of the RNA extraction procedure will also allow the assay to be used to study HCV levels in cultured cells.

5.4. Summary

1. A quantitative assay for HCV RNA was developed based on the TaqMan real-time PCR method and evaluated using the WHO International Standard.
2. The sensitivity of the assay was 50IU/ml. The standard curve was linear over a 6-$\log_{10}$ dynamic range from 10^1 - 10^7 copies/PCR giving a quantitative dynamic range of 10^2 - 10^8 in-house RNA copies/ml. The intra and inter-assay CVs were 23.77% and 29.39%, respectively.
3. The assay uses an IC for monitoring efficiency of RNA extraction, RT and PCR. The IC successfully detected PCR inhibition in heparin-spiked samples.
4. There was a significant, strong correlation between results of the TaqMan PCR assay and those of the Quantiplex HCV RNA 2.0 bDNA assay for quantitation of a panel of HCV-positive serum samples.

6. Study of viral load in symptomatic and asymptomatic patients with chronic hepatitis C

6.1. Introduction

6.1.1. HCV-related disease

The natural history of HCV infection is complex and the rate of progression and severity of disease varies between individuals (Fig.6.1). Initial infection is acute and self-resolving in some patients, however the majority develop a chronic infection which may slowly progress to serious conditions such as cirrhosis and hepatocellular carcinoma (HCC). The liver is the main site of viral replication and so liver biopsy is performed to assess the extent of HCV-related liver disease. Serum alanine aminotransferase (ALT) levels are measured as a marker of active liver disease. Chronic infection is also believed to lead to extrahepatic manifestations in some individuals. The range of disease caused by HCV has been reviewed by Houghton (1996), Hoofnagle (1997), Marcellin (1999) and Colombo (1999).

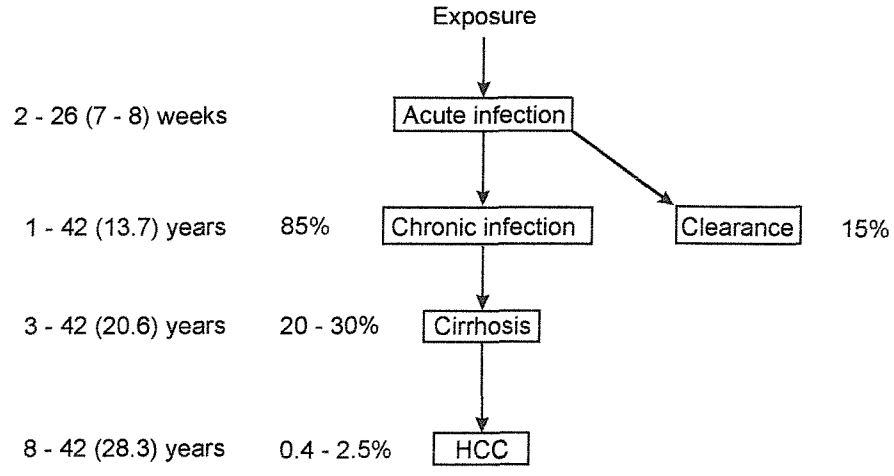


Fig.6.1 Progression of HCV-related liver disease.
Range (and mean) time from infection to clinical presentation, and percentage of infected patients developing each condition are shown (Marcellin, 1999; Tong *et al.*, 1995).

(i) Acute hepatitis C

Infection with HCV results in an initial acute phase, the clinical course of which has mainly been described in patients infected via blood transfusion. Acute infection is icteric in 20% of cases and anicteric, with no or few symptoms in 80% of cases. Symptoms are non-specific (malaise, nausea and right upper quadrant pain followed by dark urine and jaundice) and similar to other forms of viral hepatitis. Severe acute hepatitis is rare and the existence of fulminant hepatitis is controversial.

Due to the non-specific nature of the symptoms of acute hepatitis C, diagnosis is usually based on viral markers. HCV RNA appears as early as one week after exposure and increases to levels of 10^6 - 10^8 copies/ml. Anti-HCV antibodies are detectable in most cases, but the time taken to seroconvert has been found to vary from seven to 31 weeks. Serum ALT levels begin to increase shortly before clinical symptoms appear and may peak at greater than ten-fold normal level (45IU/L).

Acute hepatitis C lasts for two to 12 weeks. HCV infection is self-limiting in about 15% of infected patients, where serum ALT levels return to normal and HCV RNA becomes undetectable in the bloodstream. Anti-HCV antibody levels decrease but remain detectable for years. It is not known whether HCV infection is eradicated or remains at low levels in hepatocytes or in extrahepatic sites such as PBMCs or bone marrow.

Clearance of HCV after acute infection appears to be mainly dependent on host factors. Female sex and class II antigen alleles DQB1*0301 and DRB1*1101 have been reported to be associated with spontaneous HCV RNA clearance (Alric *et al.*, 2000).

(ii) Chronic hepatitis C

The majority (about 85 %) of patients infected with HCV remain chronically infected after the initial acute infection, with HCV RNA detectable in the blood. Most of these patients are asymptomatic. When present, symptoms include nausea, anorexia, pruritus and weight loss.

The effects of chronic hepatitis C on liver histology include appearance of lymphoid follicles and aggregates within portal tracts, steatosis, activation of lobular sinusoidal inflammatory cells, bile duct damage, and presence of eosinophilic material in the hepatocyte cytoplasm (reviewed by Houghton, 1996; Dienes *et al.*, 1999). Varying degrees of liver necrosis, scarring and fibrosis result. A gradual worsening in necroinflammatory activity is commonly observed, however, the rate of progression is highly variable, and stabilisation, or even improvement may occur.

Grading of liver disease activity can be performed by the system proposed by Knodell *et al.* (1981). The Knodell system or histological activity index (HAI) scores the biopsy according to

the extent of periportal necrosis, intralobular necrosis, portal inflammation, and fibrosis. Modified versions of the system have been described, but the Knodell score has remained the basis of grading activity. The necrosis and inflammation HAI scores are added to grade the level of disease as minimal (HAI = 1 - 3), mild (4 - 8), moderate (9 - 12) and severe (13 - 18) chronic hepatitis. Staging of chronic hepatitis relates to the time course of disease and degree of fibrosis. The fibrosis HAI score is used to describe the stage as no (fibrosis score = 0), mild (1), moderate (2), severe (3) fibrosis or cirrhosis (4). Other scoring assays have been developed but these are based on the same activity lesions (Desmet *et al.*, 1994).

Serum ALT concentration remains normal in approximately 25% of patients with chronic hepatitis C. Approximately 54% of the these patients have mild chronic hepatitis, 21% have moderate chronic hepatitis and less than 1% have cirrhosis. Most are clinically stable with no evidence of liver disease progression, steady HCV load and persistently normal serum ALT levels (Marcellin *et al.*, 1997).

Serum ALT concentration remains elevated in approximately 75% of patients with chronic hepatitis C. These patients have liver disease of varying severity and there is no correlation between the presence or degree of symptoms or the height of serum ALT elevation and the severity of liver disease. About 50% of these patients have mild chronic hepatitis. Serum ALT levels are usually elevated between one and two times the upper limit of normal and in some cases are only intermittently abnormal. However, there are exceptions, and serum ALT level is an imperfect marker of liver disease progression. The long-term outcome of mild chronic hepatitis C is not well studied but liver disease appears to progress slowly in these patients and their risk of developing cirrhosis is low.

About 50% of patients with chronic hepatitis and elevated serum ALT have moderate or severe hepatitis. Serum ALT levels are two to ten times higher than the upper limit of normal. There is an overall correlation between serum ALT level and degree of histological activity in the liver in these patients.

Progression of liver disease is slow, with an estimated mean fibrosis score increase of 0.06/year in patients with normal ALT levels and 0.11/year in patients with elevated serum ALT levels. Higher rates of fibrosis progression have also been found to be associated with age at infection greater than 40 years, alcohol consumption greater than or equal to 50g/day, and male sex (Poynard *et al.*, 1997). There is conflicting evidence as to the effect of genotype, with almost equal numbers of reports finding and not finding an association between genotype 1b and increased liver disease severity and progression. However, these studies were hampered by an inability to control for duration of infection (Mondelli and Silini, 1999).

(iii) End-stage liver disease

Chronic hepatitis C may result in a gradual worsening of liver disease over a period of 20 - 30 years. Eventually, a patient may develop the more serious conditions, cirrhosis or HCC.

(a) Cirrhosis

Cirrhosis is a severe, irreversible form of liver inflammation, necrosis and regeneration. The condition results in liver failure in approximately 20 - 25% of cases. Clinical symptoms include marked fatigue, muscle weakness and wasting, fluid retention, bruising and pruritus. Portal hypertension and hepatic failure may occur late on. End-stage HCV-related cirrhosis is the most common indication for liver transplantation.

The rate of progression to cirrhosis is lower in patients infected at a young age, in females, and those abstaining from alcohol consumption. Immunodeficient patients have a higher rate of progression to cirrhosis. Virological factors such as load and genotype have not been found to have a significant effect on rate of progression. The level of viral genetic heterogeneity is reportedly not associated with progression to cirrhosis (Lopez-Labrador *et al.*, 1999).

(b) Hepatocellular carcinoma

HCC occurs in 3 - 10% of patients with cirrhosis but rarely occurs in patients with chronic hepatitis C without cirrhosis. Patients have a single hepatic tumour nodule for several years before generation of satellite or distant nodules. Symptoms include malaise, lethargy, anorexia, fever, jaundice, pruritus, abdominal pain, altered bowel habits and hepatomegaly. Late effects include haemorrhage and hepatic encephalopathy.

Reported risk factors for developing HCC include cirrhosis, HBV co-infection, male sex, age >55 years, alcohol consumption, high baseline serum alpha-fetoprotein level and fibrosis score. Infection with genotype 1b virus has been reported to be a significant risk factor for HCC by some researchers but discounted by others (Mondelli and Silini, 1999).

(iv) Extrahepatic manifestations

HCV infection is suspected to cause a range of extrahepatic conditions including porphyria cutanea tarda, low-grade malignant lymphoma, autoimmune thyroiditis, lichen planus, Sjörgren's syndrome, aplastic anaemia, polyarteritis nodosa, erythema nodosum and idiopathic fibrosis. However, the association of these conditions with HCV infection has not been proven conclusively.

The association of mixed cryoglobulinaemia and glomerulonephritis with HCV infection is more widely accepted. Mixed cryoglobulinaemia, the formation of immune complexes of

HCV and immunoglobilins, rheumatoid factor and complement, occurs in 30 - 50% of chronic hepatitis C cases but is usually asymptomatic. The clinical symptoms, arthritis, Raynaud's disease and purpura occur in just 1 - 2% of cases.

Glomerulonephritis is a rare consequence of HCV infection which causes weakness, peripheral oedema, hypertension and hepatomegaly. The condition is thought to be caused by deposition of immune complexes in the kidney glomeruli.

Serum ferritin levels are also reported to be raised in approximately 40% of patients with chronic HCV infection. Liver iron content is also raised in about 10% of patients (Riggio *et al.*, 1997). Increased iron concentrations may be related to porphyria cutanea tarda.

6.1.2. Asymptomatic chronic hepatitis C

Chronic hepatitis C is anicteric in the majority of patients, and a large population of asymptomatic HCV carriers has been identified by routine blood donor screening. Studies of asymptomatic HCV-positive blood donors have shown that most have some degree of liver disease (87 - 100%) and raised serum ALT levels (83 - 88%) (Mutimer *et al.*, 1995; Bird *et al.*, 1995; McLindon *et al.*, 1995; Alter *et al.*, 1997). These studies show that even in HCV-positive patients with low level or absent symptoms there is still progression of liver disease.

A prospective study was carried out by the Southampton University Hospital Trust (SUHT) Liver Unit into the extent of HCV-related liver disease in asymptomatic patients identified as being HCV-positive following routine screening of donated blood (SUHT Liver Unit, unpublished data, 1998). A cohort of 77 asymptomatic HCV-positive patients were recruited. A second group consisting of 70 anti-HCV antibody positive, symptomatic patients referred to the SUHT hepatological outpatients clinic were recruited as a control cohort. These were diagnosed as a result of investigations of ill health, or suspected exposure to HCV. There were no significant differences between the two cohorts in terms of age or gender. The degree of liver disease was quantified using a modified Knodell system, which scored liver biopsies on a scale of zero to three for liver damage, inflammation and fibrosis. Serum ALT levels were measured and patients questioned as to the likely source and date of infection. No differences in the extent of liver damage, fibrosis or inflammation were found between the two groups. When the two cohorts were combined to increase the sample size for statistical analysis, total histological score was significantly correlated with estimated duration of infection and patient age.

There has not been a comprehensive study of viral load profiles in asymptomatic patients. The first objective of the current study was to compare serum HCV RNA levels in

asymptomatic and symptomatic patients and analyse potential correlations with examined host and viral factors.

6.1.3. Variation in viral load in chronically infected patients

There have been several studies of viral load over time in chronically infected HCV-positive individuals. Viral load was found to vary by <1 $\log_{10}$ copies/ml in the majority of patients (Hollingsworth *et al.*, 1996; Gordon *et al.*, 1998; Thomas *et al.*, 2000; Arase *et al.*, 2000). A recent study of a homogeneous group of female patients infected from a common source of HCV genotype 1b-contaminated anti-D immunoglobulin found a mean rate of change in load of just +0.23 $\log_{10}$ copies/ml/year over 1.2 to five years (Fanning *et al.*, 2000). These studies suggest that viral load is relatively stable in the majority of patients, however, the determinants of the level of viral load variation are not yet known. Pontisso *et al.* (1999) found that viral load was less variable in patients with normal ALT levels than in those with abnormal ALT levels, suggesting an association with the level of liver disease.

None of these studied focused on measuring viral load variation in asymptomatic patients. The second aim of the current study was, therefore, to determine the extent of HCV RNA variation during asymptomatic chronic infection by measuring viral load in serial serum samples.

6.2. HCV load in symptomatic and asymptomatic patients

6.2.1. Patient groups

HCV serum load was measured retrospectively in subsets of the asymptomatic and symptomatic chronically infected patients studied by the SUHT Liver Unit. The patients were chosen due to their HCV RNA-positive status (as determined by nested PCR). The new patient groups are shown in Table 6.1. All were untreated at time of sampling. Serum ALT levels were elevated in 80% of asymptomatic patients and 76% of symptomatic patients. Total histological scores ranged from 0 - 8 in the symptomatic patients and 1 - 8 in the asymptomatic patients.

There was no significant difference between the asymptomatic and symptomatic groups with respect to inflammation ($P = 0.348$), damage ($P = 0.330$), fibrosis ($P = 0.423$) or total histological score ($P = 0.855$). There was also no significant difference in duration of infection ($P = 0.918$; n = 48 for asymptomatics, n = 28 for symptomatics) or serum ALT concentration ($P = 0.405$, n = 52 for asymptomatics, n = 49 for symptomatics) (Table 6.1).

Table 6.1 Characteristics of patient groups.

Patient group	Symptomatic	Asymptomatic	Combined
Numbers of patients	53	57	110
Age (years) Mean	40.50	40.96	40.76
Range	23-70	20-62	20-70
Sex ratio (%), male : female	72 : 28	58 : 42	65 : 35
Duration of infection (years) Mean	19.32	19.19	19.24
Range	4-40	4-36	4-40
Suspected source of infection, n (%):			
Intra venous drug use	13 (24.5)	14 (24.6)	27 (24.5)
Blood transfusion	30 (56.6)	14 (24.6)	44 (40)
Other*	9 (17.0)	27 (47.4)	36 (32.7)
Unknown	1 (1.9)	2 (3.5)	3 (2.7)
Serum ALT level (IU/L) Mean	87.22	94.84	90.54
Range	(18-393)	(16-343)	(16-393)
Modified Knodell scores, mean:			
Inflammation	1.49	1.72	1.62
Damage	1.05	1.23	1.16
Fibrosis	0.59	0.72	0.67
Total	3.14	3.64	3.43
HCV genotype, n (%) 1	9 (17.0)	26 (45.6)	35 (31.8)
2	3 (5.7)	9 (15.8)	12 (10.9)
3	3 (5.7)	20 (35.1)	23 (20.9)
5	0 (0)	1 (1.8)	1 (0.9)
Unknown	38 (71.7)	1 (1.8)	39 (35.5)
Alcohol consumption (IU/week) Mean	11.5	13.75	13.34
Range	0-40	0-140	0-140

*Tattooing, ear piercing, human bite, vaccination, operation.

6.2.2. HCV load in asymptomatic and symptomatic patients

HCV load in stored serum samples taken as close to the date of biopsy as possible (0 - 45 months; mean = 6 months) was measured using the TaqMan PCR-based assay. HCV load ranged from 8.19×10^2 - 9.14×10^6 copies/ml (mean = 1.23×10^6 copies/ml) in the asymptomatic patients and from 1.01×10^3 - 9.58×10^6 copies/ml (mean = 1.07×10^6 copies/ml) in the symptomatic patients. No significant difference in mean viral load was found between the asymptomatic and symptomatic patient groups ($P = 0.855$).

6.2.3. HCV load in asymptomatic and symptomatic patient groups combined

Due to the lack of significant difference between the asymptomatic and symptomatic groups with respect to viral load and level of liver disease, the data from the two patient groups were combined to increase the sample size for statistical analysis. The mean viral load for the combined group was 1.15×10^6 copies/ml (range, 8.19×10^2 -9.58×10^6 copies/ml).

A significant, weak correlation (r = 0.222, $P = 0.036$) was observed between viral load and fibrosis (Fig.6.2). No significant correlation was found between viral load and inflammation, damage, or total histological score (Appendix IV; Fig.10.2). There was also no significant correlation between viral load and duration of infection, patient age, serum ALT concentration, or alcohol consumption (Appendix IV; Fig.10.3 - 10.6).

There was no significant difference in viral load between patients infected with genotype 1 and genotype 3 virus ($P = 0.490$), genotype 1 and genotype 2 virus ($P = 0.226$), or genotype 2 and genotype 3 virus ($P = 0.618$) (Appendix IV; Fig.10.7). No significant difference in viral load was found between patients infected via blood transfusion (BT) and *intra venous* drug use (IVDU) ($P = 0.830$), between those infected via BT and other routes ($P = 0.292$), or between patients infected via IVDU or other routes ($P = 0.467$) (Appendix IV; Fig.10.8).

6.2.4. Longitudinal HCV load in asymptomatic patients

Changes in serum HCV load over time were studied in 19 of the asymptomatic patients. Serum ALT was normal in 29% of the patients and total histological scores ranged from one to seven. Load was measured in two serum samples taken at least 18 months apart (mean 31.4 months; range 18 – 54 months). Of the 19 patients, eight (42%) showed an increase in load, ten (53%) showed a decrease and one (5%) showed no overall change in load (Fig.6.3). Viral load changed by a mean 17.8-fold increase (range, 57.1-fold decrease to 415.6-fold increase), a mean rate of increase of 1.73×10^3 copies/ml/month (range, 2.81×10^5 copies/ml/month decrease to 1.50×10^5 copies/ml/month increase).

There was no significant correlation between rate of change in viral load and initial viral load, inflammation, damage, fibrosis and total histological scores, initial serum ALT concentration, rate of change in serum ALT concentration, duration of infection, or patient age (Appendix IV; Fig.10.9 - 10.14).

No significant difference in rate of change in load was observed between patients with normal (≤45IU/L, n = 10) and raised (>45IU/L, n = 3) initial serum ALT concentration ($P = 0.371$). There was no significant difference in viral load rate of change between patients infected with genotypes 1 and 2 ($P = 0.699$), genotypes 1 and 3 ($P = 0.825$), or genotypes 2 and 3 ($P = 0.556$)(Appendix IV; Fig.10.15). No significant difference in rate of change in viral load was found between patients infected via BT and IVDU ($P = 0.937$), BT and all other sources ($P = 0.521$), or IVDU and all other sources ($P = 0.831$)(Appendix IV; Fig.10.16).

