HEPATITIS C VIRUS IN SALIVA

by

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To my Dad

CONTENTS

		Page
LIST OF CONTENTS		i
LIST OF TABLES		viii
LIST OF FIGURES		xii
ACKNOWLEDGEMENTS		xiv
PREFACE		xv
SUMMARY		xvi
ABBREVIATIONS		xix
CHAPTER 1: General Introduction		
	The Hepatitis Viruses	1
	Hepatitis non-A, non-B : Hepatitis C Virus	2
i)	Genomic organisation of HCV.	4
ii)	Immunodiagnostics for HCV.	7
	a) First generation tests.	7
	b) Second generation and onwards.	9
	c) Supplemental tests for HCV.	9
	d) Polymerase chain reaction for HCV RNA.	14
	e) Quantification of HCV RNA.	16
iii)	Patterns of immune response.	18
iv)	Genotyping of HCV.	19
v)	Epidemiology of hepatitis C virus infection.	23
	a) Parenteral transmission.	24

i) Recipients of blood and blood products. 24

ii) Recipients of products derived from large pools of plasma. 25

iii) Through organ transplantation. 25

iv) Through intravenous drug use. 26

v) Through occupational exposure. 26

b) Undefined routes of nonparenteral transmission. 27

i) Sexual transmission. 27

a) Prostitutes and patients attending clinics for sexually transmitted disease. 27

b) Homosexuals. 28

c) Sexual contacts of HCV positive patients. 28

ii) Vertical (mother-to-child) transmission. 30

iii) Intrafamilial (household) transmission. 31

vi) Natural course of hepatitis C virus infection. 32

vii) Treatment of hepatitis C virus with interferon. 34

a) Who to treat. 34

i) Patients with acute hepatitis C. 35

ii) Patients with decompensated liver disease. 35

iii) Children with chronic hepatitis C. 35

iv) Patients with atypical serology. 36

v) Immunosuppressed patients. 36

b) Dose and duration of treatment. 37

c) Side effects. 37

d) Monitoring treatment 38

i) Serum aminotransferase levels and liver histology. 38

ii) Serum antibodies to hepatitis C virus. 38

iii) HCV RNA qualitatively and quantitatively in serum. 39

iv) HCV RNA qualitatively and quantitatively in the liver and peripheral mononuclear cells. 39

Predictors of response. 40

viii) Other antiviral therapies. 41

a) Ribavirin. 41

b) Inosine. 42

c) Corticosteroids. 42

ix) HCV vaccines. 42

Saliva 43

i) Salivary secretion. 43

ii) Testing for salivary antibodies. 44

iii) Testing for salivary antigens. 45

A Specimen of Convenience 45

Salivary Testing for Hepatitis C Virus 46

CHAPTER 2: General Materials and Methods

i) Materials. 48

a) DNA. 48

b) Enzymes. 48

c) Chemicals. 48

d) Radioisotopes. 49

e) Miscellaneous items. 49

f) Nucleic acid purification systems 50

ii) Methods. 50

a) Sample collection. 50

i) Whole saliva. 50

ii) Oral fluid. 50

iii) Blood specimens. 50

b) Reverse transcription. 51

c) Polymerase chain reaction (nested-set). 51

i) First round. 51

ii) Second round. 51

d) Agarose gel electrophoresis. 52

e) Contamination controls 52

CHAPTER 3: Application of the Polymerase Chain Reaction for the Diagnosis of Hepatitis C Virus in a Routine Clinical Laboratory

i) Introduction. 59

ii) Materials and Methods. 62

a) Comparison of RNA extraction protocols. 62

i) RNA extraction with guanidinium isothiocycanate / acid-phenol. 62

ii) RNA extraction with QIAamp blood kit. 63

iii) RNA extraction with GLASSMAX RNA micro-isolation Spin Cartridge System. 63

b) Comparison of PCR amplification protocols. 63

i) Hot start PCR. 63

ii) Combined one-step RT-PCR. 64

c) Comparison of protocols used to detect amplified product. 64

i) Preparation of DNA probes. 65

a) 3' enlabelling with digoxigenin-11-ddUTP. 65

b) Random primed DNA labelling with Digoxigenin-11-dUTP. 66

ii) Dot blot analysis. 66

iii) Southern blot analysis. 67

iv) Hybridisation of immobilised DNA to labelled DNA probes. 68

v) Colorimetric detection of DIG-labelled probe and marker. 68

iii) Results. 81

a) Comparison of RNA extraction protocols. 81

b) Comparison of PCR amplification protocols. 81

c) Comparison of methods used to detect amplified product. 82

iv) Discussion. 83

CHAPTER 4: Detection of HCV RNA in the Saliva of Haemophiliacs and Blood Donors

i) Introduction. 90

ii) Methods and Materials. 92

a) Sample population. 92

b) Sample collection. 92

c) RNA extraction. 92

d) Reverse transcription and PCR. 93

iii) Results. 94

iv) Discussion. 100

CHAPTER 5: Serological and Salivary Markers Compared with Biochemical Markers in the Monitoring of Interferon Treatment for Hepatitis C Virus Infection

i) Introduction. 103

ii) Methods and Materials. 105

a) Sample population. 105

b) Interferon treatment. 105

c) Follow-up. 106

d) Sample collection. 106

e) RNA extraction. 106

f) Reverse transcription and PCR. 106

g) Quantitative PCR. 106

h) Genotyping. 107

i) Definition of response. 107

iii) Results. 108

iv) Discussion. 116

CHAPTER 6: Optimisation of Specimen Collection and Handling Methods for the Detection of Hepatitis C Virus in Saliva

i) Introduction. 123

ii) Methods and Materials. 125

a) Sample population. 125

b) Sample collection. 125

c) Sample processing. 126

d) RNA extraction and reverse-transcription. 127

e) Polymerase chain reaction. 127

f) DNA sequencing 127

i) Radioactive label 128

ii) Enzymatic pretreatment 128

iii) Denaturing reaction 128

iv) Annealing reaction 128

v) Polyacrylamide seqencing gel 129

g) Analysis of DNA sequencing data 130

iii) Results 134

iv) Discussion 146

CHAPTER 7: General Discussion 158

REFERENCES

APPENDICES

LIST OF TABLES

Table	Title	Page
Chapter 1		
1.1	The major hepatitis viruses.	2
1.2	Commercially available second generation screening anti-HCV ELISAs.	10
1.3	Commercially available second and third generation anti-HCV supplemental tests.	12
1.4	Proposed nomenclature and comparison with existing schemes for HCV genotypes.	20
Chapter 2		
2.1	General stock solutions and buffers.	54
2.2	Reverse transcription master mix.	56
2.3	Sequences, sources, positions and polarity of primers.	57
2.4	Polymerase chain reaction master mixes.	58
Chapter 3		
3.1	Stock solutions and buffers.	70
3.2	Viral nucleic acid extraction using QIAamp blood kit.	75
3.3	Viral nucleic acid extraction using GLASSMAX RNA micro-isolation spin cartridge system	76

3.4 Upper and lower master mixes employed in the first round of nested 'hot start' PCR. 77

3.5 Upper and lower master mixes employed in the second round of nested 'hot start' PCR. 78

3.6 Combined RT-PCR master mix. 79

3.7 Second round PCR master mix used with combined RT-PCR. 80

Chapter 4

4.1 Summary of HCV RNA positive saliva specimens from HCV seropositive haemophiliacs. 96

4.2 Summary of the effect of two freeze thaw cycles on HCV RNA positive saliva. 97

4.3 Summary of HCV RNA positive saliva samples from HCV seropositive blood donors. 98

4.4 Genotypic analysis of HCV RNA seropositive patients with detectable HCV RNA in their saliva. 99

Chapter 5

5.1 Demographic characteristics of treatment and observation patients at enrollment of the interferon drug trial. 110

5.2 Pretreatment patient characteristics according to response to interferon treatment. 111

5.3 Viral genotypes with regard to response to interferon treatment. 112

5.4 Summary of HCV RNA positive saliva samples from 7 observation patients and 2 non-responders. 113

5.5 Level of HCV viraemia in serum in relation to the presence of HCV RNA in serum and saliva by PCR. 114

5.6 Viral genotypes of HCV in patients with relation to the presence or absence of HCV RNA in their saliva. 115

5.7 Effect of interferon on HCV viraemia levels in patients receiving treatment. 122

Chapter 6

6.1 Summary of the oral health of the subjects enrolled in the study with respect to their HIV serostatus. 131

6.2 Sequencing gel stock solutions and mixes. 132

6.3 Oral characteristics of patients with respect to the presence of HCV RNA in their saliva. 137

6.4 Summary of total PCR positive saliva samples collected by the three different devices from the HCV and HIV co-infected intravenous drug users. 138

6.5 Summary of PCR on the positive saliva samples collected from HCV seropositive intravenous drug users. 139

6.6 Summary of PCR on whole saliva samples. 140

6.7 Summary of PCR on samples collected using Omnisal™ device. 141

6.8 Summary of PCR on samples collected using Salivette™ device. 142

6.9 The effects of repeat freeze-thaw cycles on the stability of HCV RNA. 143

6.10 Oral characteristics of patients with detectable HCV RNA in their saliva with respect to HCV serostatus. 144

6.11 Viral genotypes of HCV in the blood and saliva of IVDU 145

6.12 Oral characteristics of intravenous drug users with detectable HCV RNA in their saliva. 156

6.13 Oral characteristics of intravenous drug users with HCV RNA positive saliva but negative serum. 157

LIST OF FIGURES

Figure	Title	Following page
Chapter 1		
1.1	Schematic representation of the hepatitis C virus genome, its products encoded and their putative functions.	5
1.2	Serological course in acute resolving HCV infection.	15
1.3	Schematic depiction of saliva formation in the two general regions of the salivary gland.	43
Chapter 2		
2.1	Saliva collection devices for whole saliva and oral fluid used in the detection of HCV RNA by PCR.	58
Chapter 3		
3.1	Comparison of RNA extraction protocols.	82
3.2	Comparison of reverse transcription and amplification protocols.	82
3.3	Comparison of methods used to detect amplified PCR products.	82
Chapter 5		
5.1	Changes in ALT levels after 12 weeks of receiving 4.5MU IFNα-2a thrice weekly.	115

5.2 Virological relapse without biochemical relapse in patient no. 10 after 12 weeks of IFN treatment. 115

5.3 Serial changes in serum ALT levels in observation patients throughout the study. 115

5.4 Virological relapse without biochemical relapse in patient no. 11 after a complete response to IFN had been attained at week 12. 122

5.5 Relationship between genotype and the initial viral load of six patients receiving IFN. 122

Chpater 6

6.1 The Omnisal™ collection device. 133

6.2 Collection protocol for the Omnisal™. 133

6.3 Predicted cleavage patterns for digestion with HaeIII / Rsa 1 and Hinf1 / Mva 1. 133

6.4 Predicted combinations of RFLP patterns obtained from cleavage with HaeIII / Rsa 1 and Hinf1 / Mva 1 associated with sequences of HCV types 1 to 6. 133

6.5 Predicted cleavage patterns for digestion with Bst U1 or Scr F1. 133

6.6 Summary of total HCV RNA positive saliva samples from the intravenous drug users with regard to HCV serostatus. 145

6.7 Effect of sample handling conditions on the PCR result with regard to collection device. 157

6.8 Association between 5'NCR polymorphisms and virus genotype. 157

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PREFACE

The work in this thesis was carried out in the Department of Oral Sciences, Glasgow Dental School and Hospital, and the Scottish National Blood Transfusion Service Microbiology Reference Unit, Ruchill Hospital from October 1992 to January 1996, under the supervision of Dr Jeremy Bagg and Dr Eddie Follett.

These studies represent original work carried out by the author, except for the RFLP genotyping which was carried out by the South-East of Scotland Blood Transfusion Service, and the quantitative studies which were carried out by Prof Johnson Lau, and have not been submitted in any form to any other university. Where use has been made of material provided by others, due acknowledgment has been made in the text.

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SUMMARY

"The identification at the beginning of the decade of the hepatitis C virus (HCV) as the cause of a major proportion of non-A, non-B hepatitis has stimulated extensive research into the biology and clinical aspects of infection with this virus, and its epidemiology and public health importance as a cause of morbidity and mortality in human populations. The findings of epidemiological studies suggest substantial prevalence of infection in populations, together with the observation of high rates of persistence of infection, and high levels of morbidity in exposed individuals strongly supports the view that HCV represents a major clinical and public health challenge. The design and implementation of a strategy to control HCV infection is, therefore, a clear priority. However, control of HCV infection must be founded upon a knowledge of the history and dynamics of HCV transmission within the population. Such knowledge remains patchy at present and is poorly intergrated."

Viral Hepatitis Prevention Board , Barcelona, 1995.

To investigate the prevalence of hepatitis C virus in saliva, the saliva of a group of haemophiliacs and blood donors, who were previously shown to be anti HCV positive by second generation immunoassay and a recombinant immunoblot, was examined using reverse transcription and the polymerase chain reaction. Both studies indicated that HCV was present in the saliva of a proportion (47.6% and 34% respectively) of each study group.

With a view to utilizing the polymerase chain reaction on saliva as a routine diagnostic tool for the detection of HCV, a revised protocol was sought, from the numerous procedures available, to reduce the sophisticated technique to a reproducible, reliable method. All aspects of the entire protocol from beginning to end were considered. HCV RNA extraction was assessed using two commercially available kits, in addition to the more traditional lysis with a chaotrophic agent followed by organic extraction and ethanol precipitation. Two procedures involving separate reverse transcription and the polymerase chain reaction were compared with a combined one step reverse transcription and polymerase chain reaction, to establish a protocol ensuring maximum amplification while reducing the possibility of false positive results. Finally, a sensitive and specific method was sought that would enable accurate detection of the desired product in a non-laborious manner.

The appropriateness of the polymerase chain reaction performed on saliva samples as an alternative to blood was evaluated among a group of HCV infected blood donors enrolled in a trial of interferon. Observation and treatment patients were monitored using biochemical and virological markers. Blood and saliva were collected in parallel and tested for HCV RNA. Results indicated that saliva was not a suitable alternative to blood for determining response to treatment.

In every study, both whole saliva and oral fluid collected in commercially available devices were obtained. HCV RNA could be detected in both specimen types but often not in parallel. These discrepancies may have resulted from deficiencies in the sample handling protocols. It is likely that specimen processing and storage conditions may influence the stability of HCV RNA. This was evaluated by studying saliva samples collected from known HCV seropositive intravenous drug users, which had been subjected to a number of handling and storage conditions. No single method was shown to be appropriate for both saliva and oral fluid.

The evaluation of paired serum and saliva samples for HCV RNA indicated that some individuals had HCV RNA present in saliva but not in blood. DNA sequencing was employed to confirm the presence of HCV RNA in saliva and to genotype the isolates amplified. The presence of different subtypes and even genotypes in the mouth and serum was observed.

The source of HCV within the mouth and the potential risk of transmission through saliva are discussed.