Of the 19 asymptomatic patients in whom load measured over time was studied, load in three patients (AS1, AS2 and AS3) was studied in more detail. These patients had relatively mild liver disease (total histological scores of two, three and two, respectively) and all had

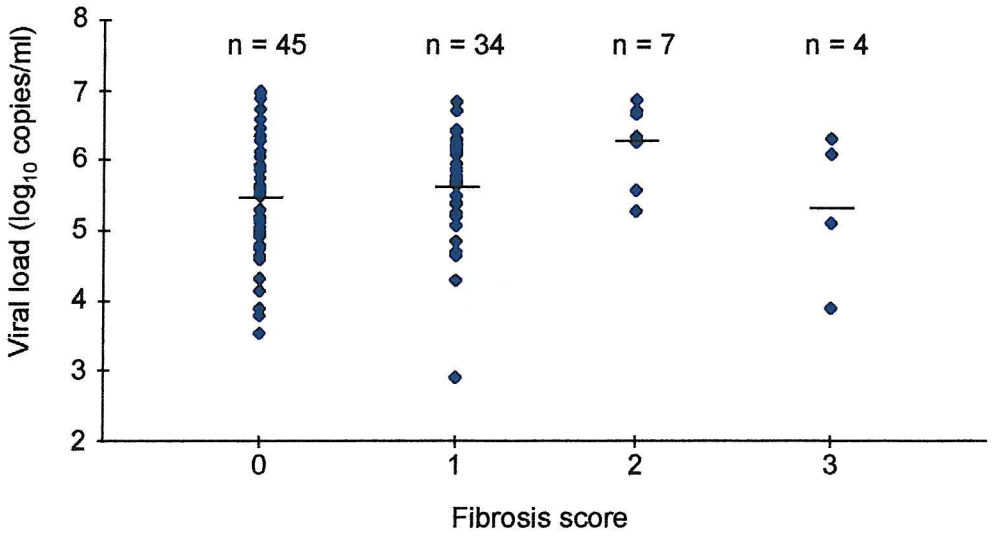


Fig.6.2 Plot of log_{10} viral load against fibrosis score for asymptomatic and symptomatic patients combined.
Sample numbers (n) and mean viral loads (horizontal lines) are shown.

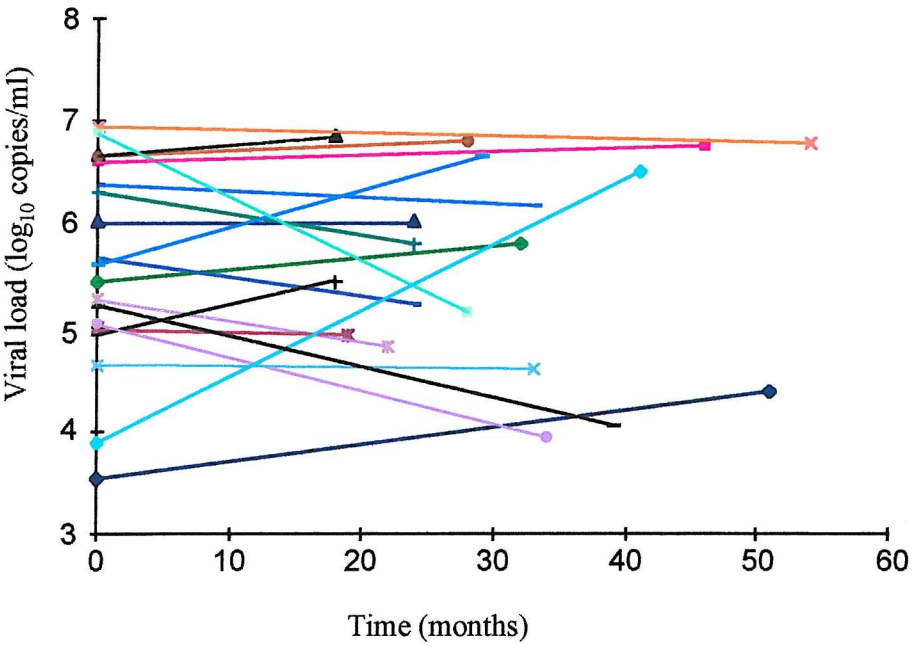


Fig.6.3 Plots of log_{10} viral load against time for asymptomatic patients.
Viral load was measured in serum samples taken at two time points for each of 19 patients.

elevated ALT levels over the study period. The patients were chosen because they had multiple stored serum samples. Viral load was analysed in multiple serum samples taken over a mean of 43.7 months (range = 32 - 59 months) (Fig.6.4).

Viral load in patient AS1 gradually increased by 36.9-fold over the 31 months studied. This corresponded to a simultaneous 1.5-fold decrease in serum ALT concentration.

Viral load in patient AS2 was more variable, showing a 3.4-fold decrease over the first 12 months studied, a 280-fold increase over the next six months, followed by a gradual 11.7-fold decrease in the final 32 months. This corresponded to a 1.4-fold decrease in serum ALT concentration over the first 12 months, a 1.6-fold increase over the next six months and a 1.4-fold decrease over the final 31 months.

Viral load in patient AS3 increased 1.63-fold over the 59 months studied. Over the same period serum ALT concentration increased 1.67-fold.

The maximum variations in viral load for patients AS1, AS2 and AS3 were 36.9-fold, 280-fold and 11.9-fold, respectively.

6.3. Discussion

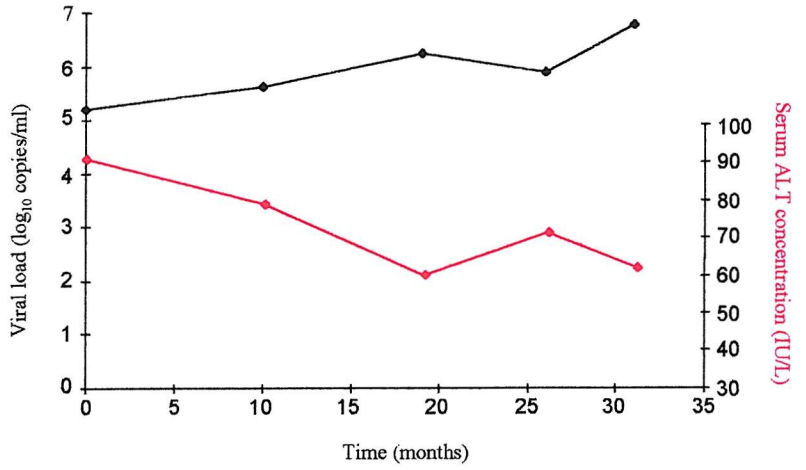
Study of viral loads in relation to liver disease severity is important for understanding the mechanisms involved in pathogenicity. Furthermore, if relationships between viral load and disease severity exist, serum viral load could be used as a surrogate marker for liver disease, reducing the need for biopsy.

The primary aim of this study was to compare viral load levels in asymptomatic and symptomatic patients. Asymptomatic HCV-positive blood donors have been found to have similar levels of liver disease as symptomatic patients (SUHT Liver Unit, unpublished data, 1998). However, there has only been one report (Gretch *et al.*, 1994) comparing viral loads between the two groups.

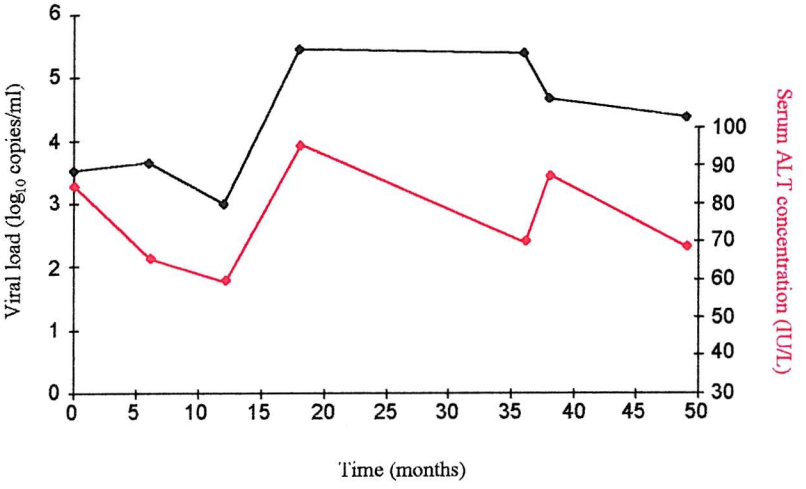
No significant difference in viral load was found between the SUHT asymptomatic and symptomatic patient groups. Gretch *et al.* (1994) also did not report any significant difference in viral load between 64 asymptomatic blood donors and 39 symptomatic patients referred for treatment of chronic hepatitis C. This indicates that asymptomatic patients pose the same infection risk as symptomatic patients and highlights the need for screening all donated blood for HCV, regardless of clinical history. It also suggests that symptoms are determined primarily by host responses to HCV infection rather than as a direct result of HCV replication.

As there were no significant differences between the asymptomatic and symptomatic patients with respect to liver histology, serum ALT levels or viral loads, the patients were

(a)



(b)



(c)

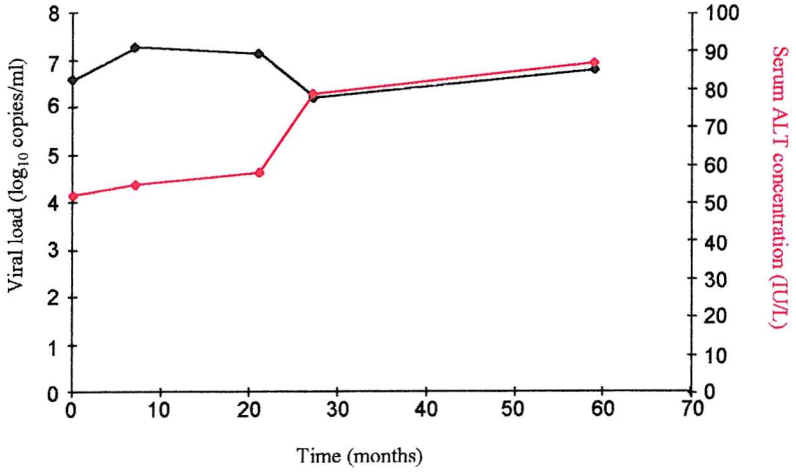


Fig.6.4 Plots of log_{10} viral load (black) and serum ALT concentration (red) against time for asymptomatic patients (a) AS1, (b) AS2 and (c) AS3.

studied as a single group to increase the statistical power of analysis.

There have been many studies of viral load in different HCV-positive patient groups using a variety of quantitative assays including the Quantiplex bDNA assays, the Amplicor Monitor assay and in-house PCR-based assays (Lau *et al.*, 1993; Montalto *et al.*, 1997; Thomas *et al.*, 2000; Fanning *et al.*, 1999; Perisco *et al.*, 2000). The limitations of these assays have been discussed (Chapter 5), making a re-evaluation of the relationship between viral load and clinical factors using the TaqMan PCR-based assay worthwhile. The range of viral loads found in the in the Southampton patients exceeded the dynamic ranges of the commercially available assays used in many previous studies, illustrating the value of the TaqMan PCR-based assay.

Viral load was found to weakly correlate with liver fibrosis score. This contradicts a study by Fanning *et al.* (1999) who reported a lack of correlation between viral load and fibrosis in a group of 77 women infected with genotype 1b HCV from a contaminated batch of anti-D immunoglobulin. Our findings also go against those of Poynard *et al.* (2001) who found that rate of fibrosis progression was dependent on age and duration of infection but not viral load in 2313 patients with chronic hepatitis C. However, the finding is supported by Gianinni *et al.* (2000) who reported a significant association between viral load and the level of fibrosis in 48 patients with chronic hepatitis C and steatosis. The correlation between viral load and level of fibrosis can be interpreted as a direct result of viral replication, or as a result of the immune response to the level of viral replication. Alternatively, as the correlation was weak and the level of statistical significance was set at $P<0.05$, the finding may have been a consequence of the large number of factors analysed.

No correlation was found between viral load and inflammation score, contradicting the results of Fanning *et al.* (1999) and Lau *et al.* (1993) who found a weak association. There was also no significant correlation between viral load and liver damage or total Knodell score which supports previous studies by Romeo *et al.* (1996) and McGuinness *et al.* (1996). However, studies of the extent of liver disease by biopsy are imperfect due to the subjective nature of the scoring system and the inherent inaccuracy of taking a single sample to be representative of the level of disease in the whole liver. In the current investigation, variation in scoring was minimised by having all biopsies examined by the same experienced hepatic pathologist.

Only 17% of the asymptomatic group had normal serum ALT concentrations, while 27% of the symptomatic group had normal ALT levels, indicating that serum ALT level does not relate to the presence or absence of symptoms. There was no significant correlation between serum ALT concentration and viral load, in agreement with findings by Choi *et al.* (1999) and

Fanning *et al.* (1999). There is disagreement over whether serum HCV RNA levels differ between patients with raised and normal ALT. There was no significant difference in viral load found between patients with normal and elevated serum ALT concentration in this study. Several other groups have reported no significant difference in viral load between the two groups (Conry-Cantelina *et al.*, 1996; Naito *et al.*, 1994; Silini *et al.*, 1995), whereas Marcellin (1999) found that mean load was slightly higher in patients with raised ALT levels.

There was no significant correlation between alcohol consumption and HCV load. This supports Anand *et al.* (2000) who found no difference in viral load between 50 chronic alcoholics and 18 completely abstinent subjects. However, the results disagree with those of Pessione *et al.* (1998) who showed a strong correlation between alcohol intake and serum HCV load in 233 patients with chronic hepatitis C. A correlation between alcohol consumption and intrahepatic HCV RNA has also been shown (Romero-Gomez *et al.*, 1998).

There was no difference in viral load between patients infected with genotype 1, 2 and 3 HCV, in agreement with the findings of Romeo *et al.* (1996), suggesting that host or other viral factors have a greater influence than genotype on HCV load. Early investigations of viral load in chronically infected HCV-positive patients were prone to genotype bias due to the genotype-dependence of the first generation of the Amplicor Monitor and Quantiplex HCV RNA assays (Mellor *et al.*, 1999). The TaqMan PCR assay was found to amplify genotypes 1 - 5 equally (Chapter 3), so the current study was probably unbiased by genotype.

There was also no significant difference in load between patients infected via different routes, in agreement with Gretch *et al.* (1994). However, this disagrees with Lau *et al.* (1993) who found higher viral load in patients who contracted HCV via BT than via other routes including *intra venous* drug use.

The number of patients studied in this project was small compared with some other reports, especially when broken down into asymptomatic and symptomatic groups. This limited the statistical power of the study and may account for the disagreements with previous reports. The clinical backgrounds of the patient groups were also very heterogeneous in terms of virus genotype, sex, mode and duration of infection compared to those described by Fanning *et al.* (1999, 2000), which also explains some of the disagreements with these reports. Homogeneous patient groups allow potentially complicating variables to be controlled for. However, such groups are rare and most studies have been performed on more heterogeneous patient groups.

Hepatitis C is a chronic disease progressing over many years. It is therefore important to understand how viral load varies over the duration of infection. Of the 19 asymptomatic patients studied, only six (32%) showed a change in viral load greater than 1 $\log_{10}$ copies/ml.

Viral load varied by more than is significant for the assay (0.5 $\log_{10}$ copies/ml) in only seven patients (37%). This supports the studies by others (Pontisso *et al.*, 1999; Hollingsworth *et al.*, 1996; Gordon *et al.*, 1998) suggesting that viral load is fairly stable over time in most patients.

The fact that there was no correlation between rate of change in viral load and initial viral load, and no significant difference in rate of change in viral load between genotypes may reflect the influence of host factors on viral replication. This disagrees with Fanning *et al.* (2000) who reported that the rate of change in viral load was negatively correlated with initial load. The lack of agreement between the results is probably due to the differences in the type of patient groups studied.

There was no significant difference in rate of change in viral load between patients infected via different routes. This contrasts with the findings of Thomas *et al.* (2000) who reported a slightly higher change in viral load in *intra venous* drug users than in patients that did not inject drugs. The population studied by Thomas *et al.* (2000) was much larger (687 patients) than the population of the current study and so had greater statistical power.

The fact that this was a retrospective study of samples stored for several years (range = 0.5 - 5 years) was not considered to have a significant affect on the results as it has been shown that viral load is stable in serum samples stored at -60°C for four years (Gordon *et al.*, 1998).

Viral load was found to be more variable in a some of the asymptomatic patients studied. The variation in load seen in these patients possibly represent temporary fluctuations in viral load that would be detected in other patients if samples were taken more frequently over time. These may relate to variation in the immune response or to changes in the viral replication rate over the duration of infection.

The extent of viral load fluctuation over time was investigated in three of the asymptomatic patients. Viral load remained quite stable in two of the patients, whereas load fluctuated over approximately 2.5 $\log_{10}$ copies/ml in one patient. This patient also had the highest degree of variation in serum ALT level. This could indicate variable levels of immune destruction of hepatocytes in response to changes in the level of virus replication. This observation agrees with those made by Pontisso *et al.* (1999) and Arase *et al.* (2000) who showed that in patients with abnormal serum ALT concentrations, viral load was more variable when ALT levels fluctuated. However, these observation need to be confirmed by following viral load and ALT level in larger groups of patients. The observation that changes in serum ALT concentration did not mirror fluctuation in viral load supports the lack of correlation between rate of change in viral load and rate of change in serum ALT in the asymptomatic patients.

The fact that load was found to vary greatly in some patients questions the validity of studies where a single measurement of viral load is taken. It has also been suggested that a

single measurement of viral load is insufficient for assessing the likelihood of a sustained response to interferon (IFN) therapy (Pontisso *et al.*, 1999). For IFN-ribavirin treatment of patients with high viral load ($\geq 3.5 \times 10^6$ copies/ml), it may be worthwhile monitoring serum HCV RNA over a period of time to assess the level of variation. Therapy could then be delayed until the load falls below this level (Arase *et al.*, 2000).

Of the 19 asymptomatic patients in whom viral load over time was studied, five subsequently received antiviral treatment. As effective treatment for HCV infection becomes more readily available, longitudinal studies of HCV load in untreated patients will become more difficult in the future.

6.4. Summary

1. No significant difference in viral load was found between 53 symptomatic and 57 asymptomatic patients with chronic hepatitis C. There was also no significant difference in serum ALT level or histological activity between the two groups.
2. In the asymptomatic and symptomatic patient groups combined, there was a weak, significant correlation between viral load and liver fibrosis. There was no significant correlation between viral load and liver inflammation, damage or total modified Knodell score, age, duration of infection, serum ALT concentration, or alcohol consumption.
3. Viral load changes were studied in 19 asymptomatic patients over 18 – 54 months. Of these, eight showed an increase in load, ten showed a decrease and one showed no overall change in load. Rate of change in viral load was not correlated with inflammation, damage, fibrosis, total histological score, initial serum ALT concentration, change in serum ALT concentration or duration of infection. No significant difference in rate of change in load was observed between patients with normal and raised initial serum ALT concentration, between patients infected with genotypes 1 and 2 and 3, or between patients infected via BT, IVDU or other routes.
4. Viral load fluctuations over time were monitored in three asymptomatic patients and were found to fluctuate by a maximum of 36.90, 280 and 11.88-fold. The most variable viral load was seen in the patient with the largest fluctuations in serum ALT concentration.

7. Virological response to treatment of hepatitis C with pegylated interferon-α plus ribavirin combination therapy

7.1. Introduction

7.1.1. Treatment of hepatitis C

The primary aim of treatment is the clearance of HCV from the patient. Serum HCV RNA load is monitored to assess efficacy of therapy. None of the currently available treatments are able to eradicate infection in all patients. There are three main categories of response to antiviral therapy currently in use (Craxe *et al.*, 1999):

(i) End of treatment response (ETR) - Normal ALT level and HCV RNA undetectable by PCR at the end of treatment.

(ii) Sustained response (SR) - Normal serum ALT level at the end of treatment and over a six-month observation period with HCV RNA undetectable by PCR six months after stopping therapy.

(iii) Non-response (NR) - abnormal serum ALT level, regardless of HCV RNA status after three months of interferon monotherapy or six months of interferon plus ribavirin combination therapy.

SR is the main measure of treatment success as, for IFN-based treatment, biochemical or virological relapse occurs maximally within six months and is negligible at one year post-treatment (Civeira and Prieto, 1999). SR appears to be a good indicator of long-term HCV serum clearance, with patients remaining negative ten years after treatment with IFN-α (Lau *et al.*, 1998).

A range of compounds has been used for treatment of HCV infection with varying degrees of success.

(i) Interferon-α

Until 1999, interferon (IFN)-α monotherapy was the only treatment for hepatitis C licensed for use in the UK. Natural IFN-α is a glycoprotein cytokine produced by PBMCs and exists as 13 subtypes. The forms of IFN-α most widely used for treatment are IFN-α2a (Intron A, Schering Plough) and IFN-α2b (Roferon-A, Roche) both of which are produced using cloned genes from human leukocytes. IFN-α induces several direct and indirect antiviral mechanisms

including intracellular RNA degradation via the 2'-5'(A) synthetase /RNase L and dsRNA-dependent protein kinase pathways, inhibition of viral RNA translation, up-regulation of MHC class I and II molecule expression, induction of IL12 receptors on Th1 cells, and prevention of viral infection of susceptible cells (Thomas *et al.*, 1999; Vilcek and Sen, 1996). IFN-α has several effects on HCV infection: reduction of virion production rate, induction of an antiviral state in uninfected cells, enhancement of infected cell lysis, and inhibition of fibrosis and HCC (Thomas *et al.*, 1999).