ABBREVIATIONS

AIDS	acquired immunodeficiency syndrome
ALT	alanine aminotransferase
AST	aspartate aminotransferase
BDA	British Dental Association
bDNA	branched deoxyribonucleic acid
cDNA	copy deoxyribonucleic acid
CAH	chronic active hepatitis
CPH	chronic persistent hepatitis
CR	complete responder
dATP	2'- deoxy adenosine 5' - triphosphate
dCTP	2' - deoxycytidine 5' - triphosphate
dGTP	2' - deoxyguanosine 5' - triphosphate
DIG	digoxigenin
DNA	deoxyribonucleic acid
dNTP	2' - deoxyribonucleoside 5' - triphosphates
dTTP	2' - deoxythymidine 5' - triphosphate
dUTP	2' - deoxyuridine 5' - triphosphate
E. coli	Escherichia coli
ELISA	enzyme linked immunoassay
EIA	enzyme immunoassay
gp	glycoprotein
HAV	hepatitis A virus
HBV	hepatitis B virus
HBsAg	hepatitis B surface antigen
HCV	hepatitis C virus
HDV	hepatitis D virus
HEV	hepatitis E virus
HIV	human immunodeficiency virus
HCC	hepatocellular carcinoma
GUSCN	guanidine isothiocyanate

IFN	interferon
IgG	immunoglobulin G
IgM	immunoglobulin M
IRES	internal ribosome entry site
IU	international units
IVDU	intravenous drug user
LiPA	line probe assay
MMLV-RT	moloney murine leukaemia virus reverse transcriptase
MU	international mega units
NANBH	non-A, non-B hepatitis
NCR	non coding region
NS	nonstructural
NR	non responder
PCR	polymerase chain reaction
PBMC	peripheral blood mononuclear cells
PR	partial responder
PT	post transfusion
PT-NANBH	post transfusion non-A, non-B hepatitis
RFLP	restriction fragment length polymorphism
RIA	radioimmunoassay
RIBA	recombinant immunoblot assay
RNA	ribonucleic acid
RP	recombinant protein
RT-PCR	reverse transcription polymerase chain reaction
sdH_2O	sterile distilled water
sIgA	secretory immunoglobulin A
SLPI	serine leukocyte protease inhibitor
SNBTS	Scottish National Blood Transfusion Services
SOD	superoxide dismutase
STD	sexually transmitted diseases

Taq pol	Thermus aquaticus polymerase
Tth pol	Thermus thermophilius polymerase
UNG	uracil - N - glycosylate
UV	ultraviolet

Chapter 1

General Introduction

The Hepatitis Viruses

Acute viral hepatitis is a common and sometimes serious viral infection of the liver, leading to inflammation and necrosis. On the basis of clinical and epidemiologic features it has been clear for many years that there are various types of this disease. In the 1960s, the different types of infectious hepatitis started to be identified (Krugman et al., 1967). The initial breakthrough came when the hepatitis B surface antigen (HBsAg) was discovered and linked to hepatitis B (Blumberg et al., 1967). This was followed in 1973 by the identification of hepatitis A (Feinstone et al., 1973). The delta agent or hepatitis D virus (HDV), which is also capable of causing viral hepatitis in conjunction with hepatitis B, was discovered in 1977 (Rizetto et al., 1977). Since then, two further agents causing non-A, non-B hepatitis have been described, hepatitis C virus (HCV) (Choo et al., 1989) and hepatitis E virus (HEV) (Reyes et al., 1990). The main features of these five viruses which cause hepatitis in man are summarised in Table 1.1

Hepatitis non-A, non-B : Hepatitis C virus

In the 1960s, before the identification of hepatitis A virus (HAV) and hepatitis B virus (HBV), it was noted that the period of incubation for transfusion-associated hepatitis was on average approximately 7 weeks. This was later recognised as being intermediate between that for HAV infection (2-6 weeks) and for HBV (2-6 months). Little weight however was given to this clinical observation. Only with the development of sensitive serological tests for identifying infection with HBV was it recognised that HBV could only be implicated in as few as 25% of transfusion-associated hepatitis cases.

TABLE 1.1

The Major Hepatitis Viruses

	Hepatitis A	Hepatitis B	Hepatitis C	Hepatitis D	Hepatitis E
Virus group	Picornavirus	Hepadnavirus	Pesti/Flavi-virus like	Plant virus-like	Calcivirus-like
Genetic material	RNA	DNA	RNA	RNA	RNA
Routes of transmission	Faecal-oral	Blood-borne Vertical Sexual	Blood-borne Vertical Sexual?	Blood-borne	Faecal-oral
Incubation period	2-6 weeks	8-24 weeks	4-20 weeks	3-12 weeks	3-7 weeks
Chronicity	No	Yes	Yes	Yes	No

The main characteristics of the major viruses known to cause hepatitis in man.

An early report by Koretz et al., (1973) described post transfusion hepatitis in patients who developed non-B hepatitis after receiving HBsAg negative blood. It was also shown that antibody to HBsAg , known to correlate with immunity to HBV when present in a recipient's blood before transfusion, did not protect that recipient from hepatitis after transfusion.

Initially it became fashionable to implicate HAV in these non-B cases following transfusion. However, once serological tests were developed to identify HAV infection, several studies demonstrated convincingly that HAV played no role in post-transfusion hepatitis (Alter et al., 1975a; 1975b; Dienstag et al., 1977; Feinstone et al., 1975). Thus, it became apparent that there existed at least one blood-borne human hepatitis virus previously unrecognised and unrelated to hepatitis A and B. By exclusion, this was termed non-A, non-B hepatitis (NANBH). Information gathered from clinical observations and studies of transmission in chimpanzees allowed certain characteristics of the virus to be deduced and a better understanding of the disease and its pathogenesis to be obtained. However, it took almost 15 years to detect the virus serologically through application of molecular biologic techniques (Choo et al., 1989).

In May 1988, the American Chiron Corporation described in a press conference the identification, cloning and expression of proteins from the agent responsible for most cases of post transfusion-non-A, non-B hepatitis (PT-NANBH). Cloning was initiated by ultracentrifugation of large volumes of infectious chimpanzee plasma known to contain the NANBH agent. From the pellet formed, the nucleic acid was recovered and denatured to allow either DNA or RNA to serve as a template for reverse transcription using random primers. The resultant cDNA was then inserted into a cloning vector, λgtll, and expressed in *E.coli* . Following lysis of the bacteria, the proteins expressed were

absorbed to an overlying filter and immunoscreened with serum from a patient with chronic NANBH which was presumed to contain antibody to the virus. After screening many clones, one single clone was reactive by radiography. The insert in this clone was then cut and used as a hybridisation probe to the original library of cDNA, and from several overlapping clones, a larger clone was obtained (c100-3). This was then expressed in yeast as a fusion protein using human superoxide dismutase sequence to facilitate expression (Choo et al., 1991). This led to dramatic advances in our understanding of the virus which was named HCV. It was now possible to detect the virus, but knowledge of the epidemiology and natural history of HCV infection remains incomplete.

i) Genomic Organisation of HCV

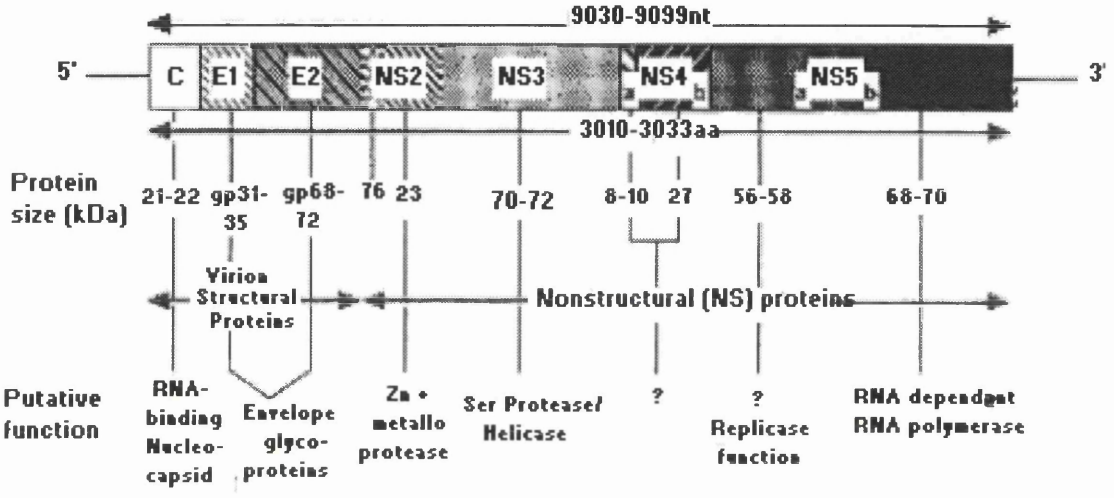
HCV contains a positive-stranded RNA genome reported to be about 9,400-10,000 nucleotides. The genome contains a 5' non-coding region (5' NCR) followed by a single open reading frame which is believed to encode a polypeptide precursor of just over 3,000 amino acids (Chan et al., 1992). Comparison of the sequence of both the genome and its encoded polypeptide with other known viral sequences revealed little homology. However, evidence showing a distant relationship to the pestiviruses and the flaviviruses has resulted in HCV, pestiviruses and flaviviruses all being considered separate genera of the Flaviviridae family (Choo et al., 1991; Miller and Purcell, 1990). As information regarding the genetic and polypeptide organisation of the HCV genome has accumulated, it has emerged that,despite poor overall primary sequence similarity between the three virus types, the basic structure and organisation of their encoded