Treatment with 3MU of IFN-α three times per week results in SR in just 6 - 21% of patients. Treatment for 12 - 24 months gives increased SR rates of 35% (Poynard *et al.*, 1996). The use of higher doses of IFN-α does not significantly improve ETR or SR rates (reviewed by Davis, 1999). Predictors of a poor virological response are advanced fibrosis or cirrhosis, longer duration of infection, male sex, high pre-treatment viral load ($>1x10^6$ copies/ml), hepatic iron deposition and infection with genotype 1 virus (Lewis and Freedman, 2000).

IFN-α treatment can result in side effects including fever, alopecia, myalgia, arthralgia, fatigue, nausea, headache, diarrhoea and depression. Approximately 10% of patients will not complete treatment because of side effects (Lewis and Freedman, 2000).

(ii) Interferon-β

Small-scale trials of treatment using IFN-β have been performed. Treatment of 21 drug-naïve patients with 9MU recombinant IFN-β three times per week gave an SR rate of 19%, suggesting efficacy similar to that of IFN-α monotherapy (Habersetzer *et al.*, 2000).

(iii) Consensus IFN

Consensus IFN was developed by scanning IFN-α subtype sequences and producing a recombinant protein with the most frequently observed amino acid at each position. The resulting 19.5kDa protein shares approximately 30% homology with IFN-β and 89% with IFN-α2b. In a comparison of consensus IFN with IFN-α2b, treatment with consensus IFN resulted in slightly higher (12.1% versus 11.3%) SR rate and similar frequency of side effects (Tong *et al.*, 1997).

(iv) Pegylated IFN-α

Chemically modified versions of interferon have recently been developed by addition of a polyethylene glycol moiety to IFN-α. There are two forms of pegylated IFN (PEG-IFN) currently under investigation: a linear 12kDa PEG-IFN-α2b (PegIntron, Schering Plough) and

a branched methoxy 40kDa PEG-IFN-α2a (PEGASYS, Roche). PEG-IFN has an approximately ten-fold increased serum half-life compared with IFN-α. This results in the drug remaining in the bloodstream for much longer than the parent molecule, and weekly doses have proven more efficacious than thrice weekly doses of IFN-α (Roche clinical study protocol, 2000; Shiffman *et al.*, 1999). SR rates of 23 - 39% have been reported, with frequency and severity of side effects similar to IFN (Trepo *et al.*, 2000; Zeuzem *et al.*, 2000).

(v) Ribavirin

Ribavirin is a guanosine analogue that is active against a broad spectrum of DNA and RNA viruses. Ribavirin monotherapy results in reduced serum ALT but does not reduce HCV RNA levels (Davis, 1999). Its mode of action against HCV is not well understood, but more than one process is believed to be involved (reviewed by Thomas *et al,*. 1999). The drug readily enters cells and undergoes phosphorylation to mono-, di- and triphosphates. The triphosphate form selectively inhibits the influenza virus RNA polymerase but has no effect on the HCV NS5B. Ribavirin phosphate is known to inhibit inosine monophosphate dehydrogenase. This may contribute to the antiviral activity by inhibiting DNA and RNA synthesis through depletion of intracellular GTP pools. This is not thought to affect viral RNA synthesis directly but probably acts through allosteric regulation of viral enzymes. It is not known why this does not significantly affect HCV replication in the absence of IFN but it is possible that IFN increases the requirements of some cells for nucleotides. Ribavirin may also affect extrahepatic HCV replication as it is concentrated in mononuclear cells. IFN may increase GTP requirement in these cells by stimulating replication.

Ribavirin has also been shown to affect the immune Th1/Th2 balance in infected animals and to enhance macrophage inhibition of viral replication *in vitro*, suggesting the drug may also act by enhancement of the immune response against HCV.

The use of IFN-α and ribavirin combined for anti-HCV therapy was licensed for use in the UK in 1999 and is the currently recommended treatment for moderate to severe hepatitis C (National Institute for Clinical Excellence, 2000). This combination has been shown to give significantly increased ETR (51 - 55% versus 29%) and SR rates (31 - 43% versus 6 - 16%) when compared with interferon alone (Poynard *et al.*, 2000). IFN-α plus ribavirin combination therapy has also been shown to give increased SR rates in patients that originally relapsed within six months of IFN monotherapy when compared with a second treatment of IFN alone (49% versus 5%) (Davis *et al.*, 1998). Predictors of SR to combination therapy as initial treatment are (in descending order of predictive value): infection with genotype 2 or 3 virus, pre-treatment viral load $<3.5x10^6$ copies/ml (by NGI SuperQuant assay), no or only

portal fibrosis, female gender and age <40 years (Poynard *et al.*, 2000). Viral load and genotype are also predictive of SR in patients who relapsed after IFN monotherapy (Davis *et al.*, 1998).

The main side effect of ribavirin monotherapy and IFN/ribavirin combination therapy is a reversible haemolytic anaemia due to accumulation of the drug in erythrocytes (Lewis and Freedman, 2000). The frequency and severity of other side effects of combination therapy are similar to IFN monotherapy (Christie and Chapman, 1999).

(vi) Amantadine

Amantadine was developed for the treatment of influenza A virus infection and has also been used for treatment of Parkinson's disease. It is thought to act against influenza virus by blocking viral uncoating and therefore prevents infection of new cells, but no inhibition of the virus-associated RNA-dependent RNA polymerase has been described (Torre *et al.*, 2001). Amantadine taken orally showed early promise as a monotherapy for HCV infection (Smith, 1997), but in larger trials only gave a 2% SR rate (El-Zayadi *et al.*, 1998). In combination with IFN-α, amantadine gave ETR rates of 9 - 14% in IFN non-responders (El-Zayadi *et al.*, 1998; Younossi *et al.*, 1998).

(vii) Iron depletion

HCV infection frequently leads to increases in serum ferritin, iron and transferrin saturation and liver biopsies may contain excess iron deposits (Lewis and Freedman, 2000). Treatment of these symptoms by venesection has been shown to significantly improve serum aminotransferase concentrations (Hayashi *et al.*, 1994). There is also evidence to suggest that iron depletion by venesection or intravenous desferrioxamine may also improve SR rates in patients treated with IFN (Van Thiel *et al.*, 1996; Bayraktar *et al.*, 1995). However, the exact mechanism of hepatic iron depletion in treatment of hepatitis C has yet to be defined (Lewis and Freedman, 2000).

(viii) Ursodeoxycholic acid

Ursodeoxycholic acid (UDCA) is a non-toxic bile acid used to treat gallstones. It may act as a membrane-protective agent as it is capable of preventing erythrocytes and hepatocyte damage induced by noxious agents and may have immunomodulatory effects. UCDA in combination with IFN has been shown to give higher rates of serum ALT normalisation than IFN monotherapy, but does not alter virological response rate (Boucher *et al.*, 1995; Angelico *et al.*, 1995).

7.1.2. Roles of HCV RNA quantitation in antiviral research and patient management

Measurement of serum HCV load has several important applications with respect to treatment of HCV:

(i) Prediction of treatment response

IFN-based treatments are expensive, can have significant side effects and are not effective in all patients. It is therefore important to assess the likelihood of such treatment to benefit the patient (Davis and Lau, 1997).

Sequential quantitation of patient serum HCV RNA has shown viraemia levels to be relatively stable in most untreated, chronically infected patients (Gordon *et al.*, 1998; Chapter 6), allowing pre-treatment viraemia level to be correlated with IFN response. Sustained IFN response is more common in patients with pre-treatment serum viral loads of $<1x10^6$ genomes/ml than in patients with pre-treatment loads $>1x10^6$ genomes/ml (50.5% versus 17.3%; Davis and Lau, 1997). Patients with pre-treatment viral loads of $<3.5x10^6$ copies/ml reportedly show a higher SR rate to IFN-ribavirin combination therapy than those with higher viraemia levels (40-44% versus 26-38%, respectively; Poynard *et al.*, 2000).

(ii) Monitoring of treatment responses

Monitoring HCV viral load throughout treatment is important to assess the efficacy of the therapy. Viral load monitoring has proved valuable in assessing responses to IFN monotherapy and IFN plus ribavirin combination therapy (Davis *et al.*, 1998; McHutchinson *et al.*, 1998). Reduction in viral load through IFN therapy has been correlated with improved liver histology (Shiffman *et al.*, 1997). Measurement of a treatment's success (i.e. reduction of serum viral load to undetectable levels) depends upon the detection limit of the assay used. Although quantitative assays offer the clinician more information on the rate of response to therapy, qualitative assays such as the Bayer VERSANT TMA assay which has superior sensitivity (10 IU/ml) are still important for determining HCV RNA clearance (Sarrazin *et al.*, 2001).

(iii) Study of viral kinetics

The study of HCV RNA levels after treatment has provided insights into the natural history of HCV infection and the action and efficacy of therapeutic agents. Mathematical evaluation of decreases in viral RNA levels in response to IFN monotherapy (Zeuzem *et al.*, 1996; Lam *et al.*, 1997; Neumann *et al.*, 1998), and combination therapy with IFN and ribavirin (Zeuzem *et*

al., 1998) has provided information on the mode of action of these drugs and on viral turn-over rates. Treatment with IFN and ribavirin resulted in a biphasic fall in viral load, with an initial rapid IFN dose-dependent decrease within the first day, followed by a less rapid decrease thought to be due to host responses against infected cells. Zeuzem *et al.* (1998) also deduced that ribavirin has an immunomodulatory effect and has no direct synergy with the antiviral activity of IFN. Neumann *et al.* (1998) reported that pre-treatment HCV turn-over is approximately 10^{12} virions/day and that IFN acts by blocking viral production or release.

It has also been reported that a rapid decrease in viral load over the first 12 weeks of therapy (Weiland *et al.*, 1999) and HCV RNA-negativity at week 12 (Castro *et al.*, 2000) are predictive of SR to IFN-α. A decline in viral load of ≥ 1.2 $\log_{10}$ copies/ml over the first two months of treatment with IFN/ribavirin combination therapy in IFN non-responders was found to be predictive of SR (Castro *et al.*, 2000).

7.1.3. PEG-IFN plus ribavirin combination therapy

Monotherapy using PEG-IFN and IFN/ribavirin combination therapy have given significant improvements in SR rates over IFN-α monotherapy. It was therefore hypothesised that treatment with a combination of PEG-IFN and ribavirin would give still higher SR rates. Manns *et al.* (2001) recently reported the results of a randomised open-label trial comparing Schering Plough 12kDa PEG-IFN plus ribavirin with IFN plus ribavirin for initial treatment of hepatitis C in 1530 patients and found sustained virological response rates of 47 - 54% for PEG-IFN plus ribavirin.

Researchers have recently begun to study the dynamics of viral load in response to PEG-IFN plus ribavirin. Cornberg *et al.* (2001) recently found no significant difference in mean reduction in viral load over four and 12 weeks of therapy between 40 patients treated with 12kDa PEG-IFN and ribavirin and the same number treated with IFN-α and ribavirin. However, a higher proportion of patients that showed a rapid decline in load (greater than two logs over four weeks) were treated with PEG-IFN plus ribavirin. It remains to be seen whether the rapid decline in load relates to SR in these patients.

A phase III, randomised, multicentre efficacy and safety study is currently being carried out to examine the effects of duration of treatment and daily dose of ribavirin in patients with chronic HCV infection treated with the combination of Roche 40kDa PEG-IFN-α2a (PEGASYS) and ribavirin (Roche clinical study protocol, 2000). Of 1280 patients enrolled in the international trial, eight were treated at SUHT and studied for this thesis.

7.2. Viral load in HCV-positive patients treated with PEG-IFN and ribavirin

7.2.1. Patient treatment and sample collection

Virological response was monitored in eight patients (P1 - 8) treated with Roche PEG-IFN and ribavirin (Table 7.1). The primary objective of the trial was to compare the efficacy and safety of PEG-IFN and ribavirin given for 24 weeks with the same treatment given for 48 weeks. The secondary objective was to compare efficacy and safety of two dose regimens of ribavirin (800mg/day or 1000-1200mg/day) in combination with PEG-IFN.

The patients were selected according to the following criteria:

(i) Age ≥18 years.

(ii) Serologically proven chronic hepatitis C.

(iii) Received no prior antiviral therapy.

(iv) Quantifiable HCV load (>2000 copies/ml by Amplicor Monitor).

(v) Abnormal serum ALT levels in at least two samples taken ≥14 days apart in the six months prior to treatment (>45 IU/L).

(vi) Biopsy performed within 15 months of start of treatment showing chronic liver disease.

(vii) Not suffering from other forms of liver disease, HCC, decompensated liver disease, severe depression or other psychological disorders, cardiac or renal disease, seizure disorders, anaemia, severe retinopathy, HIV or HBV infection.

Table 7.1 Characteristics of patients prior to treatment with PEG-IFN and ribavirin.

Patient	Sex	Age (years)	Viral load (copies/ml)	HCV genotype	ALT (IU/L)	Histological scores			
						I	D	F	Total
P1	Female	47	$9.7x10^5$	3a	59	3	1	2	6
P2	Male	52	$3.9x10^5$	1a	44	2	1	1	4
P3	Male	35	$1.7x10^6$	1a	71	2	2	2	6
P4	Male	45	NK	1a	111	2	2	2	6
P5	Female	44	$1.6x10^6$	1b	28	2	1	1	4
P6	Male	44	$6.1x10^6$	1b	80	2	1	2	5
P7	Male	42	$5.2x10^6$	3a	104	2	0	2	4
P8	Male	49	$1.6x10^6$	3a	70	2	1	1	4

NK = not known, I = inflammation, D = damage, F = Fibrosis.

Patients were treated for either 24 or 48 weeks with 180 μg/week PEG-IFN and either 1000 - 1200 or 800mg/day ribavirin (Table 7.2). Departures from this schedule were necessitated by clinical responses such as low white cell count or development of significant side effects. Unless altered, the dose of ribavirin was blinded until completion of the trial. Hence, ribavirin doses were unknown at the time of submission of this thesis. Duration of treatment was blinded until week 24. Serum samples were taken immediately before administering the first dose (week zero) and after one, two, four, six, and 12 weeks and at the end of treatment (24 or 48 weeks). Serum HCV RNA concentration was quantified using the TaqMan PCR-based assay. Serum was also sent for biochemical analysis (including measurement of ALT concentration) by the hospital pathology department.

7.2.2. Response to treatment

All of the patients showed some degree of virological response to treatment (Fig.7.1). The least change in viral load was observed for patient P6 with only a 6.7-fold maximum decrease in load. HCV RNA remained detectable throughout the treatment period in this patient and returned to approximately the same concentration as before treatment. In all of the other patients HCV RNA became undetectable within 12 weeks.

The rate of initial decline in viral load and serum ALT concentration differed between the patients (Table 7.3, Fig.7.3). All patients except P6 and P4 (no baseline sample available) showed a decrease in load over the first week of therapy. All except P5 showed a decrease in ALT over the first week. Overall, serum ALT levels decreased in all patients except P6 in whom levels remained elevated (Fig.7.2). ALT levels had normalised by week 12 in all patients except P4 and P6. ALT levels in patient P5 were found to have normalised prior to treatment and remained normal throughout the study period.

At the end of treatment, all patients except P6 were negative for HCV RNA (87.5% end of treatment virological response rate) (Table 7.3). Six patients had normal serum ALT levels at the end of treatment, while levels in patient P4 were 1.2-fold above normal and patient P6 1.9-fold above normal (75% end of treatment biochemical response rate). The ETR rate in these patients was therefore 75%.

Higher ETR rates were obtained for patients with the following baseline characteristics (Table 7.4): age <40 years, male gender, genotype 3 virus, load $<3.5 \times 10^6$ copies/ml, serum ALT concentration <100 IU/L. However, sample sizes were too small to test statistical significance.

Table 7.2 Schedule of PEG-IFN and ribavirin treatment and doses.

Patient	Drug*	Week of treatment																			
		0	1	2	3	4	5	6	7	8	9	10	11	12	13-14	15-16	17-24	25-29	30-36	36-42	42-48
P1	I	180		135	90			0	45		90		0	45							
	R	B																			
P2	I	180											135								
	R	B														0	600				
P3	I	180																0			
	R	B	0																		
P4	I	180				135						90									
	R	B																			
P5	I	180																			
	R	B														0	200	0			
P6	I	180																0			
	R	B																0			
P7	I	180		0	180	0		90		0		45						0			
	R	B																0			
P8	I	180																0			
	R	B													0	600		0			

*PEG-IFN dose (I) is shown in μg/week; ribavirin (R) dose is shown in mg/day. B = blinded

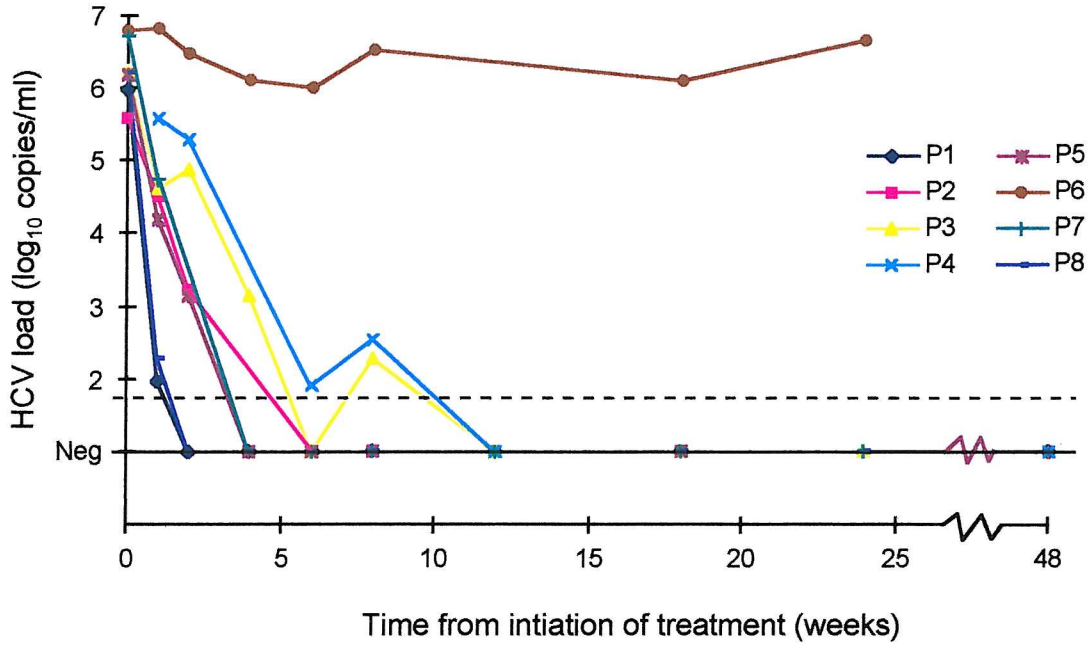


Fig.7.1 Plots of viral load over total treatment periods.
Dashed line = limit of detection for TaqMan assay. Neg = HCV RNA undetectable.

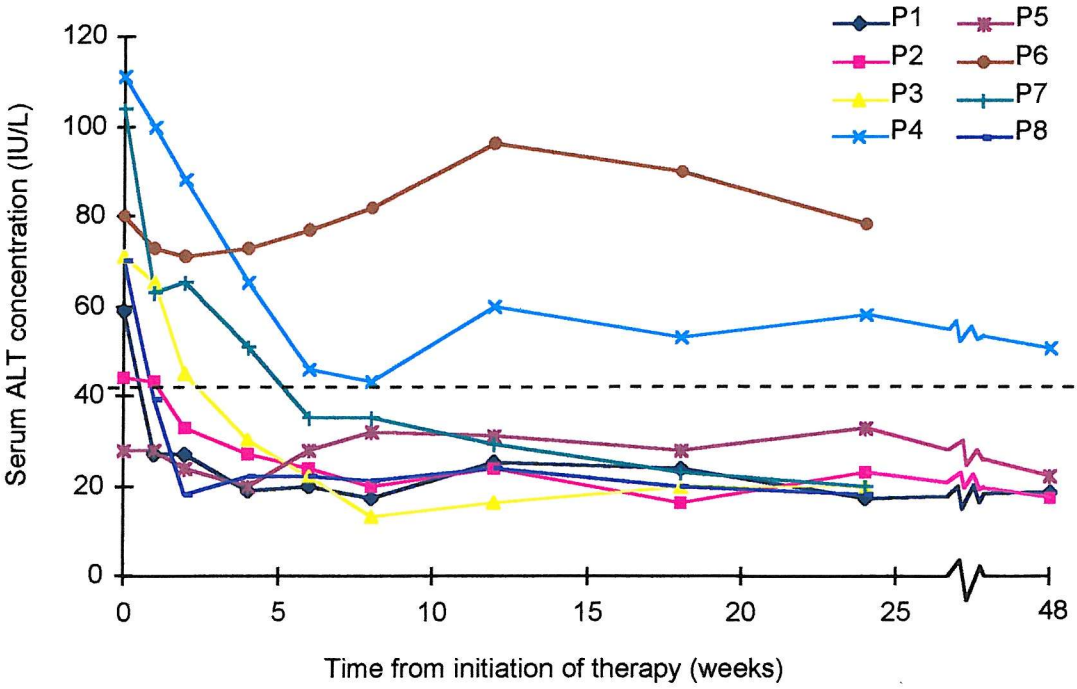


Fig.7.2 Plots of serum ALT concentration over total treatment periods.
Dashed line = upper limit of normal serum ALT concentration.

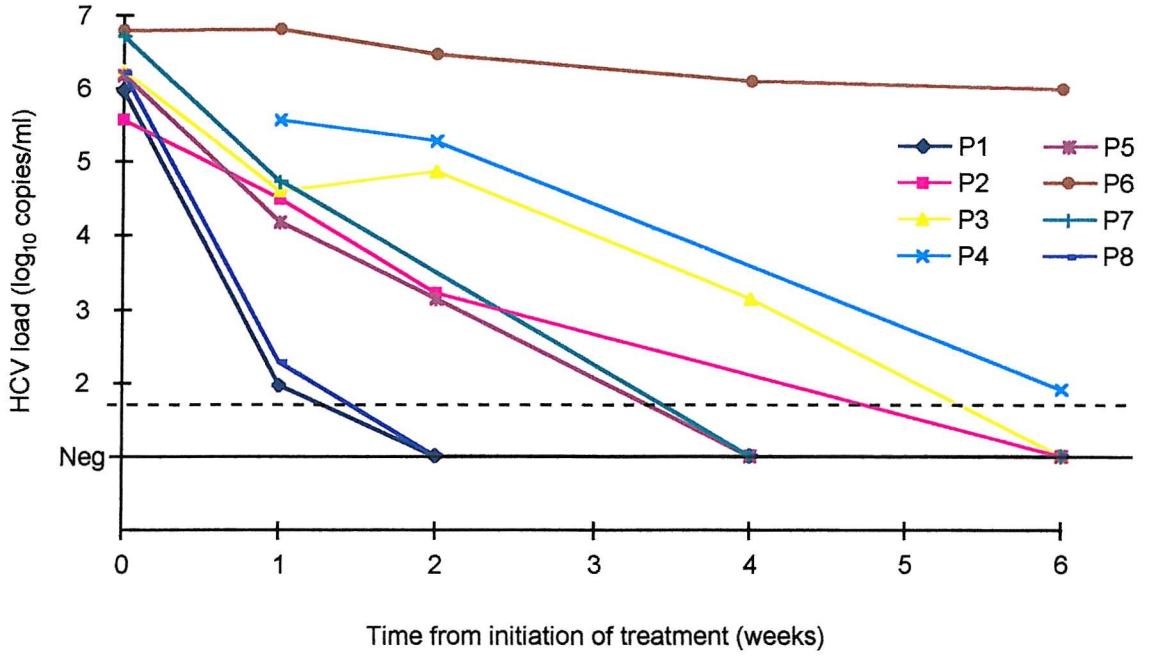


Fig.7.3 Plots of viral load over first six weeks of therapy.
Dashed line = limit of detection. Neg = HCV RNA undetectable.

Table 7.3 Initial serum HCV load and ALT responses in patients treated with PEG-IFN and ribavirin.

Patient	Response in first week of therapy			Week of serum HCV RNA clearance*	End of treatment serum ALT concentration, IU/L (Week of normalisation)
	Fold HCV load change	Rate of HCV load change (copies/ml/day)	Fold ALT change		
P1	-10312	$-1.38x10^5$	-2.19	4	19 (1)
P2	-12	$-5.15x10^4$	-1.02	6_4	18 (2)
P3	-42	$-2.40x10^5$	-1.09	12	20 (4)
P4	NK_0	NK_0	-1.11	12	51
P5	-101	$-2.22x10^5$	1.00	4	22 (0)
P6	+1.1	$+9.11x10^4$	-1.10	-	78
P7	-93	$-6.37x10^5$	-1.65	4_2	20 (6)
P8	-8508	$-2.32x10^5$	-1.79	2	18 (1)

Decrease and increases are indicated by - and + symbols, respectively.
Weeks where samples were not obtained are shown in subscript. NK = not known.
*HCV RNA was undetectable at end of treatment in all patients except P6 ($4.43x10^6$ copies/ml).

Table 7.4 ETR rates to PEG-IFN and ribavirin according to baseline characteristics.

Baseline characteristic		ETR rate, % (number of responders/total)
Age (years)	≥40	71 (5/7)
	<40	100 (1/1)
Sex	M	67 (4/6)
	F	100 (2/2)
Genotype	1	60 (3/5)
	3	100 (3/3)
HCV load (copies/ml)*	$\geq 3.5 \times 10^6$	50 (1/2)
	$< 3.5 \times 10^6$	100 (5/5)
ALT (IU/L)	≥100	50 (1/2)
	<100	83 (5/6)
Duration of treatment (weeks)	24	75 (3/4)
	48	75 (3/4)

*Baseline load unattainable for patient P4.

7.3. Discussion

Quantitative HCV RNA assays have proven invaluable for the study of virological responses to therapy in patients with HCV infection. Most clinicians advocate tests with detection limits down to 100 copies/ml for monitoring treatment response (Weiland, 1998). The TaqMan PCR-based assay has a detection limit of 50 copies/ml and has a 6-log dynamic range. This makes the assay ideal for quantifying changes in viral load and determining viral clearance from the serum in treated patients.

A combination of Roche PEG-IFN and ribavirin is currently under trial for treatment of chronic HCV infection. In this small-scale study of viral load in patients treated with this combination, one of the patients (P6) showed no improvement in viral load or ALT concentration. One patient (P4) showed a virological clearance but their serum ALT level did not normalise. Overall, treatment resulted in a high ETR rate (75%). This compares with ETR rates of 29% and 51 - 55% for IFN monotherapy and IFN-ribavirin combination therapy, respectively (Poynard *et al.*, 2000), suggesting PEG-IFN and ribavirin may be suitable for treatment of chronic hepatitis C. The findings that higher ETR rates were given by patients with age <40 years, male gender, genotype 3 virus, and load $< 3.5 \times 10^6$ copies/ml agrees with the findings of Poynard *et al.* (2000) for IFN and ribavirin combination therapy. Duration of treatment had no effect on SR rate. Unfortunately ribavirin doses were blinded and not

available at the time of submission of this thesis. The effect of ribavirin dose, therefore, can not be assessed.

Decline in HCV load corresponded to a decrease in serum ALT concentration, suggesting reduced liver inflammation and necrosis, consistent with findings for IFN monotherapy and IFN-ribavirin combination treatment. Post-therapy biopsies have not yet been performed so it is not known whether treatment resulted in improved liver histology in these patients.

Rate of viral load decrease varied considerably between the patients. There appeared to be four sub-sets of virological response rate within these patients: rapid responders, intermediate responders, slow responders and non-responders. The two fastest responders were patients with genotype 3 virus. The slow responders and the non-responder were all infected with genotype 1 virus. This suggests a relationship between response rate and HCV genotype. This is consistent with the higher IFN monotherapy SR rate of genotype 3 virus compared with genotype 1 virus. The rate of load decrease did not appear to relate to any other baseline characteristic. Neumann *et al.* (2000) reported higher rates of primary and secondary phase viral load decline in patients infected with genotype 2 HCV than those with genotype 1 virus during treatment with IFN-α supporting the findings that rate of response to PEG-IFN and ribavirin may also be genotype-dependent.

Manns *et al.* (2001) reported sustained virological response rates of 47 - 54% for 12kDa PEG-IFN plus ribavirin and found higher rates in patients infected with HCV genotypes 2 and 3 than those with genotype 1 (80% versus 33 - 42%). These results are consistent with those of the current study with respect to the effects of genotype. From the results of this small-scale study, it is reasonable to suggest that the performance of 40kDa Roche PEG-IFN plus ribavirin will provide similarly increased SR rates compared with currently available treatments.

Of the six patients whose viral loads were measured at week four of treatment, load decreased by >2 $\log_{10}$ copies/ml relative to baseline load in five (83%). This compares with 68% of 40 patients in the study of 12kDa PEG-IFN plus ribavirin described by Cornberg *et al.* (2001).

It is only possible to draw limited conclusions from the results of this study due to several factors: The number of patients enrolled in the study was small, making statistical analysis impossible. The number of different treatments regimens administered decreased the number of patients per treatment further. Deviations from the treatment schedule made necessary by side effects also make analysis of the data difficult. For example, patient P3 received ribavirin for only one week which may explain why viral load in this patient increased by 1.9-fold between weeks one and two. Problems were also caused by the nature of the patients studied,

with blood samples being unattainable at several time points due to difficulties in extracting blood from sunken veins of IVDUs. These results need to be confirmed by studying a larger number of patients. Analysis of the data from all patients enrolled in the multicentre drug trial should provide stronger evidence for any difference in ETR and SR rates between PEG-IFN and ribavirin combination therapy and currently used treatments.

It was not possible to measure loads on a daily basis in these patients because of the constraints of the clinical study protocol. However, the kinetics of viral load decline during the first one or two weeks of treatment also needs to be studied in more detail. This should provide insights into the mode of action of the PEG-IFN and ribavirin combination, as has been the case for IFN and IFN plus ribavirin therapies.

Because of the debilitating side effects and cost of treatment with IFN-based therapies, improved algorithms for prediction of SR are continually being sought. The study of viral loads during treatment may allow more accurate and earlier prediction of SR. Rate of initial decline in viral load has been found to be of greater value than HCV genotype or pre-treatment viral load for prediction of SR to IFN-α (Zeuzem *et al.*, 1998). Further studies are needed to determine predictive factors for SR after PEG-IFN plus ribavirin treatment.

7.4. Summary

1. Virological and biochemical responses were studied in eight patients with chronic hepatitis C treated with PEG-IFN and ribavirin.
2. End of treatment virological and biochemical response rates were 87.5% and 75%, respectively. Overall ETR rate was 75%.

8. Effects of immunomodulatory compounds on HCV levels in cultured peripheral blood mononuclear cells

8.1. Introduction

The study of HCV biology has been hampered by the lack of an efficient, convenient, reproducible model for virus replication. HCV replication systems described so far have been based on animal models, infection of cultured cells, propagation of cells from infected patients, or transfection of cell lines and animals with cloned HCV sequences (reviewed recently by Bartenschlager and Lohmann, 2000).

8.1.1. Model systems for the study of HCV replication

(i) Animal models

The only reproducible animal model available for the study of HCV infection is the chimpanzee (*Pan troglodytes*). Several groups in the 1970s showed the transmission of NANBH to chimpanzees after intravenous administration of various human inocula. Direct intrahepatic transfection of chimpanzees with full-length cloned HCV RNA transcripts has also been described. However, use of the chimpanzee model is limited by ethical reasons, its scarcity and high maintenance costs.

Experimentally inoculated tree shrews (*Tupaia belangeri chinensis*) were studied as a potential small animal model. However, only about a quarter of animals became infected and viraemia was transient or intermittent and at low levels. Recently, transgenic SCID mice carrying human hepatocyte engraftments were reported to be susceptible to HCV infection and produce high levels of virus (Mercer *et al.*, 2001).

(ii) Cell culture systems

Several cell culture systems for HCV have been described. These have either been based on infection or transfection of cultured cells *in vitro* or cultivation of infected cells from HCV-positive patients.

Infection of human and chimpanzee primary hepatocytes has been reported, as has infection of the hepatoma cell line PH5CH, T- and B-cell lines. HCV has also been shown to productively infect African green monkey Vero and Mosquito AP61 cells (Germi *et al.*, 2001). The most success has been found using HPBMa10-2 T-cell and Daudi B-cell lines in which

virus was detectable for over one year post-infection and could be transmitted to naïve cells and a chimpanzee.

HepG2 cells transfected with a full-length cloned cDNA have been shown to produce particles infectious for Daudi cells. Subgenomic RNAs have also been created and shown to replicate in Huh-7 cells. Transfection systems will allow manipulation of the viral genome for mutational analysis of viral functions.

HCV replication has also been demonstrated in primary hepatocytes and PBMCs taken from infected patients. Cell culture systems described so far for HCV suffer from poor reproducibility and low level virus production.

8.1.2. HCV replication in PBMCs

There have been several reports of HCV replication in PBMCs. Negative-strand HCV RNA has been detected in PBMCs, indicating the possibility of viral RNA replication. In addition, cultured PBMCs have been infected *in vitro* resulting in viral replication (Cribier *et al.*, 1995) although at a very low level. The level of HCV RNA in PBMCs cultured from infected patients is also reported to be low, with only about 0.2 - 8.1% of cells infected (Muratori *et al.*, 1996).

In an attempt to stimulate higher levels of viral replication in PBMCs cultured from HCV-infected patients, the effects of several immunomodulatory compounds were assessed. It was hoped that this would also give insights into the possible effects of using such compounds for therapy of HCV. The effects of the following immunomodulatory compounds on HCV RNA levels in PBMCs were assessed:

(i) Phytohaemagglutinin

Phytohaemagglutinin (PHA) is a plant lectin, which is able to agglutinate many mammalian red blood cell types, alter cell membrane transport systems, alter cell permeability to proteins, and interfere with cellular metabolism. PHA is also able to trigger DNA synthesis in T-cells, and has been shown to activate latent HIV-1 in PBMCs (Chun *et al.*, 1997).

(ii) Hydrocortisone

Hydrocortisone is a glucocorticoid immunosuppressant. These act mainly via their effects on antigen-presenting cells in which they influence production of cytokines as well as expression of cell surface molecules (Girndt *et al.*, 1998). They also inhibit T-cell proliferation and IL-2 production (Goodwin *et al.*, 1986).

(iii) Cyclosporin A

Cyclosporin is a cyclic polypeptide consisting of 11 amino acids and is a potent immunosuppressive agent. It acts specifically and reversibly on lymphocytes, inhibiting production and release of lymphokines including interleukin-2 and -3, blocking lymphocyte activation at the G_0 or G_1 phase of the cell cycle, and inhibiting antigen-triggered release of lymphokines by activated T-cells (Nair *et al.*, 1999).

8.2. Effects of immunomodulatory compounds on the level of HCV RNA in cultured PBMCs

8.2.1. Patient PBMC samples

PBMCs were extracted from whole blood of three HCV RNA-positive patients with chronic hepatitis C (C1, C2, C3) (Table 8.1). None of the patients were receiving antiviral therapy and none were infected with HBV or HIV.

Table 8.1. Characteristics of PBMC donors.

Patient	Age (years)	Sex	Serum HCV load (copies/ml)	HCV genotype	Histological activity scores			
					I	D	F	Total
C1	35	M	$4.64x10^4$	1b	2	1	1	4
C2	56	M	$2.13x10^5$	3a	3	1	3	7
C3	43	F	$1.19x10^5$	3a	2	1	2	5

M = male, F = female, I = inflammation, D = damage, F = fibrosis.

8.2.2. HCV RNA level in cultured PBMCs treated with immunomodulatory compounds

The efficiency of detection of HCV RNA in the presence of PBMCs was assessed in a preliminary experiment (data not shown). PBMCs were collected from an HCV-negative individual and RNA was extracted after the addition of 100 and 1000 IUs of the WHO International Standard. Quantitation using the TaqMan PCR-based assay gave values within three-fold of the value expected from the titre published by Saldanha *et al.* (1999). From the assay detection limit for HCV RNA from serum (50 copies/ml, equivalent to 14 copies /extraction), the limit of detection of HCV RNA from cells was estimated as being approximately 14 copies/cell pellet.

PBMCs from patient C1 were cultivated for seven days at a density of $1x10^6$ cells in each culture well. The cells were either untreated, or treated with cyclosporin A (0.5μg/ml),

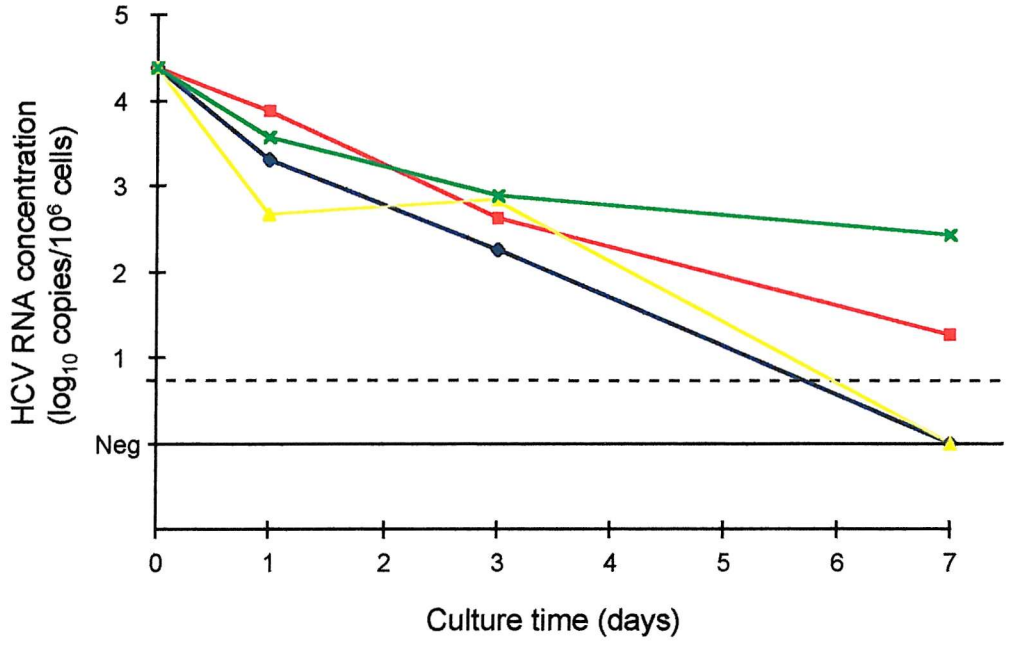
hydrocortisone (1μM) or PHA (0.5% v/v). The cells and medium were collected, separated and stored at -80°C after one, three, four, six and seven days. RNA was then extracted from the cells and media and HCV RNA quantified using the TaqMan PCR-based assay. RNA extraction and amplification efficiencies were monitored using IC C_Ts and there was no evidence of inhibition. HCV RNA was only detected in the uncultured PBMCs (day zero), but was at a concentration of less than 14 copies/cell pellet, the nominal detection limit of the assay. HCV was not detected in any of the culture media.

The experiment was repeated except for each culture being seeded with $2.5x10^6$ PBMCs from patient C2 who had a 4.6-fold higher viral load than patient C1 (Fig.8.1). HCV RNA in the uncultured PBMCs was found to be at a concentration of $2.40x10^4$ copies/10^6 cells and was undetectable in the culture medium. After one day of culture, the HCV RNA concentration in the cells had decreased by 3.2 to 51.3-fold. The HCV RNA concentration in the hydrocortisone-treated cells increased by 1.5-fold between days one and three, whereas the HCV RNA concentration in the other cells decreased by 4.7 to 17.6-fold. HCV RNA was detectable at low levels in the cyclosporin A and PHA-treated cells (23.6 and 2.9-fold decreases, respectively) after seven days, but was undetectable in the untreated and hydrocortisone-treated cells. HCV RNA became detectable in the culture media after one day. HCV RNA concentrations then decreased in the media by 3.1 to 5.2-fold after three days and a further 1.3 to 3.5-fold after seven days.