polypeptides are comparable with regard to their hydropathicity profiles (Choo et al., 1991). However, an exception is the 5' NCR, upstream from the sequence encoding the large polyprotein which is highly homologous with the equivalent region in the pestiviruses (Choo et al., 1991; Takamizawa et al., 1991). An important role for this region in some aspect of viral replication has been suggested. Comparisons have been made to the lengthy 5' NCR present in the RNA genomes of picornaviruses (Inchauspe et al., 1991). These allow for cap-independent translation at internal ribosome entry sites (IRES) and also contain cis-acting sites which allow internal entry of ribosomes. Although no significant nucleotide sequence relatedness exists, reports of such structures in the 5' NCR of the HCV genome have been made (Brown et al., 1992; Selby et al., 1993; Yoo et al., 1992). Which structure, if indeed either, is important to viral replication and polyprotein translation remains to be determined.

HCV encodes a single polyprotein precursor from which individual proteins are processed. Whether this occurs co-translationally and/or post-translationally through the action of host and viral encoded protease has not yet been resolved. Schematically, four domains can be identified: two NCR found at the 5' and 3' end, the 5' structural region (containing the core and envelope proteins), and a 3' nonstructural region (encoding a variety of proteins, NS1-NS5). The structural proteins form the viral particle, whereas the nonstructural proteins are involved in replication of the virus.

By transfecting cloned cDNA into mammalian cells via a range of expression vectors it has been possible for some protein products to be identified (Figure 1.1). A nucleocapsid protein of about 22kD and two glycoproteins (gp) of approximately 33kD and 72kD are processed from the 5' terminal region of the genome. By comparison with the

FIGURE 1.1



Schematic representation of the hepatitis C virus genome, its products encoded and their putative functions.

pestiviruses and flaviviruses, gp33 is probably equivalent to a matrix glycoprotein in the virion while gp72 is a viral envelope glycoprotein corresponding to gp53/55 of the pestiviruses. Gp72 may also represent the first NS protein analogous to the NS1 glycoprotein of the flaviviruses (Houghton et al., 1994). It has been indicated that gp72 is more likely to be equivalent to the pestiviral protein as,unlike the flaviviral glycoprotein, it is not secreted. However, it has also been shown that in contrast to the pestiviral protein, the flaviviral NS1 and HCV NS1 contain potential glycosylation sites (Takamizawa et al., 1991). Comparison of the available E2/NS1 sequences revealed the presence of a hypervariable region. This region seems to lack a conserved secondary structure and resembles the V3 loop of HIV gp120 (Weiner et al., 1992). Specific antibody reactions have been detected against peptides corresponding to linear epitopes in this region, indicating that this part of the E2 region encodes antigenically distinct variants subject to immune selection (Weiner et al., 1992; Lesniewski et al., 1993; Taniguchi et al., 1993). The variability of gp72 is likely to have significant implications for vaccine strategies. From the nonstructural region, proteins of 23 and 60kD, corresponding to the flaviviral NS2 and NS3 proteins, have been identified. Both the NS2 and NS3 of HCV encodes different proteases. The NS3 protein appears to contain a nucleotide triphosphate-binding helicase enzyme and a serine protease that is presumably involved in the unwinding of the RNA template during replication, translation and /or assembly of the polyprotein precursor into the mature proteins. Selby et al, (1993), report that NS3 cleaves itself from the polyprotein precursor before catalysing cleavage reactions to liberate NS4 and NS5. Less is known about NS4 and NS5 proteins. NS4 appears hydrophobic and codes for two proteins (4a,4b). Both contain highly immunogenic epitopes and may be associated with cellular membranes (Houghton et al., 1994). NS5 is thought to contain the RNA dependent RNA

polymerase needed for replication (Takamizawa et al., 1991). It has been shown that NS5 is processed into two proteins NS5a and NS5b, by NS3 (Selby et al., 1993). The processing of the NS5 domain into the 2 proteins resembles the situation for the pestiviruses and is in contrast to that of the flaviviruses. This reaction implies that HCV more closely resembles the pestiviruses (Collett et al., 1988). NS5b has been reportedly shown to encode the viral replicase. The function of NS5a remains unknown.

ii) Immunodiagnostics for HCV

a) First Generation Tests

The cloning of the HCV genome facilitated the production of a panel of recombinant antigens. Large quantities produced in *E. coli* and yeast formed the basis of the widely used immunodiagnostic assays aimed at detecting antibody to HCV. Sequence data have also enabled production of synthetic peptides for use in these assays as well. The original antibody test developed incorporated the c100-3 polypeptide expressed in yeast as a fusion protein with human superoxide dismutase (SOD). This polypeptide corresponded to nearly all of the NS4 protein of the original isolate (Choo et al., 1989; Houghton et al., 1991), but represented only about 12% of the coding capacity of HCV (Chien et al., 1992). This recombinant antigen was attached onto the surface of microwells for use in a solid-phase capture assay.

The original test developed was a radioimmunoassay (RIA).This was succeeded by a commercially available enzyme-linked immunoassay (ELISA) developed by Ortho Diagnostic Systems (Raritan, NJ, USA) with recombinant antigens provided by Chiron. Subsequently, another anti-HCV first generation ELISA assay was produced by Abbott Laboratories (Nth Chicago, Ill, USA). The presence of antibody to c100-3 proved a good

marker for infection with HCV and was detected in about 80% of chronic HCV cases whether sporadic or PT-NANBH (Courouce and Janot, 1994). However, following the introduction of these assays as a routine clinical and screening tool it became apparent that the tests were unsatisfactory with regard to sensitivity and specificity. False positive results were encountered in patients with superoxide dismutase antibodies, rheumatoid factors and autoantibodies (M^{c}Farlane et al., 1990). Such reports created controversy over the widespread implementation of the first generation assay, as the frequency of false positives increased significantly in low-risk populations such as blood donors and those with sporadic disease (Contreras and Barbara, 1989; Dodd, 1992; Flegg, 1989). It should be remembered, however, that the introduction of these first generation assays was an improvement from the use of surrogate markers, and represented a very important step in the diagnosis of HCV infection, reducing the incidence of PT-NANBH by 50-85% or more (Dodd, 1992; Courouce and Janot, 1994).