The experiment was repeated except for each culture being seeded with $5x10^6$ PBMCs from patient C3 (2.56-fold higher viral load than patient C1) and incubating for 14 days (Fig.8.2). HCV RNA in the uncultured PBMCs was found to be at a concentration of 451 copies/10^6 cells and undetectable in the culture medium. After one day of culture, the HCV RNA concentration in the cells decreased by 2.3 to 31.8-fold and by 10.5 to 23.7-fold between days one and three. HCV RNA was detectable at a low level in the untreated cells (1.6-fold decrease) after seven days, but was undetectable in the other cells. After 14 days incubation HCV RNA was undetectable in all of the PBMC cultures. HCV RNA became detectable in the culture media after one day and decreased by 1.6 to 8.6-fold after three days. HCV RNA was undetectable in the medium of the hydrocortisone-treated PBMCs after seven days and had decreased by 2.5 to 10.9-fold in the media of the other cells. After 14 days incubation, HCV RNA was undetectable in the medium of the PHA-treated cells. HCV RNA was detected after 14 days in the media of the untreated, cyclosporin A and hydrocortisone-treated PBMCs, but was below the assay detection limit.

Stability of serum HCV RNA in the culture medium over 14 days incubation was assessed (Fig.8.2b). HCV RNA concentration decreased by 118-fold over days one to seven and was

(a)



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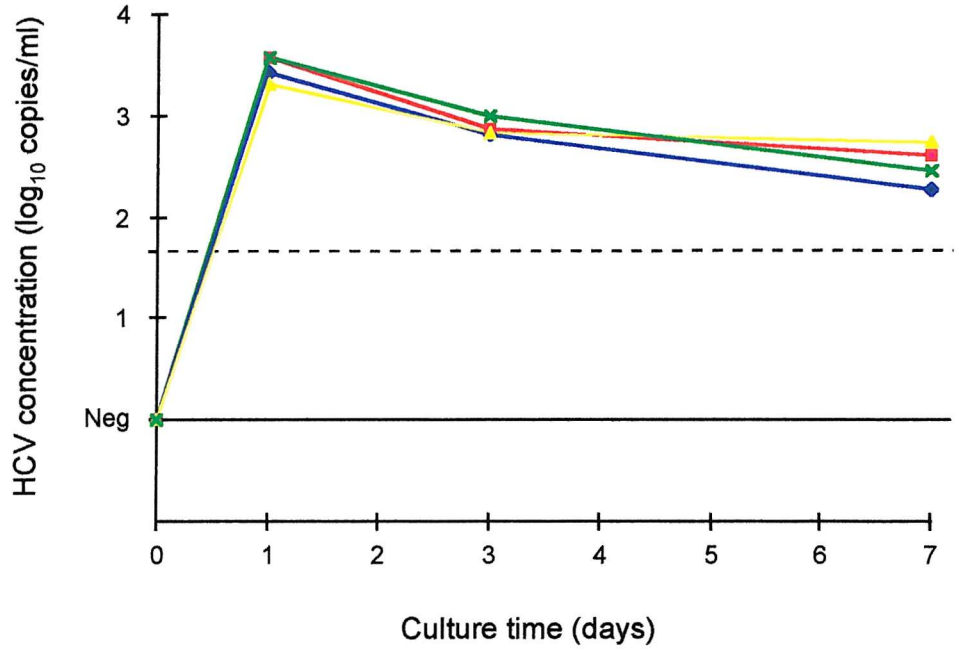


Fig.8.1 Plots of HCV RNA concentration in (a) cultured PBMCs and (b) culture supernatants for patient C2.
PBMCs were cultured at 2.5×10^6 cells per culture plate well. Cultures were then treated with cyclosporin A (red), hydrocortisone (yellow) or PHA (green), or were untreated (blue). Dashed line = limit of detection. Neg = HCV RNA undetectable.

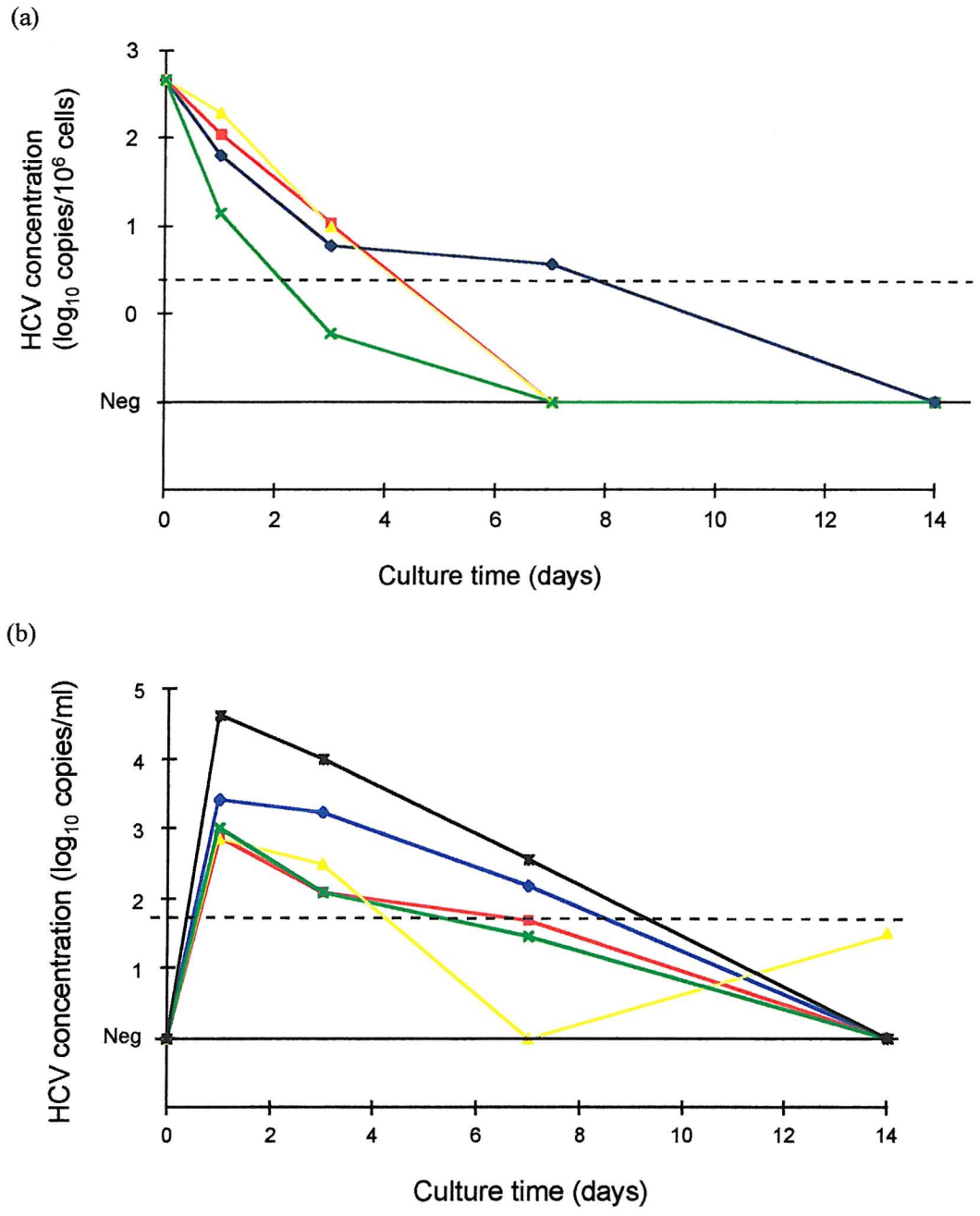


Fig.8.2 Plots of HCV RNA concentration in (a) cultured PBMCs and (b) culture supernatants and serum control for patient C3.
PBMCs were cultured at $5x10^6$ cells per culture plate well. The cells were then treated with cyclosporin A (red), hydrocortisone (yellow) or PHA (green), or were untreated (blue). Serum from patient C3 (black) was diluted 1:5 in culture medium and incubated under the same conditions. Dashed line = limit of detection. Neg = HCV RNA undetectable.

undetectable after 14 days.

Patient C3 cells were counted by microscopy using a counting chamber at each time-point to assess the effect of each treatment on cell replication (Fig.8.3). PHA-treated cells were not counted because they had blasted and therefore could not be counted accurately. The number of cells was found to fluctuate by 1.3 to 1.6-fold over the first three days of culture and showed a 1.2 to 2.5-fold decrease compared with the pre-culture values over the 14 days of culture. However, these variations were probably due to the subjective nature of the counting method.

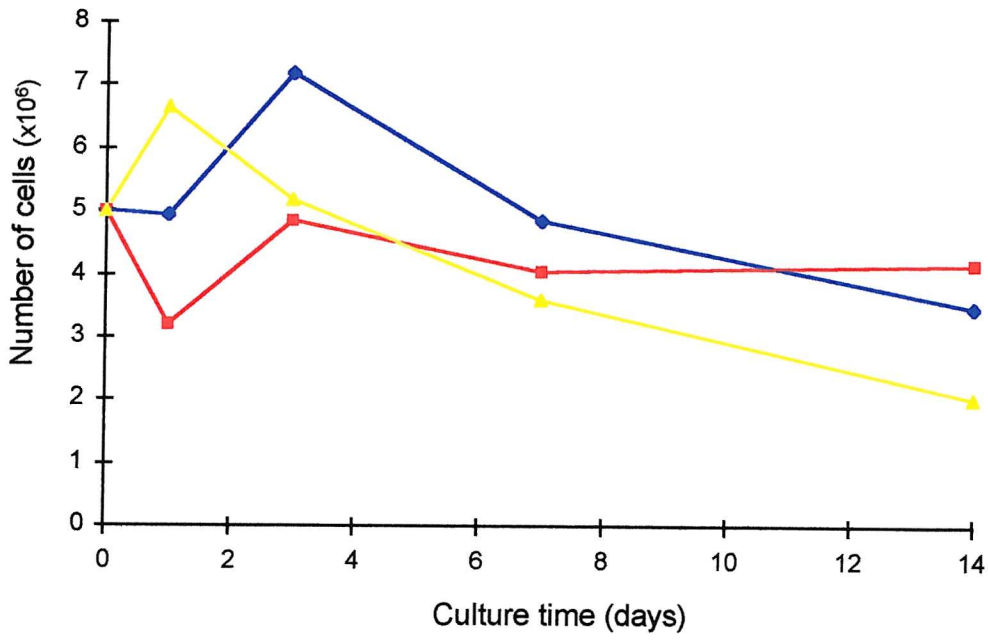


Fig.8.3 Plot of number of cultured PBMCs over time for patient C3.
Total cell numbers were quantified for the untreated (blue) PBMC cultures and those treated with cyclosporin A (red) or hydrocortisone (yellow).

8.3 Discussion

HCV replication has been reported in PBMCs by a number of investigators. These have mainly used RT-PCR to detect the negative-strand RNA replicative intermediate (Bartenschlager and Lohmann, 2000). Results of these studies have been questioned due to the poor strand-specificity of early assays. False-priming of positive-strand RNA, self-priming due to the secondary structures in the 5'UTR of positive-strand RNA, failure to inactivate RT activity and random-priming by cellular nucleic acids have been put forward as possible causes (McGuinness *et al.*, 1994; Gunji *et al.*, 1994). Later reports have employed different strategies to improve strand-specificity, such as the use of tagged RT primers, chemical modification of RNA 3' ends before RT, use of thermoresistant reverse transcriptases with higher reaction temperatures, and the use of primers that amplify the core region which has less extensive secondary structure than the 5'UTR (Negro and Levrero, 1998). The use of more strand-specific RT-PCRs has resulted in contradicting reports as to the presence of negative-strand RNA in PBMCs (Mellor *et al.*, 1998; Laskus *et al.*, 1997; Lerat *et al.*, 1996; Cribbier *et al.*, 1995). It has been suggested that the increase in strand-specificity has resulted in assays being less sensitive (Negro and Levrero, 1998).

Other methods used for investigation of virus replication include quantitation of positive-strand RNA (Magy *et al.*, 1999), immunofluorescence (Bertolini *et al.*, 1993), and transmission of virus to naïve cells or chimpanzees (Shimuzu and Yoshikura, 1994; Shimuzu *et al.*, 1998). For the current project, longitudinal measurements of positive-strand RNA concentration were performed to investigate whether HCV was replicating in cultured PBMCs from infected individuals.

HCV was detected in PBMC samples from all three patients tested. The lowest concentration of viral RNA was detected in PBMC from patient C1, who had a viral load of only 4.64×10^4 copies/ml. The highest concentrations of HCV RNA were found in PBMCs from patient C2, who had the highest serum load of the three patients tested. These findings are consistent with those of Trimoulet *et al.* (2000) who reported a correlation between the level of HCV RNA in serum and PBMCs. The fact that HCV RNA was at almost undetectable levels in PBMCs from patient C1 supports the low level of HCV replication in these cells as suggested by Negro and Levrero (1998). It also supports the findings of Muratori *et al.* (1996) who reported that only approximately 0.2 - 8.1% of an HCV-positive patient's PBMCs are infected as detected by *in-situ* RT-PCR. Other than serum load, it is possible that viral factors such as genotype and quasispecies diversity, or host factors such as HLA type and immune status may also have affected the level of HCV in the PBMCs.

Importantly, HCV RNA was not detected in supernatants before PBMCs culture, suggesting that cell washing removed extracellular virus and that RNA detected in the pre-culture cell pellets was intracellular. It does not, however, eliminate the possibility that the HCV RNA was passively adsorbed to outer surfaces of the cells. After one day of incubation, HCV RNA was detected in the culture media indicating that either the release of viral particles from infected cells or shedding of adhered viruses from cell surfaces. Treatment of the cells with RNase and trypsin, followed by washing before culture could be used to remove external HCV as performed by Cribier *et al.* (1995), however it could not be guaranteed that traces of RNase would not be left in the samples and degrade any RNA released from the cells during culture. It was assumed that as HCV can only replicate intracellularly, any peak in HCV RNA concentration would be due to intracellular replication.

HCV RNA levels were found to decrease in cells and media over the period of cell culture incubation and become undetectable after 14 days in PBMCs from patient C3. This suggests that HCV RNA was either not replicating in the cultured cells, or that HCV RNA was degraded by cellular nucleases at a faster rate than that of replication. HCV RNA degraded at a similar rate when serum was incubated for 14 days, possibly indicating the inherently instability of HCV RNA under the conditions used, or the presence of nucleases in the serum.

Cells treated with the immunosuppressive compounds cyclosporin A and hydrocortisone showed no detectable difference in the level of HCV RNA over time from the untreated PBMCs. These findings appear to contradict those of Magy *et al.* (1999) who found that when HCV-positive serum (mean load = $4x10^7$ GEq/ml by Quantiplex HCV RNA 2.0) was incubated with PBMCs cultured from HCV-negative individuals there was a peak of viral replication after 11 days in culture. When corticosteroid was added to the culture media, peaks of viral replication were detected after seven and 15 days. They also detected increases in viral load in some HCV-positive liver transplant recipients after anti-rejection treatment using corticosteroids. These differences may be due to the fact that in the present study PBMCs from HCV-positive patients were cultured as opposed to addition of high-titre HCV-positive serum to HCV-negative cells. The cells may therefore have been in different metabolic states due to the presence of persistent HCV infection, or PBMCs may be more susceptible to infection *in vitro* resulting in higher proportions of cells being infected. The differences in the immunosuppressive agents used may also have led to the discrepancies.

PHA appeared to have no significant effect on the level of viral replication in HCV-infected PBMCs even though the cells had been stimulated to blast. This is in contrast to the findings for HIV-infected PBMCs in which viral replication is stimulated by addition of PHA

(Chun *et al.*, 1997). This supports the hypothesis that that the genome of HCV, unlike that of HIV, does not integrate into the host cell genome as part of its life cycle.

There was no detectable pattern as to which of the treated cultures showed the lowest rate of HCV RNA depletion in the cells or culture media. Also, taking experimental error into account for the C3 cell counts, the addition of cyclosporin A and hydrocortisone did not have a significant effect on cell numbers, compared with the untreated cells. However, statistical analysis could not be performed because of the limited number of patients studied due to difficulties in recruiting untreated HCV-positive patients. Experiments were also limited to one test per treatment for each time-point because of low yields of PBMCs from patient blood. Therefore, the results of this study need to be confirmed by testing larger numbers of patients.

In conclusion, most recent studies on replication of HCV in PBMCs suggest that the virus either does not replicate in these cells or does so at very low levels. The results of this investigation support previous reports of the presence of HCV RNA in PBMCs, but the experiments would not have enabled very low levels of HCV RNA replication to be detected. Addition of PHA, hydrocortisone or cyclosporin A to cultured infected PBMCs did not result in increased levels of HCV compared with the untreated cells. Therefore, it appears that these immunomodulatory compounds were unable to boost virus replication in PBMCs under the conditions used to levels which may be useful for further research into areas such as studies of virus replication, morphology and vaccine production.

8.4. Summary

1. HCV RNA was detected in PBMCs isolated from three chronically HCV-infected patients. HCV RNA concentrations in cells and culture media decreased over time and became undetectable in cells after 14 days incubation.
2. Addition of the immunomodulatory compounds PHA, cyclosporin A and hydrocortisone had no significant effect on the level of HCV RNA in cells or culture media.

9. Discussion

A TaqMan PCR-based assay has been developed for quantitation of HCV. The assay was found to be sensitive, accurate, to amplify sequences from the most common UK HCV genotypes equally and to quantify HCV RNA over a wide dynamic range. The IC allowed the RNA extraction, RT and PCR efficiencies to be monitored. No inhibition was detected in any of the clinical samples tested, possibly due to the high efficiency of the RNA extraction method used. Assay performance also compared well with those of the commercially available assays and other TaqMan PCR assays reported. The assay proved capable of quantifying HCV RNA in all samples tested in the three clinical studies undertaken. This would not have been the case if a commercially available assay such as the Roche Amplicor Monitor 2.0 or Bayer Quantiplex HCV RNA 2.0 assay was used, due to their limited sensitivities and dynamic ranges.

The wide dynamic range of the TaqMan PCR allowed viral load to be quantified in all asymptomatic and symptomatic HCV-positive patients studied and to assess viral load variation over time. The TaqMan PCR assay will, therefore, be ideal for carrying out further studies of viral load in chronic HCV infection.

The TaqMan PCR assay was used to monitor virological responses in patients treated with the new combination therapy of PEG-IFN and ribavirin. Viral loads varied over a large range (<50 to $6x10^6$copies/ml), highlighting the importance of using an assay with a wide dynamic range. The assay's limit of detection (50 IU/ml) was low enough to suggest that patients in whom HCV RNA was undetectable had cleared the virus from the bloodstream. However, very low level viraemia could not be discounted and the presence of HCV RNA in the liver was not investigated. The new combination therapy resulted in an ETR rate of 75%. This compares with ETR rates of 29% and 51 - 55% for IFN monotherapy and IFN-ribavirin combination therapy, respectively (Poynard *et al.*, 2000) and, although the number of patients was very small, suggests PEG-IFN and ribavirin may be more efficient for treatment of chronic hepatitis C. However, the SR rate has yet to be determined in these patients. If SR rate relates to the rate of decline in viral load in these patients, the data may be useful for creating treatment algorithms for PEG-IFN plus ribavirin therapy. However, accurate assessment of factors predictive of SR will require analysis of the combined data from the other sites participating in the trial.

The TaqMan PCR assay developed for this project and other real-time PCR assays will be valuable for studies of future anti-HCV therapies. As the molecular biology of HCV becomes better understood, more rational approaches to drug development will be applied to specifically target stages in the viral life cycle (reviewed by Koff, 1998; Lewis and Freedman, 2000; Rosen and Gretch, 1999). These are likely to include inhibitors of the viral protease, helicase and polymerase activities. Other potential therapies include ribozymes which have been shown to specifically cleave HCV RNA sequences *in vitro* (Lieber *et al.*, 1996). Antisense oligonucleotides designed to bind HCV RNA have been shown to inhibit HCV gene expression (Wakita and Wands, 1994). Compounds such as thymosin-α1, interleukins or other cytokines may be used for modulation of the immune response to HCV infection (Sherman *et al.*, 1996). Future therapies will probably be used in combination with currently available drugs to maximise SR rates. Quantitation of HCV loads will be of importance for studying viral response kinetics to new drugs. This may offer insights into the mechanisms of antiviral activity and into the HCV replication cycle.

As well as being a valuable tool for research, the assay can also be used for routine quantitation of HCV loads in infected patients, pre-treatment and in response to therapy. Increased knowledge of factors involved in response to treatment will allow treatment to be tailored to the individual patient.