The cause of the false positive results with the first generation assays remains unresolved. Nonspecific serum 'stickiness' to the solid phase has been considered (M^{c}Farlane et al., 1990). The assay also lacked sensitivity in that not all persons with HCV seroconvert according to the first generation antibody test. Some never produce detectable levels of anti c100-3, while in others anti c100-3 disappears rapidly (Bonino et al., 1993). The test is also limited by the delay in antibody production often observed. This suggests that there is a lengthy 'window period' during which a patient may have a negative test result using first generation assays. It is interesting to note that even in direct comparison, the Abbott and Ortho first generation ELISAs do not identify the same anti-HCV positive specimens. Both use the solid phase antigen licensed by Chiron Corporation but the tests have different assay designs, Ortho using the microtiterplate,

and Abbott a latex bead. How much of an effect the design has on the test has not been assessed or considered by other researchers. Perhaps incorporation into the different test designs presents epitopes in a slightly different conformation. More often, differences resulting from individual workers are held responsible for these discrepancies.

b) Second Generation and Onwards

The deficiencies in the first generation tests led to the development of second generation tests. These assays incorporated additional recombinant antigens from the HCV genome (Table 1.2). At present, the Ortho HCV 3.0 ELISA test system is widely used in blood donor screening. Sensitivity is further increased by addition of antigen derived from the NS5 region. This shows an even greater increase in sensitivity than the second generation assay when compared to the original tests. The format of the third generation assay is however the same as the first two and therefore has the same risk of false positive results in low risk populations, such as blood donors.

c) Supplemental Tests for HCV

In order to confirm results obtained by any ELISA, for any viral antibody, there should be a test that is of equal sensitivity and specificity, and utilises a different assay format and uses different antigens. Most of the available confirmatory tests only fulfil two of these criteria, and as a result they should be considered as supplementary rather than truly confirmatory.

The first confirmatory test was a recombinant immunoblot assay (RIBA) purchased from the Chiron Corporation through Ortho Diagnostic Systems. This first

TABLE 1.2

		HCV proteins	
Manufacturer	Nature	Name	Region
Ortho D. S.	recombinant	C 22-3	core
		C 200: C 33c	NS3
		C100-3	NS4
Abbott	recombinant	C 22-3	core
		C 33c	NS3
		C100-3	NS4
Sanofi Pasteur	recombinant	NC 450	core
		4091-1	NS3
UBI	synthetic peptides	3K 243E	core
		3K 204H	NS4
Murex-Wellcome	recombinant	/	core
			NS3
			NS5
Behring	synthetic peptides	/	core
			NS4

Commercially available second generation screening anti-HCV ELISA assays. The table summarises the nature, name, and source of the HCV proteins used as antigens in the assays.

generation test used the three recombinant antigens: the composite C100-3 produced in yeast, the original 5-1-1 produced in *E. coli*, and SOD. The C100-3 and the 5-1-1 antigens are superoxide dismutase-fusion polypeptides of HCV hence the SOD antigen is present as a control to detect the presence of antibody to SOD. All three antigens are blotted onto nitrocellulose strips along with two levels of human IgG as internal controls. For this assay, a positive result requires reactivity to two HCV antigens with bands of intensity at least equal to the low IgG control band. Confirmatory HCV neutralisation assays were also developed by Abbott Laboratories. These are based on the principle that HCV antigens in solution may block or neutralise the binding of antibodies to HCV-coated polystyrene beads in the HCV ELISA. Second and third generation supplemental tests are available from a number of manufacturers (Table 1.3).

Evaluation of these newer assays is still ongoing. With the introduction of additional antigens, the newer assays do exhibit better reproducibility, specificity and sensitivity than the first generation assays , owing to the presence of core and NS3 proteins. Antibodies to C22-3 and C33c are detectable earlier , more regularly and for a longer period than C100-3 (Courouce and Janot, 1994). Some patients develop antibodies to C22-3 alone or C33 and C22-3 but not to C100-3 (Lelie et al., 1992).

On the above evidence, the strategy to assess true HCV status in low risk populations involves initial anti-HCV detection by a screening ELISA followed by an alternative ELISA and a supplemental assay. Because the available anti-HCV supplemental assay systems employ different methods from the screening ELISA, it is reasoned that their agreement can be used to classify anti-HCV status (Dow et al., 1993). Problems arise, however, when RIBA-indeterminate results are obtained. Some patients develop antibodies to c22-3 only or c33c and can be viraemic. The significance of such RIBA-2 patterns may

TABLE 1.3

Name of test	Core	NS3	NS4	NS5	Manufacturer
RIBA-2	C22-3 RP	C33-c RP	5-1-1 RP C100-3 RP		Chiron
RIBA-3	peptide	C33-c RP	peptide	RP	Chiron
Matrix	RP	C33-c RP	C100-3 (yeast) C100-3 (E. coli)		Abbott
Deciscan	C1=NC450 RP C2= peptides	409.1 RP	peptide		Sanofi -Pasteur
Inno-Lia	four peptides		peptide	peptide	Innogenetics

Commercially available second and third generation anti-HCV supplemental tests, and the HCV epitopes used in the immunoblots. RP= recombinant protein.

differ between blood donors and high risk individuals. In patients with symptoms, or those who are asymptomatic but fall into a recognised risk group, a positive result in the screening ELISA is likely to be specific, even if only an indeterminate result is obtained with a supplemental test (Skidmore, 1994). Indeterminate results obtained from normal low risk individuals are an important problem for the Blood Transfusion Service. Such donors need to be confirmed as true positives to prevent the unnecessary loss of units for transfusion. The introduction of third generation supplemental tests shows good correlation with viraemia and has improved the above situation.

It is important to consider here that available assays, while measuring antibody against epitopes of non-assembled HCV proteins, may miss those against tertiary and quaternary structure dependent epitopes (Bonino et al., 1993). It has also to be remembered that a sample giving a positive reaction in one assay may be negative in another due to different combinations of antigen being employed in different assays. A further important consideration is that currently employed assays use antigens derived from HCV type 1 sequences. As a result, diagnosis of infections with other HCV genotypes relies on detecting cross-reacting antibodies. However, the degree of cross-reactivity will depend on the degree of sequence similarity between genotypes. It is known that sequence similarity varies throughout the genome and one would expect this to affect cross-reactivity. For example, the c100-3 recombinant protein used in first generation assays was derived from the NS4 region. This part of the genome varies significantly between types 1,2 and 3. Studies have shown weak (or absent) reactivity to c100-3 from individuals infected with variants other than type 1 (M^{c}Omish et al., 1993; Nagayama et al., 1993). The use of c22-3 reduces the risk of missing infections by other types due to the higher degree of amino acid

sequence conservation (Tsukiyama-Kohara et al., 1993). All donors infected with types 2-6 react strongly with the recombinant core protein (M^{c}Omish et al., 1994).

Routine screening of blood donations with the aforementioned assays has had a direct impact on reducing the incidence of post transfusion hepatitis. Important information on epidemiology and knowledge of the disease has also been provided indirectly.

d) Polymerase Chain Reaction (PCR) for HCV

Initially it was reported that antibody, once formed, persisted throughout the course of chronic hepatitis, suggesting that loss of previously detected antibody was a good indicator of recovery (Alter et al., 1989). This is not always the case. Later studies show that antibody response varies from person to person and depends on the immune responsiveness of the host. In young children, HCV infection is often associated with an incomplete serological response possibly as a result of the immaturity of the immune system at the time of infection (Borlotti et al., 1994). Antibody can be intermittently positive and disappear at variable intervals during follow up (Puoti et al., 1992).