Real-time quantitation may also be used to study HCV RNA concentrations in the liver or in other suspected sites of replication in response to treatment. This would be most effective if a convenient animal model for hepatitis C could be developed allowing multiple biopsies to be performed.

The real-time PCR assay allowed quantitation of HCV RNA in cultured PBMCs and the study of the effects of treating cells with immunomodulatory compounds. Although none of the compounds used was able to boost HCV levels in these cells under the conditions used, the assay will be useful for studying HCV RNA replication kinetics in cell culture or cell-free replication systems that may be developed in the future. This will allow the effects of antiviral drugs on viral replication to be studied in detail and improve our understanding of the regulation of RNA replication. By using different primers and probes, real-time TaqMan PCR assays may also be used to quantify host cell mRNA to study the effects of HCV replication on cellular gene expression in infected cell-cultures or *in vivo*.

Quantitation using current and future technologies must be standardised. The WHO International Standard is now the accepted standard for assay assessment and recently released commercial assays have been calibrated in IU/ml. This allows more accurate comparison of assay performance by researchers and clinicians. Standardisation is especially important for

developing treatment algorithms based on pre-treatment viral load, as clinicians must be able to apply viral load values to an algorithm regardless of the assay used. It will also allow consistent measurement of viral load if a change to another assay is made during ongoing patient management.

The sensitivity of real-time PCR assays do not yet meet that of the most sensitive qualitative assay, the VERSANT TMA assay (Bayer) which has a limit of detection of <10 IU/ml. The goal for development of real-time PCR quantitative assays should now be to increase their sensitivity. This may be achieved by increasing the sample volumes tested or by improving the RNA extraction, RT or amplification efficiencies. Improved sensitivity has been reportedly attained by extracting RNA from whole blood, possibly due to the association of HCV with cryoglobulins (Schmidt *et al.*, 2000), but these finding are disputed (Cook *et al.*, 2001). RNA extraction using the QIAamp viral RNA extraction kit (Qiagen) used here has recently been reported to be less efficient for recovery of HCV RNA from serum than the High Pure viral nucleic acid kit (Roche) (Read, 2001). The Roche extraction kit could, therefore, be incorporated into the method to increase sensitivity.

The field of real-time PCR quantitation is fast moving and new detection instruments are being developed which may increase sensitivity. Recently, the DNA Engine Opticon (MJ Research) and MX4000 (Stratagene) systems have been released. Roche are also developing an automated real-time PCR product detection system (COBAS TaqMan) which will incorporate an automated sample preparation stage (Roche, 1999).

New fluorescent dyes are also being released which may improve assay sensitivities. For example, the Black Hole Quencher dyes recently released by Biosearch Technologies provide improved quenching of reporter dye fluorescence, resulting in increased signal to noise ratios which will allow more sensitive PCR product detection in FRET-based assays.

The efficiency of target detection using TaqMan PCR depends upon the efficiency of binding and cleavage of a probe. Therefore, use of systems such as molecular beacon primers, in which FRET occurs within the primer, may increase assay sensitivity as long as the level of non-specific amplification is avoided. Scorpions primers have been developed to overcome the problem of detection of non-specific amplification (Whitcome *et al.*, 1999). These have reporter and quencher dyes on opposite ends of a 5' tail. The tail has regions of self-complementarity which, when annealed, bringing the dyes close enough together for quenching of reporter fluorescence to occur. The tail also contains an amplicon-complementary region between the dye molecules. Separation of reporter and quencher occurs when the amplicon-complementary region binds to the amplicon after primer extension. This means reporter fluorescence is detected only when target-specific amplification occurs.

The next generation of real-time PCR-based HCV quantitative assay systems are likely to involve automation of the RNA extraction, RT and amplification steps. This should lead to improved reproducibility and sample throughput, and reduced hands-on time. Multiplex PCR may be employed to quantify more than one template by using different reporter dyes. This strategy has been used for simultaneous detection of HBV and HCV genomes (Mercier *et al.*, 1999). However, for quantitation, assays would need to be carefully optimised to avoid the accuracy being compromised through competition. Real-time PCR systems may also be miniaturised to increase portability for use in mobile laboratories or 'near-patient' testing.

In summary, real-time PCR allows accurate quantitation of HCV loads over a wide dynamic range. Although these tests are not yet as sensitive as the best qualitative assays, future advances will allow development of real-time assays with comparable sensitivities. Real-time PCR assays will play an important role in future research and management of hepatitis C.

Appendix I: Product suppliers

Table 10.1 Addresses of suppliers used in this project.

Supplier	Address
Adolf Kuhner AG Schweiz	Birsfelden, Switzerland
Ambion	Austin, USA
Amersham	Little Chalfont, UK
Applied Biosystems	Foster City, USA
Apple	Cupertino, USA
Bayer	Newbury, UK
Bio-101	Carlsbad, USA
Bioline	Kenilworth, USA
BioRad	Hercules, USA
Bio-Stat	Stockport, UK
Biowhittaker	Wokingham, UK
Cruachem	Aston, USA
Difco	Detroit, USA
DNA Star	Madison, USA
Flowgen	Lichfield, UK
Glaxo Wellcome	Uxbridge, UK
Hastings Software	Hastings-on-Hudson, USA
Hellma	Southend on Sea, UK
Hitachi	Tokyo, Japan
Invitrogen	Groningen, The Netherlands
Jencons	Leighton Buzzard, UK
Labsystems	Helsinki, Finland
Life Technologies	Rockville, USA.
Merck	Poole, UK
Microsoft	Redmond, USA
Millipore	Bedford, USA
Molecular Probes	Eugene, USA
National Diagnostics	Atlanta, USA
NIBSC	Potters Bar, UK
Nalgene	Rochester, USA
Novagen	Madison, USA
Nycomed	Oslo, Norway
Oswel DNA Service	Southampton, UK
Polaroid	Cambridge, USA
Promega	Madison, USA
Qiagen	Crawley, UK
Sarstedt	Numbrecht, Germany
Scandinavian Gene Synthesis AB	Köping, Sweden
Scottlab	Petaluma, USA
Sigma-Aldrich	Poole, UK
SPSS	Chicago, USA
Tecan	Goring-on-Thames, UK
Ultra-Violet Products	San Gabriel, USA

Appendix II: Oligonucleotides

Table 10.2 Oligonucleotides used in this project

Oligonucleotide	Sequence (5' - 3')
UTR1	GAGGAACTACTGTCTTCACG
UTR2	TATCAGGCAGTACCACAAGG
UTR3	GTCTAGCCATGGCGTTAG
UTR4	ACTCACCGGTTCCGCAG
HCV 939	CTGTGAGGAACTACTGTCTT
HCV 209	ATACTCGAGGTGCACGGTCTACGAGACCT
HCV 209T	ATACTCGAGGTGCACGGTC
HCV 209TH	TGGTAAGCTTATACTCGAGGTGCACGGTC
HCV 211T	CACTCTCGAGCACCCTATC
HCV939E	CTAGAATTCCTGTGAGGAACTACTGTCTT
5 PUTc-1a	CCCAACACTACTCGGCTAG
5 PUT1-s	AACTACTGTCTTCACGCAGAAAGC
5 PUT2FAM	T_FGGCGTTAGTATGAGTGTCGT$_T$GCAG
pGEM3Z-SP6	ATTACGCCAAGCTATTTAGG
pGEM3Z-T7	TTCCCAGTCACGACGTTG
pGEM3Z-T72	TCGGTGCGGGCCTCTTC
TMF1G	GAGTGTCGTGCAGCCTCCA
TMF1AT	AGTATGAGTGTCGTACAGCCTCCA
TMF1AC	ACGAGTGTCGTACAGCCTCCA
TMF2.1	GGAACTACTGTCTTCACGCAGAAAG
TMF2.3	ACTTCTGTCTTCACGCGGAAAG
TMF3.1	ACGCAGAAAGCGTCTAGCCAT
TMF3.3	CGGAAAGCGCCTAGCCAT
TMR1.1	CTTTCGCGACCCAACACTACT
TMR1.2	TTCGCAACCCAACGCTACT
TMR2	GCAAGCACCCTATCAGGCAGTA
TMP1	T_FGTACTCACCGGTTCCGCAGACCACTAT$_T$
TMP1RC	A_FTAGTGGTCTGCGGAACCGGTGAGTACAT$_T$
2DF	CTAGAATTCGAATAGATCAGTAGCAAACATTTGTA
2DR	TACGGATCCACAGCCAGAATCGG
HF	GATCTACTGCCTGATAGGGTGCTTGC
HR	CTACTGCAGGCAAGCACCCTATCAGG
2DR2A	ACGCGGCCAGAACAATGG
2DR2B	AGAGCCAAAGGAAACAAGTG
2DTMF	TGGCAGCCACGAATATGAAAC
2DTMP	A_FGGTTCCCAGACCTGCACGTGCAAA$_T$

Fluorophores are represented subscript F (FAM) and T (TAMRA) after the nt to which they are attached.

Appendix III: Results of assay development experiments

Table 10.3 Percentage identities between a 255nt section (nt -277 to -22) of 5'UTRs of Wessex and published HCV isolates.

	HCV-1	HCV-BK	HCV-J6	HPC-J8G	HPC-K3a	ED43	HCV 1480
Sot1-1a	99.2	98.8	94.1	92.2	93.8	95.3	96.5
Sot2-1a	99.6	99.2	95.3	93.4	92.6	96.1	97.7
Sot3-1a	99.6	99.2	95.3	93.4	92.6	96.1	97.7
Sot4-1a	99.6	99.2	95.3	93.4	92.6	96.1	97.7
Sot5-1a	99.2	98.8	94.9	93.0	93.0	95.7	97.3
Sot6-1a	98.8	98.4	94.1	92.2	93.4	95.7	96.5
Sot7-1a	98.8	98.4	94.5	92.6	93.4	95.7	96.9
Sot8-1a	99.2	98.8	94.1	92.2	93.8	95.3	96.5
Sot9-1a	**100.0**	99.6	94.9	93.0	93.0	96.1	97.3
Sot10-1b	99.2	99.6	94.1	92.2	93.8	95.3	97.3
Sot11-1b	99.2	99.6	94.5	92.6	93.0	96.1	97.7
Sot12-1b	99.6	**100.0**	94.5	92.6	93.4	95.7	97.7
Sot13-1b	99.6	99.2	94.9	93.0	92.6	96.5	97.3
Sot14-1b	99.2	99.6	94.5	92.6	93.0	96.1	97.7
Sot15-1b	99.6	**100.0**	94.5	92.6	93.4	95.7	97.7
Sot16-1b	99.2	99.6	94.5	92.6	93.0	96.1	97.7
Sot17-2a	94.9	94.5	99.6	97.7	90.2	94.5	95.7
Sot18-2a	94.9	94.5	99.6	97.7	90.2	94.5	95.7
Sot19-2a	94.9	94.5	99.6	97.7	90.2	94.5	95.7
Sot20-2a	94.9	94.5	99.2	97.3	90.2	94.5	95.7
Sot21-2a	94.9	94.5	**100.0**	98.0	90.2	94.5	95.7
Sot22-2a	94.9	94.5	99.6	97.7	90.2	94.5	95.7
Sot23-2b	93.4	93.0	98.4	**98.8**	89.1	93.0	94.1
Sot24-2b	93.4	93.0	98.4	98.0	88.7	93.0	94.1
Sot25-2b	93.4	93.0	97.7	98.0	89.8	92.2	93.4
Sot26-3a	93.0	93.4	90.2	88.3	99.2	93.0	93.4
Sot27-3a	93.4	93.8	90.6	88.7	99.2	93.4	93.8
Sot28-3a	93.4	93.8	90.6	88.7	99.6	93.4	93.8
Sot29-3a	93.4	93.8	90.6	88.7	99.6	93.4	93.8
Sot30-3a	96.9	96.5	95.7	93.8	93.8	**98.8**	98.0
Sot31-3a	93.4	93.8	90.6	88.7	**99.6**	93.4	93.8
Sot32-3a	92.2	92.6	89.5	87.5	98.0	92.2	92.6
Sot33-3a	93.0	93.4	90.6	88.7	98.8	93.8	93.8
Sot34-3a	93.4	93.0	91.0	89.1	98.0	94.1	93.4
Sot35-4	96.9	97.3	95.3	93.3	93.7	97.3	99.6
Sot36-5	97.3	97.7	95.7	93.8	93.4	96.9	**100.0**

Highest percentage identities for each Wessex sequence are shown in bold type.

Table 10.4 Percentage identities between a 255nt section (nt-277 to -22) of the 5'UTRs of Wessex HCV isolates.

	Sot1-1a	Sot2-1a	Sot3-1a	Sot4-1a	Sot5-1a	Sot6-1a	Sot7-1a	Sot8-1a	Sot9-1a	Sot10-1b	Sot11-1b	Sot12-1b	Sot13-1b	Sot14-1b	Sot15-1b	Sot16-1b	Sot17-2a	Sot18-2a
Sot1-1a	100	98.8	98.8	98.8	99.2	99.6	99.6	100	99.2	98.4	98.4	98.8	98.8	98.4	98.8	98.4	94.1	94.1
Sot2-1a	98.8	100	100	100	99.6	98.8	98.4	98.8	99.6	98.8	99.2	99.2	99.6	99.2	99.2	99.2	95.3	95.3
Sot3-1a	98.8	100	100	100	99.6	98.8	98.8	98.8	99.6	98.8	99.2	99.2	99.6	99.2	99.2	99.2	95.3	95.3
Sot4-1a	98.8	100	100	100	99.6	98.8	98.4	98.8	99.6	98.8	99.2	99.2	99.6	99.2	99.2	99.2	95.3	95.3
Sot5-1a	99.2	99.6	99.6	99.6	100	99.2	98.8	99.2	99.2	98.4	98.8	98.8	99.6	98.8	98.8	98.8	94.9	94.9
Sot6-1a	99.6	98.8	98.8	98.8	99.2	100	99.2	99.6	98.8	98	98.8	98.4	99.2	98.8	98.4	98.8	94.1	94.1
Sot7-1a	99.6	98.4	98.4	98.4	98.8	99.2	100	99.6	98.8	98	98	98.4	98.4	98	98.4	98	94.5	94.5
Sot8-1a	100	98.8	98.8	98.8	99.2	99.6	99.6	100	99.2	98.4	98.4	98.8	98.8	98.4	98.8	98.4	94.1	94.1
Sot9-1a	99.2	99.6	99.6	99.6	99.2	98.8	98.8	99.2	100	99.2	99.2	99.6	99.6	99.2	99.6	99.2	94.9	94.9
Sot10-1b	98.4	98.8	98.8	98.8	98.4	98	98	98.4	99.2	100	99.2	99.6	98.8	99.2	99.6	99.2	94.1	94.1
Sot11-1b	98.4	99.2	99.2	99.2	98.8	98.8	98	98.4	99.2	99.2	100	99.6	99.6	100	99.6	100	94.5	94.5
Sot12-1b	98.8	99.2	99.2	99.2	98.8	98.4	98.4	98.8	99.6	99.6	99.6	100	99.2	99.6	100	99.6	94.5	94.5
Sot13-1b	98.8	99.6	99.6	99.6	99.2	99.2	98.4	98.8	99.6	98.8	99.6	99.2	100	99.6	99.2	99.6	94.9	94.9
Sot14-1b	98.4	99.2	99.2	99.2	98.8	98.8	98	98.4	99.2	99.2	100	99.6	99.6	100	99.6	100	94.5	94.5
Sot15-1b	98.8	99.2	99.2	99.2	98.8	98.4	98.4	98.8	99.6	99.6	99.6	100	99.2	99.6	100	99.6	94.5	94.5
Sot16-1b	98.4	99.2	99.2	99.2	98.8	98.8	98	98.4	99.2	99.2	100	99.6	99.6	100	99.6	100	94.5	94.5
Sot17-2a	94.1	95.3	95.3	95.3	94.9	94.1	94.5	94.1	94.9	94.1	94.5	94.5	94.9	94.5	94.5	94.5	100	100
Sot18-2a	94.1	95.3	95.3	95.3	94.9	94.1	94.5	94.1	94.9	94.1	94.5	94.5	94.9	94.5	94.5	94.5	100	100
Sot19-2a	94.1	95.3	95.3	95.3	94.9	94.1	94.5	94.1	94.9	94.1	94.5	94.5	94.9	94.5	94.5	94.5	100	100
Sot20-2a	94.1	95.3	95.3	95.3	94.9	94.1	94.5	94.1	94.9	94.1	94.5	94.5	94.9	94.5	94.5	94.5	99.6	99.6
Sot21-2a	94.1	95.3	95.3	95.3	94.9	94.1	94.5	94.1	94.9	94.1	94.5	94.5	94.9	94.5	94.5	94.5	99.6	99.6
Sot22-2a	94.1	95.3	95.3	95.3	94.9	94.1	94.5	94.1	94.9	94.1	94.5	94.5	94.9	94.5	94.5	94.5	100	100
Sot23-2b	92.6	93.8	93.8	93.8	93.4	92.6	93	92.6	93.4	93	93	93	93.4	93	93	93	99.8	99.8
Sot24-2b	92.6	93.8	93.8	93.8	93.4	92.6	93	92.6	93.4	92.6	93	93	93.4	93	93	93	99.8	99.8
Sot25-2b	92.6	93.8	93.8	93.8	93.4	92.6	92.2	92.6	93.4	93	93	93	93.4	93	93	93	97.3	97.3
Sot26-3a	93.8	92.6	92.6	92.6	93	93.4	93.4	93.8	93	93.8	93	93.4	92.6	93	93.4	93	90.2	90.2
Sot27-3a	94.1	93	93	93	93.4	93.8	93.8	94.1	93.4	93.8	93.4	93.8	93	93.4	93.8	93.4	90.6	90.6
Sot28-3a	94.1	93	93	93	93.4	93.8	93.8	94.1	93.4	94.1	93.4	93.8	93	93.4	93.8	93.4	90.6	90.6
Sot29-3a	94.1	93	93	93	93.4	93.8	93.8	94.1	93.4	94.1	93.4	93.8	93	93.4	93.8	93.4	90.6	90.6
Sot30-3a	96.1	97.3	97.3	97.3	96.9	96.1	96.5	96.1	96.9	96.1	96.5	96.5	96.9	96.5	96.5	96.5	95.7	95.7
Sot31-3a	94.1	93	93	93	93.4	93.8	93.8	94.1	93.4	94.1	93.4	93.8	93	93.4	93.8	93.4	90.6	90.6
Sot32-3a	93	91.8	91.8	91.8	92.2	96.6	92.6	93	92.2	92.6	92.2	92.6	91.8	92.2	92.6	92.2	89.5	89.5
Sot33-3a	93.8	93	93	93	93.4	94.1	93.4	93.8	93	93.4	93.8	93.4	93.4	93.8	93.4	93.8	90.6	90.6
Sot34-3a	94.1	93.4	93.4	93.4	93.8	94.5	93.8	94.1	93.4	92.6	93.4	93	93.8	93.4	93	93.4	91	91
Sot35-4	96.1	97.3	97.3	97.3	96.9	96.1	96.5	96.1	96.9	96.9	97.3	97.3	96.9	97.3	97.3	97.3	95.3	95.3
Sot36-5	96.5	97.7	97.7	97.7	97.3	96.5	96.9	96.5	97.3	97.3	97.7	97.7	97.3	97.7	97.7	97.7	95.7	95.7

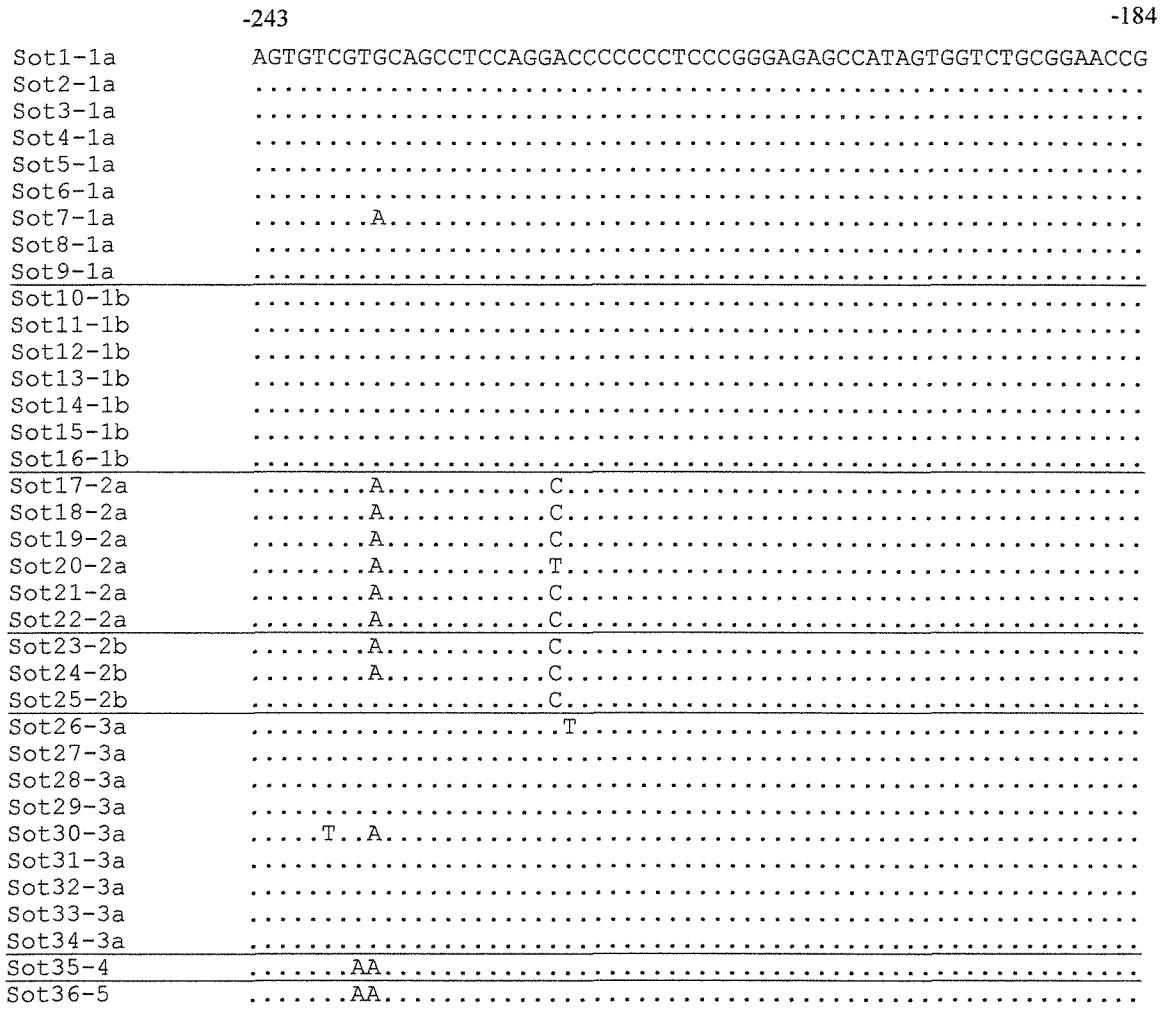
	Sot19-2a	Sot20-2a	Sot21-2a	Sot22-2a	Sot23-2b	Sot24-2b	Sot25-2b	Sot26-3a	Sot27-3a	Sot28-3a	Sot29-3a	Sot30-3a	Sot31-3a	Sot32-3a	Sot33-3a	Sot34-3a	Sot35-4	Sot36-5
Sot1-1a	94.1	94.1	94.1	94.1	92.6	92.6	92.6	93.8	94.1	94.1	94.1	96.1	94.1	93	93.8	94.1	96.1	96.5
Sot2-1a	95.3	95.3	95.3	95.3	93.8	93.8	93.8	92.6	93	93	93	97.3	93	91.8	93	93.4	97.3	97.7
Sot3-1a	95.3	95.3	95.3	95.3	93.8	93.8	93.8	92.6	93	93	93	97.3	93	91.8	93	93.4	97.3	97.7
Sot4-1a	95.3	95.3	95.3	95.3	93.8	93.8	93.8	92.6	93	93	93	97.3	93	91.8	93	93.4	97.3	97.7
Sot5-1a	94.9	94.9	94.9	94.9	93.4	93.4	93.4	93	93.4	93.4	93.4	96.9	93.4	92.2	93.4	93.8	96.9	97.3
Sot6-1a	94.1	94.1	94.1	94.1	92.6	92.6	92.6	93.4	93.8	93.8	93.8	96.1	93.8	92.6	94.1	94.5	96.1	96.5
Sot7-1a	94.5	94.5	94.5	94.5	93	93	92.2	93.4	93.8	93.8	93.8	96.5	93.8	92.6	93.4	93.8	96.5	96.9
Sot8-1a	94.1	94.1	94.1	94.1	92.6	92.6	92.6	93.8	94.1	94.1	94.1	96.1	94.1	93	93.8	94.1	96.1	96.5
Sot9-1a	94.9	94.9	94.9	94.9	93.4	93.4	93.4	93	93.4	93.4	93.4	96.9	93.4	92.2	93	93.4	96.9	97.3
Sot10-1b	94.1	94.1	94.1	94.1	93	92.6	93	93.8	93.8	94.1	94.1	96.1	94.1	92.6	93.4	92.6	96.9	97.3
Sot11-1b	94.5	94.5	94.5	94.5	93	93	93	93	93.4	93.4	93.4	96.5	93.4	92.2	93.8	93.4	97.3	97.7
Sot12-1b	94.5	94.5	94.5	94.5	93	93	93	93.4	93.8	93.8	93.8	96.5	93.8	92.6	93.4	93	97.3	97.7
Sot13-1b	94.9	94.9	94.9	94.9	93.4	93.4	93.4	92.6	93	93	93	96.9	93	91.8	93.4	93.8	96.9	97.7
Sot14-1b	94.5	94.5	94.5	94.5	93	93	93	93	93.4	93.4	93.4	96.5	93.4	92.2	93.8	93.4	97.3	97.3
Sot15-1b	94.5	94.5	94.5	94.5	93	93	93	93.4	93.8	93.8	93.8	96.5	93.8	92.6	93.4	93	97.3	97.7
Sot16-1b	94.5	94.5	94.5	94.5	93	93	93	93	93.4	93.4	93.4	96.5	93.4	92.2	93.8	93.4	97.3	97.7
Sot17-2a	100	99.6	99.6	100	98	98	97.3	90.2	90.6	90.6	90.6	95.7	90.6	89.5	90.6	91	95.3	97.7
Sot18-2a	100	99.6	99.6	100	98	98	97.3	90.2	90.6	90.6	90.6	95.7	90.6	89.5	90.6	91	95.3	95.7
Sot19-2a	100	99.6	99.6	100	98	98	97.3	90.2	90.6	90.6	90.6	95.7	90.6	89.5	90.6	91	95.3	95.7
Sot20-2a	99.6	100	99.2	99.6	97.7	97.7	96.9	90.2	90.6	90.6	90.6	95.7	90.6	89.5	90.6	91	95.3	95.7
Sot21-2a	99.6	99.2	100	99.6	98.4	98.4	97.7	90.2	90.6	90.6	90.6	95.7	90.6	89.5	90.6	91	95.3	95.7
Sot22-2a	100	99.6	99.6	100	98	98	97.3	90.2	90.6	90.6	90.6	95.7	90.6	89.5	90.6	91	95.3	95.7
Sot23-2b	99.8	97.7	98.4	99.8	100	98.4	99.2	89.1	89.5	89.5	89.5	94.1	89.5	88.3	89.5	89.5	93.7	94.1
Sot24-2b	99.8	97.7	98.4	99.8	98.4	100	97.7	88.7	89.1	89.1	89.1	94.1	89.1	87.9	89.1	89.5	93.7	94.1
Sot25-2b	97.3	96.9	97.7	97.3	99.2	97.7	100	89.8	90.2	90.2	90.2	93.4	90.2	89.1	90.2	90.2	92.9	93.4
Sot26-3a	90.2	90.2	90.2	90.2	89.1	88.7	89.8	100	99.2	99.6	99.6	93.8	99.6	98	98.8	98	93.7	93.4
Sot27-3a	90.6	90.6	90.6	90.6	89.5	89.1	90.2	99.2	100	99.6	99.6	94.1	99.6	98.8	99.6	98.4	94.1	93.8
Sot28-3a	90.6	90.6	90.6	90.6	89.5	89.1	90.2	99.6	99.6	100	100	94.1	100	98.4	99.2	98.4	94.1	93.8
Sot29-3a	90.6	90.6	90.6	90.6	89.5	89.1	90.2	99.6	99.6	100	100	94.1	100	98.4	99.2	98.4	94.1	93.8
Sot30-3a	95.7	95.7	95.7	95.7	94.1	94.1	93.4	93.8	94.1	94.1	94.1	100	94.1	93	94.1	94.5	98.4	98
Sot31-3a	90.6	90.6	90.6	90.6	89.5	89.1	90.2	99.6	99.6	100	100	94.1	100	98.4	99.2	98.4	94.1	93.8
Sot32-3a	89.5	89.5	89.5	89.5	88.3	87.9	89.1	98	98.8	98.4	98.4	93	98.4	100	98.4	97.3	92.9	92.6
Sot33-3a	90.6	90.6	90.6	90.6	89.5	89.1	90.2	98.8	99.6	99.2	99.2	94.1	99.2	98.4	100	98.8	94.1	93.8
Sot34-3a	91	91	91	91	89.5	89.5	90.2	98	98.4	98.4	98.4	94.5	98.4	97.3	98.8	100	93.7	93.4
Sot35-4	95.3	95.3	95.3	95.3	93.7	93.7	92.9	93.7	94.1	94.1	94.1	98.4	94.1	92.6	94.1	93.7	100	99.6
Sot36-5	95.7	95.7	95.7	95.7	94.1	94.1	93.4	93.4	93.8	93.8	93.8	98	93.8	92.6	93.8	93.4	99.6	100

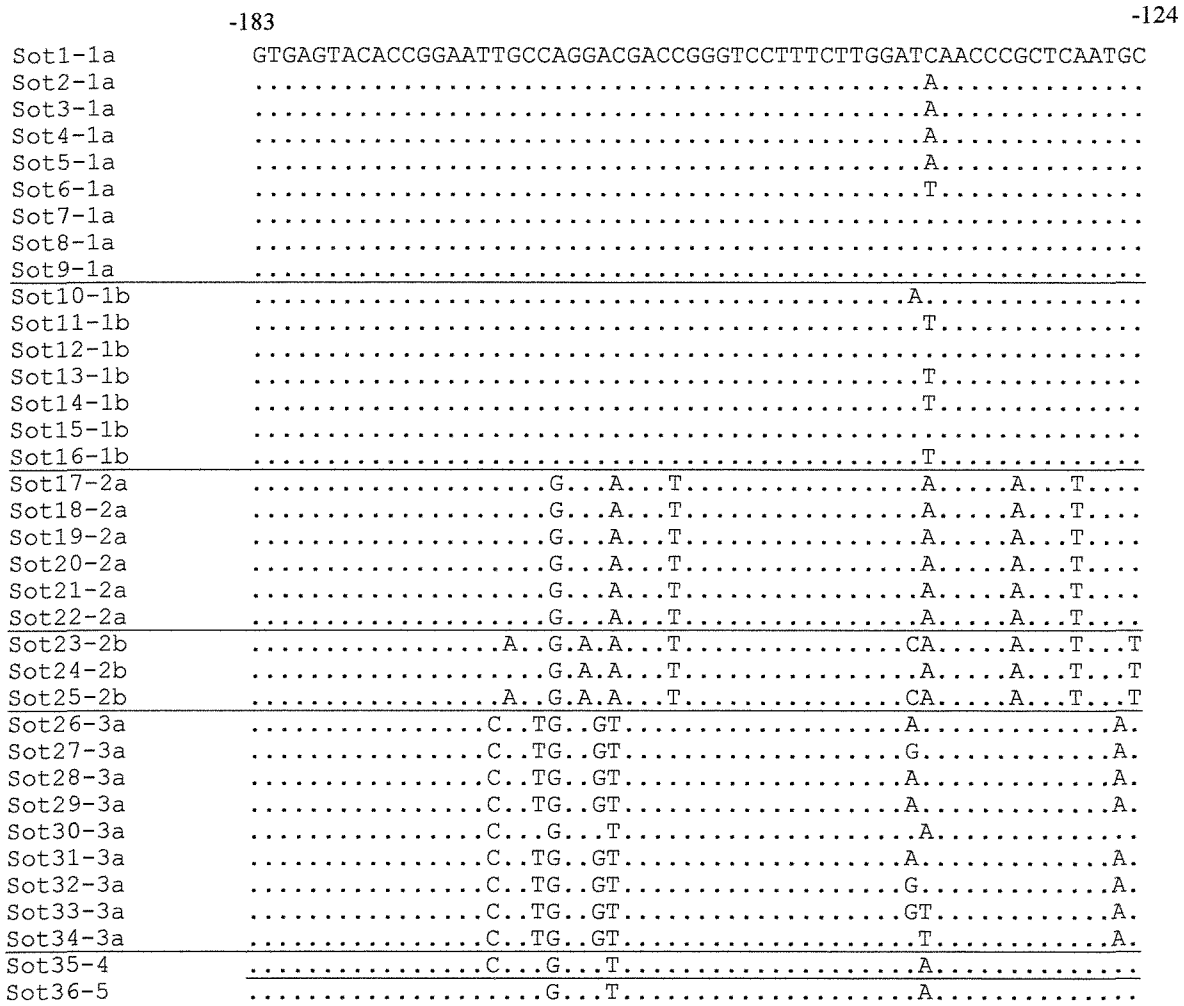
Table 10.5 Results of TaqMan PCR experiment to evaluate primer-probe combinations.

Primers	Probe	Genotype 1a (C_T)		Genotype 3a (C_T)		Mean C_T
TMF1	TMP1	28.14	26.93	28.51	28.18	27.94
TMR1	TMP1RC	29.20	28.85	28.79	29.37	29.05
TMF1	TMP1	27.06	27.76	27.35	27.49	27.42
TMR2	TMP1RC	29.74	28.39	30.04	30.29	29.62
TMF2	TMP1	28.17	28.01	28.33	27.75	28.07
TMR1	TMP1RC	29.26	28.73	28.91	29.17	29.02
TMF2	TMP1	29.24	29.44	29.27	28.94	29.22
TMR2	TMP1RC	30.18	29.74	30.16	29.82	29.98
TMF3	TMP1	28.19	27.71	28.34	28.45	28.17
TMR1	TMP1RC	28.48	28.61	28.40	28.52	28.50
TMF3	TMP1	28.93	28.21	28.24	28.49	28.47
TMR2	TMP1RC	29.11	29.49	29.10	29.02	29.18
5PUTc-1a	TMP1	28.79	29.35	32.64	31.84	30.66
5PUT1-s	TMP1RC	29.54	29.70	33.19	32.60	31.26
	5PUT2FAM	30.95	30.25	35.04	34.48	32.67

Fig.10.1 Alignment of sequence data for Wessex HCV 5'UTRs.
Sequences represent sense strand of cDNA. Nucleotides identical to the reference strand are represented as a dot (.). Nucleotide co-ordinates are shown and correspond to those of the prototype strain, HCV-1 (Choo *et al.*, 1991).

```
                -277                                    -244
Sot1-1a      5' CACGCAGAAAGCGTCTAGCCATGGCGTTAGTATG
Sot2-1a         ....................................
Sot3-1a         ....................................
Sot4-1a         ....................................
Sot5-1a         ....................................
Sot6-1a         ....................................
Sot7-1a         ....................................
Sot8-1a         ....................................
Sot9-1a         ....................................
Sot10-1b        ....................................
Sot11-1b        ....................................
Sot12-1b        ....................................
Sot13-1b        ....................................
Sot14-1b        ....................................
Sot15-1b        ....................................
Sot16-1b        ....................................
Sot17-2a        ....................................
Sot18-2a        ....................................
Sot19-2a        ....................................
Sot20-2a        ....................................
Sot21-2a        ....................................
Sot22-2a        ....................................
Sot23-2b        ....................................
Sot24-2b        ....................................
Sot25-2b        ............C.......................
Sot26-3a        .....G.......C..................C.
Sot27-3a        .....G.......C..................C.
Sot28-3a        .....G.......C..................C.
Sot29-3a        .....G.......C..................C.
Sot30-3a        ....................................
Sot31-3a        .....G.......C..................C.
Sot32-3a        .....G.......C..........G.......C.
Sot33-3a        .....G.......C..................C.
Sot34-3a        .....G.......C..................C.
Sot35-4         T...................................
Sot36-5         ....................................
```


-243
-184
Sot1-1a AGTGTCGTGCAGCCTCCAGGACCCCCCCTCCCGGGAGAGCCATAGTGGTCTGCGGAACCG
Sot2-1a
Sot3-1a
Sot4-1a
Sot5-1a
Sot6-1a
Sot7-1a .....A.....
Sot8-1a
Sot9-1a
Sot10-1b
Sot11-1b
Sot12-1b
Sot13-1b
Sot14-1b
Sot15-1b
Sot16-1b
Sot17-2a .....A.....C.....
Sot18-2a .....A.....C.....
Sot19-2a .....A.....C.....
Sot20-2a .....A.....T.....
Sot21-2a .....A.....C.....
Sot22-2a .....A.....C.....
Sot23-2b .....A.....C.....
Sot24-2b .....A.....C.....
Sot25-2b .....C.....
Sot26-3a .....T.....
Sot27-3a
Sot28-3a
Sot29-3a
Sot30-3a .....T..A.....
Sot31-3a
Sot32-3a
Sot33-3a
Sot34-3a
Sot35-4 .....AA.....
Sot36-5 .....AA.....


-183
-124
Sot1-1a GTGAGTACACCGGAATTGCCAGGACGACCGGGTCCTTTCTTGGATCAACCCGCTCAATGC
Sot2-1a .....A.....
Sot3-1a .....A.....
Sot4-1a .....A.....
Sot5-1a .....A.....
Sot6-1a .....T.....
Sot7-1a .....
Sot8-1a .....
Sot9-1a .....
Sot10-1b .....A.....
Sot11-1b .....T.....
Sot12-1b .....
Sot13-1b .....T.....
Sot14-1b .....T.....
Sot15-1b .....
Sot16-1b .....T.....
Sot17-2a .....G...A...T.....A.....A...T....
Sot18-2a .....G...A...T.....A.....A...T....
Sot19-2a .....G...A...T.....A.....A...T....
Sot20-2a .....G...A...T.....A.....A...T....
Sot21-2a .....G...A...T.....A.....A...T....
Sot22-2a .....G...A...T.....A.....A...T....
Sot23-2b .....A..G.A.A...T.....CA.....A...T...T
Sot24-2b .....G.A.A...T.....A.....A...T...T
Sot25-2b .....A..G.A.A...T.....CA.....A...T...T
Sot26-3a .....C..TG..GT.....A.....A.
Sot27-3a .....C..TG..GT.....G.....A.
Sot28-3a .....C..TG..GT.....A.....A.
Sot29-3a .....C..TG..GT.....A.....A.
Sot30-3a .....C...G...T.....A.....
Sot31-3a .....C..TG..GT.....A.....A.
Sot32-3a .....C..TG..GT.....G.....A.
Sot33-3a .....C..TG..GT.....GT.....A.
Sot34-3a .....C..TG..GT.....T.....A.
Sot35-4 .....C...G...T.....A.....
Sot36-5 .....G...T.....A.....

```
                -123                                                    -66
Sot1-1a         CTGGAGATTTGGGCGTGCCCCCGCAAGATCGCTAGCCGAGTAGTGTTGGGTCGCGAAA
Sot2-1a         ............................CT............................
Sot3-1a         ............................CT............................
Sot4-1a         ............................CT............................
Sot5-1a         ............................C.............................
Sot6-1a         ............................................................
Sot7-1a         ............................................................
Sot8-1a         ............................................................
Sot9-1a         ............................CT............................
Sot10-1b        ........................G...CT............................
Sot11-1b        ........................G...CT............................
Sot12-1b        ........................G...CT............................
Sot13-1b        ............................CT............................
Sot14-1b        ........................G...CT............................
Sot15-1b        ........................G...CT............................
Sot16-1b        ........................G...CT............................
Sot17-2a        .C..CC......................CT............C.......T......
Sot18-2a        .C..CC......................CT............C.......T......
Sot19-2a        .C..CC......................CT............C.......T......
Sot20-2a        .C..CC......................CT............C.......T......
Sot21-2a        .C..TC......................CT............C.......T......
Sot22-2a        .C..CC......................CT............C.......T......
Sot23-2b        .C..TC......................CT............C.......T......
Sot24-2b        .C..TC......................CT.......T.....C.......T.....C
Sot25-2b        .C..TC......................CT............C.......T......
Sot26-3a        .CA..A..................G.....A.............................
Sot27-3a        .CA..A..................G.....A.............................
Sot28-3a        .CA..A..................G.....A.............................
Sot29-3a        .CA..A..................G.....A.............................
Sot30-3a        .C...A......................CT............................
Sot31-3a        .CA..A..................G.....A.............................
Sot32-3a        .CA..A..................G.....A............G......A.....
Sot33-3a        .CA..A..................G.....A.............................
Sot34-3a        .CA..A........................A...........T................
Sot35-4         .C........................G...CT............................
Sot36-5         .C........................G...CT............................
```

```
                    -65                                                -22
Sot1-1a             GGCCTTGTGGTACTGCCTGATAGGGTGCTTGCGAGTGCCCCGGG 3'
Sot2-1a             .....................................................
Sot3-1a             .....................................................
Sot4-1a             .....................................................
Sot5-1a             .....................................................
Sot6-1a             .....................................................
Sot7-1a             .....................................................
Sot8-1a             .....................................................
Sot9-1a             .....................................................
Sot10-1b            .....................................................
Sot11-1b            .....................................................
Sot12-1b            .....................................................
Sot13-1b            .....................................................
Sot14-1b            .....................................................
Sot15-1b            .....................................................
Sot16-1b            .....................................................
Sot17-2a            .....................................................
Sot18-2a            .....................................................
Sot19-2a            .....................................................
Sot20-2a            .....................................................
Sot21-2a            .....................................................
Sot22-2a            .....................................................
Sot23-2b            .....................................................
Sot24-2b            .....................................................
Sot25-2b            .....................................................
Sot26-3a            .....................................................
Sot27-3a            .....................................................
Sot28-3a            .....................................................
Sot29-3a            .....................................................
Sot30-3a            .....................................................
Sot31-3a            .....................................................
Sot32-3a            .....................................................
Sot33-3a            .....................................................
Sot34-3a            .....................................................
Sot35-4             .....................................................
Sot36-5             .....................................................
```

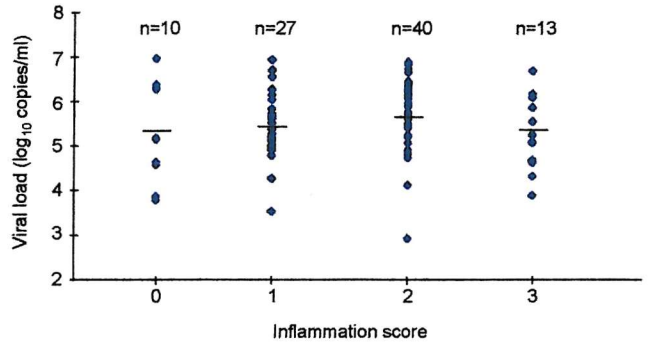
Table 10.6. Yields of pSotIC and pTRI-xef *in vitro* transcription reactions for different incubation times.