Even with the available serological assays, it is unclear whether a positive anti-HCV result implies infectivity or simply a past infection. Assessment of potential infectivity depends on direct detection of HCV either by testing for HCV antigens or HCV RNA. Antigenic markers for HCV are being developed, but concentrations of HCV antigen in serum are at the lower limit of detectability using the available immunoassay techniques. However, the ability to detect HCV nucleic acid by amplifying reverse transcribed complementary DNA has been developed (Kato et al., 1990; Weiner et al., 1990). The principles of PCR have been described elsewhere (Ehrlich and Sirko, 1994;

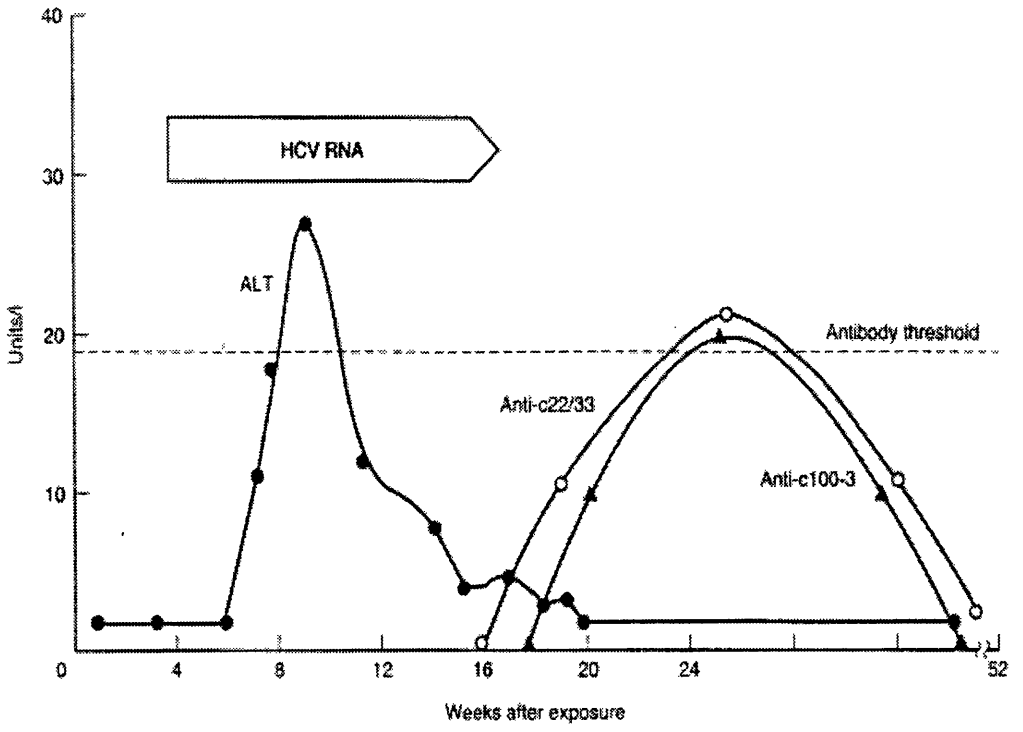
Saiki et al., 1988). The application of PCR has provided a sensitive and specific assay for viral RNA present in the bloodstream and other tissues.

Detection of viral RNA can now be made within 1-2 weeks of exposure, long before antibody can be detected using conventional assays (Figure 1.2). At present, the only definitive test for diagnosis of HCV infection is a positive PCR result (Brown and Dusheiko, 1993; Rubin et al., 1994; Dow et al., 1994). A complicated and expensive technique, PCR is unavailable in the majority of diagnostic laboratories. However, the introduction of PCR has yielded valuable information regarding the pattern of HCV replication, its relation to antibody levels and the clinical course of NANBH.

Theoretically there remain potentially infectious individuals, who have become infected in the recent past, which the best tests currently available may not detect. Some PCR positive blood donations may not be detected even when using third generation serological assays (Dow et al 1994). On the other hand, not all anti-HCV positive sera are PCR positive. There are several possible explanations for this: patterns of fluctuating viraemia, low grade viraemia below the level of detection by current PCR assays, infection with a strain of HCV significantly different in nucleic acid sequence so as not to be recognised by the primers employed, or the deleterious effect of storage and treatment on the samples (Cuypers, 1994).

Standardisation and quality assurance between laboratories does need improvement, and a positive result, though indicative of viremia and potential infectivity, like a negative, should be interpreted with care (Van der Poel, 1994a; Zaaijer et al., 1993). The clinical value of PCR depends on the sensitivity and reproducibility of applied procedures. The importance of primer selection and its effect on PCR results is a

FIGURE 1.2



Serological course in acute resolving HCV infection

Adapted from Brown and Dusheiko, 1994

recognised source of inconsistencies. Investigators have shown significant heterogeneity at several regions of different HCV strains which may lead to false negative results in PCR assays when primer-template mismatch occurs (Bukh et al., 1992; Chan et al., 1992; Okamoto et al., 1992a; b). It is believed that primers derived from the 5' noncoding and core regions are highly conserved and provide the best sensitivity. PCR sensitivity may vary among different clones owing to minor sequence variations in the 5' NCR. A recent study by Smith and colleagues (1995) compared 314 5'NCR sequences of viruses of genotypes 1 to 6 and identified primers that would be equally efficient in detecting RNA from different virus genotypes. Sensitivity can be maximised by using double or nested PCR. However, such an approach increases the risk of assay contamination (Cuypers, 1994). Recent reports have indicated that the use of one-stage PCR amplification combined with sensitive detection methods, for example Southern blot or liquid hybridization with radioactive oligonucleotide probes, is sensitive enough, with less risk of contamination (Geiger and Caselmann, 1992; Gretch et al., 1993; Hu et al., 1992; 1993). However, potentially hazardous radiolabelled probes are also unwanted, therefore, 'nested' PCR remains the most popular way of detecting HCV. The impact of specimen handling and storage has also been considered to have dramatic effects on the validity of PCR results. To ensure maximum sensitivity specimens should ideally be processed immediately and frozen in aliquots to prevent repeated freeze-thawing (Busch et al., 1992; Cuypers et al., 1992; Wang et al., 1992b).

e) Quantification of HCV RNA

Rather than merely determining the presence or absence of viral RNA, quantifying the amount of HCV RNA may be used as an aid in defining the key features of the disease

process. Additionally, quantitative analysis of HCV in serum may provide a marker of response to therapeutic intervention. Different methods to quantify HCV RNA have been used:

1) The limiting dilution PCR assay estimates the HCV RNA titre through the performance of serial ten fold dilutions of extracted RNA prior to PCR. The titre is calculated from the highest dilution giving a positive result (Sherman et al., 1993; Simmonds et al., 1990)

2) The competitive Reverse Transcription (RT) - PCR assay measures the HCV RNA concentration by mixing the cDNA with ten fold serial dilutions of a competitor DNA that differs from cDNA by having a small deletion (Hagiwara et al., 1993; Kato et al., 1993; Kumar et al., 1994).

Both the above have limited use in clinical practice as they are expensive, time-consuming, laborious and difficult to reproduce.

3) A competitive PCR method with colorimetric detection of the amplified products has been developed. (Lundeberg et al., 1991; Yun et al., 1994; Shindo et al., 1994). After RT, one tenth of the resulting cDNA, and the same volume of serially diluted cloned competitor DNA, are coamplified by nested PCR. The competitor DNA consists of the amplified region of the wild type cDNA with an internal region substituted with the lac operator sequence.

4) Recently a branched DNA (bDNA) signal amplification method has been developed. This assay is based upon hybridisation of HCV RNA by synthetic oligonucleotides to the highly conserved 5' noncoding and core regions immobilised on the surface of a microwell plate. Oligonucleotides complementary to different HCV genotype variations are incorporated in regions with minor genomic variation. Synthetic bDNA

amplifier molecules and multiple copies of a probe are hybridised to the complex. The complex is incubated with a luminescent substrate and the light emission is measured, the signal being proportional to the level of target nucleic acid. Quantification of HCV RNA is then determined from a standard curve (Davies et al., 1992; Urdea., 1993). Unfortunately, the bDNA assay is not yet as sensitive as PCR for the detection of HCV (Bresters et al ., 1994), but for quantification it is easier to handle and less time consuming than the aforementioned techniques.

iii) Patterns of Immune Response to HCV

With the array of antigens in the current tests, researchers have sought to relate an epidemiological or clinical feature of chronic infection, or viral replication with a particular antibody response to individual antigens (Chemello et al., 1993; Lelie et al., 1992; Puoti et al., 1992). Apart from NS5, PCR positivity can be seen in blood donors with only single antigen reactivity to either C100, C22-3, or C33 in RIBA-3 (Follet et al., 1992). A strong correlation has been reported between the presence of core antibodies and viral carrier status (De Beenhouwer et al., 1992; Lelie et al., 1992; Tanaka et al., 1993). The lack of reactivity against c22 antigens in PCR negative blood donors has also been observed (Perrons and Garson, 1993). It is, however, apparent that even with the many tests available, no combination can identify viraemic donations exclusively. It is likely that it is the extreme heterogeneity of the anti-HCV immune response, which has been reported to be dependent on the immune responsiveness of the host, that causes the various antibody patterns recorded.

iv) Genotyping of HCV

With the increase of information in the literature regarding the nucleotide sequence of different HCV isolates, it has become apparent that many differ strikingly from the original prototype HCV (HCV-1) (Choo et al., 1991). Indeed, comparison of the original American isolate with Japanese and European isolates reveals significant heterogeneity in both nucleotide and polypeptide sequence. Such heterogeneity even exists within most persistently infected individuals (Simmonds, 1994). There are at least five different systems of nomenclature that differ in the degree of sequence divergence required to assign a new virus type (Houghton et al., 1991; Cha et al., 1992; Chan et al., 1992; Simmonds et al., 1993; Okamoto et al., 1992b; Enomoto et al., 1990; Tsukiyama-Kohara et al.,1991). The European and USA group1 isolates of Chan et al.(1992) are classified as Type 1 by Houghton et al.(1991), and as group K1 by Enomoto et al.(1990). Far East group 1 isolates of Chan et al.(1992) are equivalent to Type II by Houghton et al.(1991), and group 2 are equivalent to Type III. Group 2 also correspond to group K2 as described by Enomoto et al.(1990). Chan et al.(1992) have also recently differentiated two clusters of group 2 sequences. Controversy remains over which method of classification is the most appropriate to accommodate the growing number of distinct sequences identified. The lack of consensus leads to confusion when work from different laboratories is compared. In consequence, a system for the nomenclature of hepatitis C viral genotypes, believed to reflect the observed two-tiered nature of HCV sequence variation found in the coding regions of the genome, was proposed and accepted at the 2nd International Conference of HCV and Related Viruses (Aug 1994, San Diego, California) (Table 1.4) (Simmonds et al., 1994a).

TABLE 1.4

Proposed names	Alternative names[a]				
	Ch	Si	Ok	En	Ts
1a	I	1a	I	PT	NC
1b	II	1b	II	K1	I
1c	NC	NC	NC	NC	NC
2a	III	2a	III	K2a	II
2b	III	2b	IV	K2b	II
2c	III	NC	NC	NC	NC
3a	IV	3	V	NC	NC
3b	IV	NC	VI	NC	NC
4a	NC	4	NC	NC	NC
5a	V	NC	NC	NC	NC
6a	NC	NC	NC	NC	NC

Proposed nomenclature and comparison with existing schemes for HCV types.

NC, not classified; [a] Nomenclature according to existing schemes: *Ch*, Houghton et al and Cha et al; *Si*, Chan et al and Simmonds et al; *Ok*, Okamoto et al and Mori et al; *En*, Enomoto et al; *Ts*, Tsukiyama-Kohara et al.

Various methods of genotyping HCV have been described. Direct sequencing, with analysis of consensus sequences, is the gold standard for genotype analysis but is not suitable for large numbers of clinical samples. Sequence information has also enabled the development of type-specific primers and probes (Okamoto et al., 1992b; 1993; Enomoto et al., 1990; Stuyver et al., 1993; Simmonds et al., 1993; Zhang et al., 1995). Although widely used, it is predictable that such primers and probes would fail to amplify or hybridise to sequences unrecognised when the primers/probes were designed. For example, the primers described by Okamoto et al (1992b; 1993), for identifying genotypes I, II, III, IVand V (Types 1a, 1b, 2a, 2b and 3a respectively), would fail to amplify type 3b, 4, 5 and 6 sequences. Recently a commercially available test for HCV typing has been developed by Innogenetics (Zwijnaarde, Belgium). This assay uses oligonucleotides derived from the 5'NCR as specific probes for each genotype. Although published data suggest that this line probe assay (LiPA) is able to identify HCV subtypes, in its present form, the test would fail to distinguish between subtypes 3a and 3b, and to identify gentoype 6 (Andonov and Chaudhary, 1995; Stuyver et al., 1993). The use of restriction fragment length polymorphism (RFLP) appears more accommodating to new types (Nakao et al., 1991; McOmish et al., 1994). With the available sequence data, it is possible to predict a range of electropherotypes. This can be used reliably to differentiate genotypes 1, 2, 3, 4, 5 and 6, and provisionally for subtypes 1a, 1b, 2a, 2b, 3a and 3b (Murphy et al., 1994; McOmish et al., 1994; Dusheiko et al., 1994; Davidson et al., 1995). Both RFLP and LiPA are methodologically simple and are able to be continuously modified to accommodate new major genotypes and subtypes.

Information to date indicates that types 1, 2 and 3 are the predominant genotypes infecting blood donors throughout Europe. In particular, type 1a and 3a

infections are more common in Northern countries. Types 2 and 3 are not found in Eastern European countries. Interestingly, North America and Australia show a remarkably similar genotype distribution to Northern Europe (Simmonds, 1994; Lau et al., 1995; Davidson et al., 1995). In contrast with Europe and North America, type 1a is notably absent in the Far East (Japan and Taiwan). Only types 1b, 2a and 2b have been detected in Japanese blood donors. Type 6a has only been found in Hong Kong and neighbouring Macau, although it is believed that future studies will identify a greater frequency of type 6 in the Far East. Unlike the Far East, South East Asia shows a predominance of type 3 with 1a and 1b rather than 2. Sequence analysis of type 3 here has also revealed several new subtypes as yet untypable. In South Africa, type 5a prevails among both black and white blood donors. In contrast, type 4 has been established as the predominant genotype in central and North Africa. In particular, HCV infection among Egyptians is only by type 4 (Simmonds, 1994; Davidson et al., 1995).

Whether distinct clinical features of type C hepatitis or the oncogenicity of HCV are associated with a particular viral type has not yet been clarified. The issue of genotype pathogenicity is complex. Other host and viral factors such as viral load, host immunity, age, mode of acquisition or coexistent infection may determine the outcome (Dusheiko et al., 1994., Dusheiko and Simmonds, 1994). It has been suggested that infection by 'Japanese-type HCV' (HCV type 1, genotype 1a) is more frequently associated with chronic active hepatitis or cirrhosis. Likewise, reports of patients infected with HCV type II (genotype 1b, HCV K1), having a lower response rate to interferon alpha than patients with HCV type III and IV (genotype 2 and 3, HCV K2a) have been made (Simmonds, 1994).

These are important areas of research. The effect of HCV genotype may be interdependent on other factors. Should these findings be confirmed, then the existence of geographical variation in the predominant circulating genotypes in different countries would lead to the adoption of different treatment policies for patient management. Furthermore, the documented antigenic differences between genotypes will have important implications for blood donor screening and for the design and composition of future candidate vaccines for HCV in different countries

v) Epidemiology of Hepatitis C Virus Infection

Epidemiologic studies show that HCV is efficiently transmitted through the transfusion of blood or blood products, the transplantation of organs from infected donors and through the sharing of contaminated needles amongst injecting-drug users. However, up to 50% of patients with HCV infection report no history of percutaneous exposure and are classified as sporadic cases. A number of studies suggest that perinatal, sexual, household and occupational transmission does occur but our knowledge of transmission in these settings remains incomplete.

There are a number of points to remember when assessing earlier studies on transmission. First, many original studies from which prevalence rates for HCV are quoted, used first generation assays. Up until 1992, published literature mainly describes the epidemiology of HCV based on results from ELISA-1 assays. In a limited number of studies, ELISA-1 results were confirmed by RIBA-1. Introduction of second generation assays and supplemental tests in later studies are likely to have predicted prevalence rates more accurately. Secondly, results obtained from anti-HCV and PCR tests have produced

discrepancies. However, it should be remembered that the presence of HCV RNA in the chronic carrier state does not always correlate with the presence of anti-HCV, and vice-versa. The possibility of such discrepancies resulting from false negatives must always be considered when comparing prevalence rates between these tests.