Incubation time (hr.)	Yield (μg/20μl reaction)			
	pSotIC		pTRI-xef	
	A_{260}	RiboGreen	A_{260}	RiboGreen
2	58.0	46.0	66.0	56.0
4	74.0	63.0	80.0	62.0
6	92.0	67.0	102.0	63.5
21	108.0	78.0	88.0	63.0

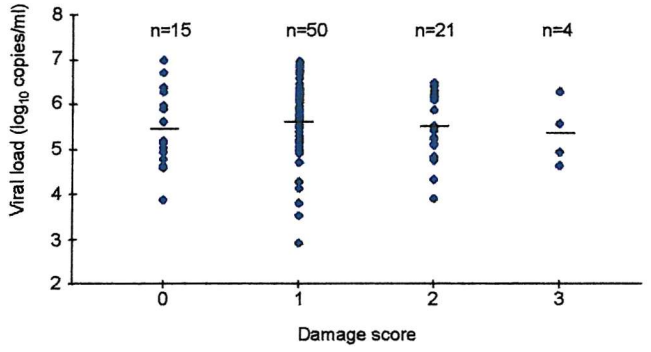
Single A_{260} and triplicate RiboGreen measurements (mean shown) were performed on *in vitro* transcription reaction samples taken after 2, 4, 6 and 21hr incubation.

Appendix IV: Results of a study of viral load in asymptomatic and symptomatic HCV-infected patients

(a) r = 0.072, $P = 0.501$



(b) r = -0.017, $P = 0.874$



(c) r = 0.119, $P = 0.265$

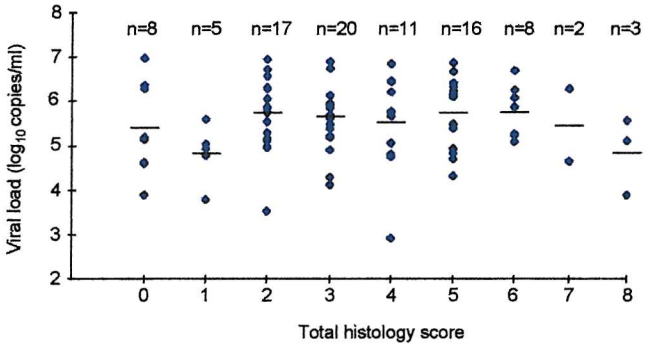


Fig.10.2 Plots of $\log_{10}$ viral load against (a) inflammation, (b) damage, (c) total histology score for asymptomatic and symptomatic patients combined.
Correlation coefficients (r) with probabilities (p) and sample numbers (n) and mean viral loads (horizontal lines) are shown.

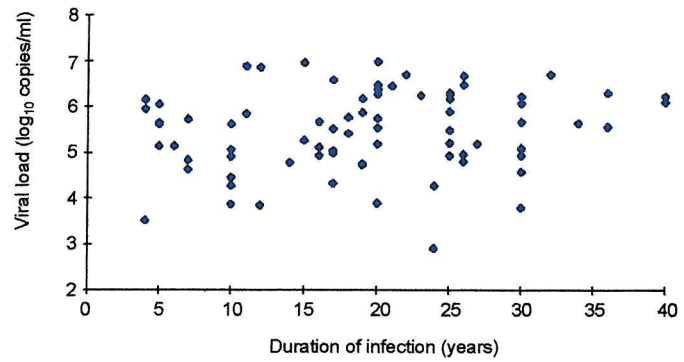


Fig.10.3 Plot of viral load versus estimated duration of infection for asymptomatic and symptomatic patients combined (r = 0.201, *P* = 0.081, n = 76).

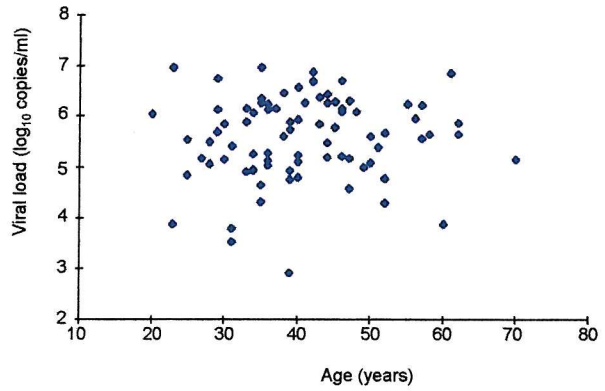


Fig.10.4 Plot of log_{10} viral load against age for asymptomatic and symptomatic patients combined (r = 0.156, *P* = 0.370, n = 83).

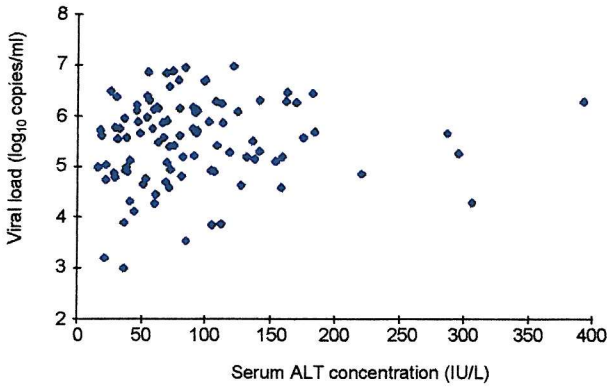


Fig.10.5 Plot of log_{10} viral load against serum ALT level for asymptomatic and symptomatic patients combined (r = 0.142, *P* = 0.155, n = 101).

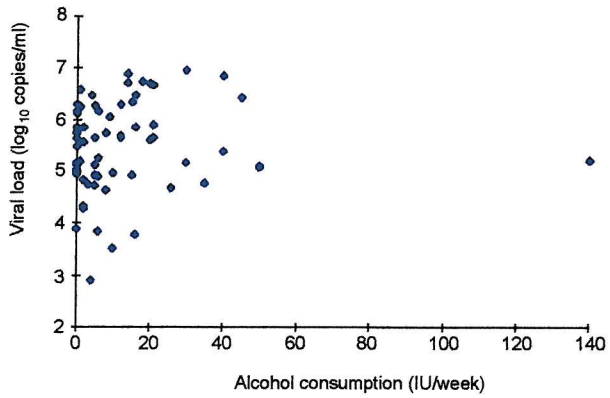


Fig.10.6 Plot of log_{10} viral load against alcohol consumption for asymptomatic and symptomatic patients combined (r = 0.152, *P* = 0.235, n = 65).

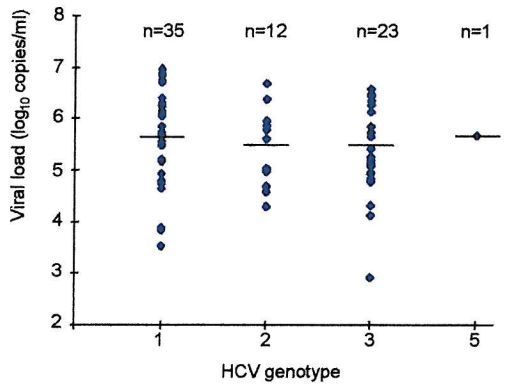


Fig.10.7 Plot of log_{10} viral load against infecting HCV genotype for asymptomatic and symptomatic patients combined.
Mean log_{10} viral loads are shown (horizontal lines).

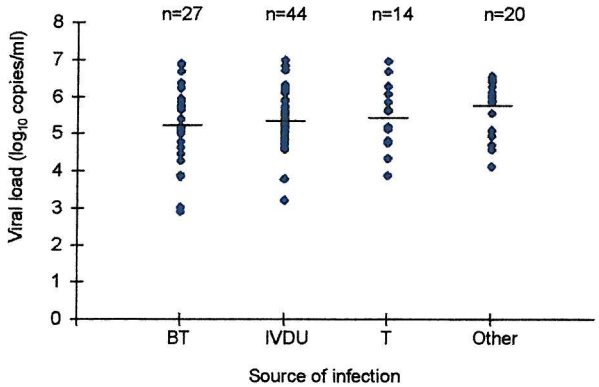


Fig.10.8 Plot of log_{10} viral load against suspected source of infection for asymptomatic and symptomatic patients combined.
Mean log_{10} viral load is shown (horizontal lines) for each suspected source (BT, blood transfusion; IVDU, in vitro drug use; T, tattoo).

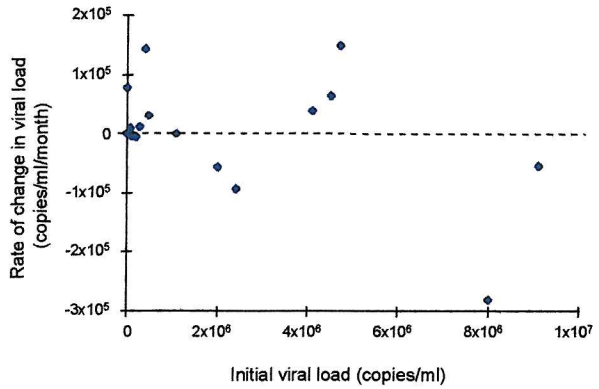


Fig.10.9 Plot of rate of change in viral load against initial viral load for asymptomatic patients (r=-0.140, $P = 0.567$, n = 19).
Dashed line = zero change in load.

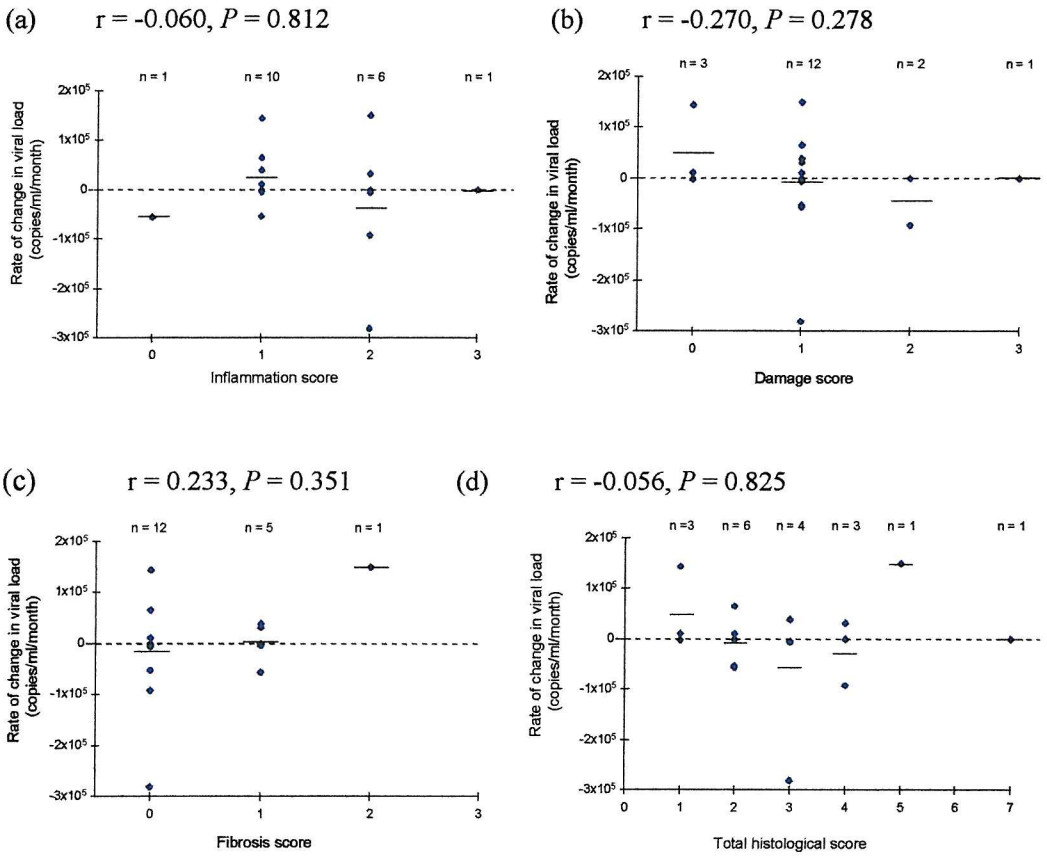


Fig.10.10 Plots of rate of change in viral load against inflammation (a), damage (b), fibrosis (c), and total histological score (d) for asymptomatic patients (n = 18).
Sample numbers (n) and mean viral load changes (horizontal lines) are shown.
Dashed line = zero change in load.

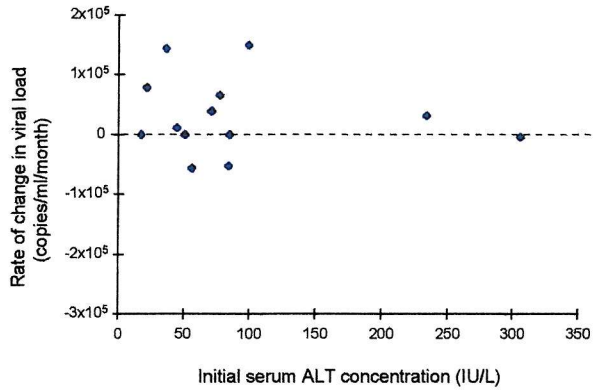


Fig.10.11 Plot of rate of change in viral load against initial serum ALT concentration for asymptomatic patients (r = -0.093, *P* = 0.762, n = 13).
Dashed line = zero change in load.

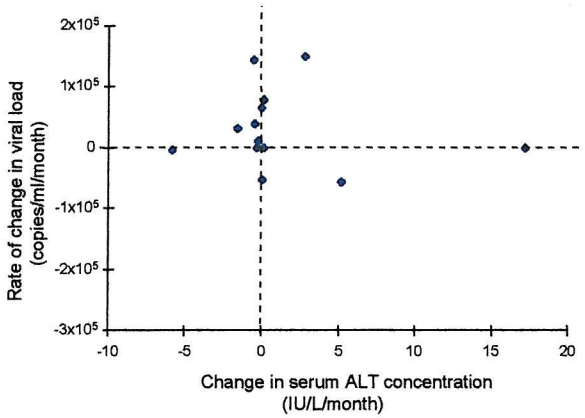


Fig.10.12 Plot of rate of change in viral load against rate of change in serum ALT concentration for asymptomatic patients (r = -0.143, *P* = 0.642, n = 13).
Dashed lines = zero change.

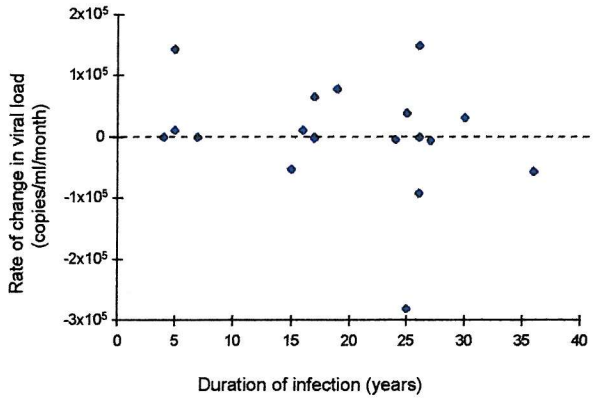


Fig.10.13 Plot of rate of change in viral load against duration of infection for asymptomatic patients (r = -0.248, *P* = 0.307, n = 19).
Dashed line = zero change in load.

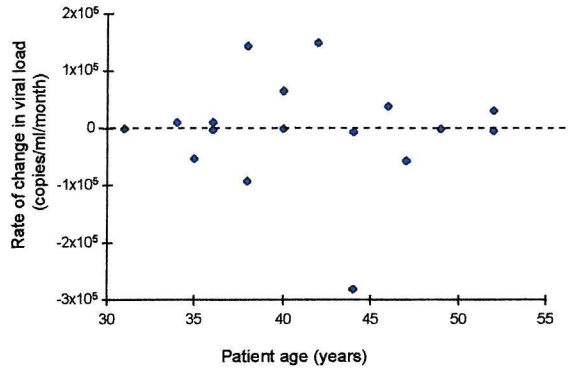


Fig.10.14 Plot of rate of change in viral load against patient age at biopsy for asymptomatic patients (r = -0.074, *P* = 0.778, n = 17).
Dashed line = zero change in load.

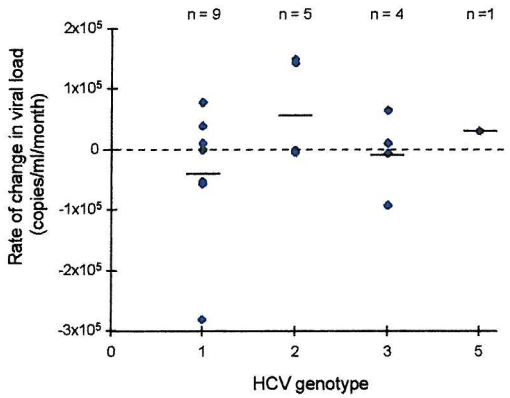


Fig.10.15 Plot of rate of change in viral load against infecting genotype for asymptomatic patients.
Sample numbers (n) and mean viral load changes are shown (horizontal lines).
Dashed line = zero change in load.

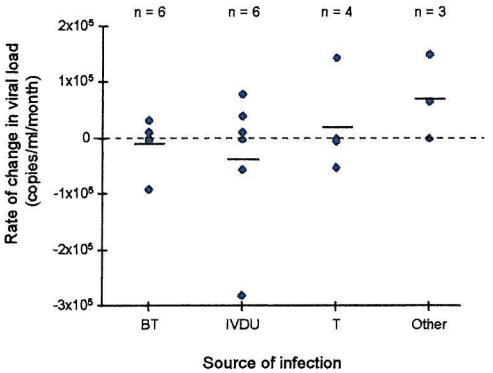


Fig.10.16 Plot of rate of change in viral load against suspected source of infection for asymptomatic patients.
Mean rates of change in viral load are shown (horizontal lines) for each suspected source (BT, blood transfusion; IVDU, *in vitro* drug use; T, tattoo). Dashed line = zero change in load.

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