The transmission of HCV can conveniently be considered in relation to specific routes of infection and the particular groups of individuals at risk of infection.

a) Parenteral Transmission

i) Recipients of Blood and Blood Products

Before the advent of anti-HCV testing (1989), the frequency of post-transfusion (PT)-hepatitis in recipients of blood was 10-12% in the USA, 2-4% in Northern Europe and 6-18% in the South of Europe (Van der Poel, 1994b). Prospective studies indicated that 50-100% of these cases were due to HCV (Van der Poel , 1994b; Kitchen and Barbara, 1993). Transmission was linked to the number of units of blood transfused and specifically to those units with HCV RNA (Farci et al., 1991; Takano et al., 1992). Routine screening of blood donors for anti-HCV is now performed in almost every country that can afford the cost of testing.

Haemodialysis patients are a high risk group because of the dangers of long term intensive transfusion therapy. The prevalence of anti-HCV in this group of patients varies widely (2.4%- 52%) (Van der Poel, 1994b). Studies of anti-HCV antibodies among patients attending dialysis centres have shown a higher prevalence among patients in areas with a high prevalence of HCV infection in the general (blood donating) population (Tamura et al., 1990). As yet the mechanism of transmission in haemodialysis units remains undefined. Anti-HCV appears to correlate with the duration of the dialysis

period (Schlipkoter et al., 1990), and the number of units transfused (Mondelli et al., 1990). Patients may have acquired HCV via the blood transfusion or via the dialysis procedure itself. However, with the duration of treatment, the chances increase that a patient has received transfusions (Van der Poel et al., 1994b).

ii) Recipients of Products Derived from Large Pools of Plasma

Treatment of haemophilia, thalassaemia and other acquired coagulation disorders with untreated blood and blood products is a well recognised route of transmission. With the relatively insensitive first generation assays, between 60 and 100% of haemophiliacs who had at any time received untreated products were shown to be anti-HCV positive (Roggendorf et al., 1989; Makris et al., 1990; Lim et al., 1991). With second generation assays, the prevalence of HCV infection in these patients was later recognised as being approximately 100%. These patients have a particularly short incubation period. This may reflect a higher dose of virus introduced in the pooled concentrate. The introduction of heat-inactivated factor VIII and IX concentrates in 1985 has resulted in a dramatic decrease in the number of seroconversions (Kitchen and Barbara, 1993; Van der Poel., 1994b).With the successful treatment of plasma concentrates to inactivate any virus, the problem of HCV infection in patients with blood clotting disorders should be solved. However, there will remain a residuum of patients who have been infected already.

iii) Through Organ Transplantation

There is now evidence to suggest that HCV infection may be transmitted through organ transplantation (Belli et al., 1993; Chan et al., 1993; Diethelm et al., 1992; Konig et al., 1992; Muller et al., 1992; Periera et al., 1993; Shah et al., 1992; Silvain et al., 1992;).

Whether the same risk applies with all organs (liver, heart, kidney and pancreas), remains to be seen. Chronic liver disease can be related to HCV positivity in both donors and recipients. Screening of potential donors for anti-HCV is now routine. However, disagreement exists over whether to accept organs from donors who have anti-HCV but no viraemia (Skidmore 1993). As mentioned earlier, not all anti-HCV positive sera are PCR positive. However, the problems asssociated with PCR discussed earlier must be considered. Rejection of organs on the basis of anti-HCV alone is likely to eliminate a number of potential donors who may not transmit HCV infection.

iv) Through Intravenous Drug Use

Exposure through parenteral drug use is now considered to be the major route of transmission. This was expected as individuals sharing syringes or needles for intravenous drug use have a high risk of infection by parenterally transmitted blood-borne infectious agents. Studies worldwide indicate a seroprevalence of approximately 80% among intravenous drug users (Kitchen and Barbara, 1993; Van der Poel, 1994b).

v) Through Occupational Exposure

Needlestick injury poses less of a risk of infection with HCV than intravenous drug use, probably because there is only a single exposure, and very little blood is transferred. Seven cases of HCV transmission to health care workers following needlestick injuries have been documented (Seef, 1991; Suzuki et al., 1994; Tsude et al., 1992; Vaglia et al., 1990). In general, there is a low incidence of HCV infection in medical personnel even in high prevalence areas (Van der Poel, 1994b). Dentists are

exposed to blood of numerous patients in the course of their work. One study in New York City using first generation assays demonstrated a higher prevalence of seropositivity among dentists (1.75%) compared with controls (0.14%) (Klein et al., 1991). The level of seropositivity was especially high among oral surgeons (9.3%), and was also greater in those providing dental treatment for large numbers of drug addicts (Klein et al., 1991). A smaller study of 94 dentists in South Wales, UK, failed to detect any who were HCV seropositive (Herbet et al., 1992). Although HCV seroconversion following needlestick exposure has been reported, the seroprevalence of HCV among healthcare personnel at risk for blood exposure is no greater than that of the general population (Hernandez et al., 1992; Norrgren et al., 1992; Zuckerman et al., 1994).

b) Undefined Routes of Nonparenteral Transmission

i) Sexual Transmission

By analogy with HBV, sexual transmission seemed a likely mode of transmission for HCV. Alter and co-workers were the first to suggest that sexual transmission had an important role in the spread of HCV (Alter MJ et al., 1982; 1989; 1990). Since then other published literature has provided controversial evidence for the existence of sexual transmission of HCV in groups with different sexual behaviour.

a) Prostitutes and Patients Attending Clinics for Sexually Transmitted Diseases

Prevalence studies in patients attending clinics for sexually transmitted diseases (STD) and prostitutes have shown anti-HCV at a greater prevalence than in blood donors (Corona et al., 1991; Nakashima et al., 1992; Thomas, 1995; Weinstock et al ., 1993; Wu

et al ., 1993). Transmission in prostitutes and individuals with multiple partners may indicate that the risk factor for sexual transmission is the number of partners.

b) Homosexuals

The prevalence of HCV in homosexual men has provided further mixed evidence for and against sexual transmission. Early studies prior to the identification of HCV could not identify homosexuality as a risk factor for NANBH (Alter MJ et al., 1989). This is in sharp contrast to the transmission of HBV and HIV since this is more likely to occur during homosexual than during heterosexual intercourse. Later reports on the seroprevalence of anti-HCV in homosexual men ranged from 0-50% (Melby et al., 1990; Osmond et al., 1993a; Tedder et al., 1991). Although accepted as being low, the prevalence is higher than that observed in the normal blood donor population.

c) Sexual Contacts of HCV Positive Patients

Reports showing sexual transmission to contacts of HCV seropositive patients have so far been controversial. (Akahane et al., 1992; 1994; Brackmann et al., 1993; Bresters et al ., 1993; Hallam et al., 1993; Kao et al., 1992; Kolho et al., 1991; Osmond et al., 1993b; Pachuki et al., 1991; Peano et al., 1992; Scaraggi et al ., 1993; Seef and Alter, 1994). Evidence for transmission is supported by reports of an increase in anti-HCV prevalence with increasing age (Nishioka et al., 1991; Lee et al., 1991). More recently, the frequency of HCV infection has been reported to correlate with the duration of relationship (Akahane et al ., 1994; Oshita et al., 1993). Whether a preference exists for the direction of transmission, from male to female or vice-versa, is undecided. Osmond and co-workers reported transmission from male to female as being more

efficient (Osmond et al., 1993b). On the other hand, Kao et al found the converse true (Kao et al., 1992).

Overall, sexual transmission of HCV does occur to some extent although it does not seem to be an efficient route of transmission.

It is not clear whether the divergent results are due to differences in susceptibility of different populations to HCV infections, differences in the level of infectivity in patients, or infection with different strains of HCV that may have different degrees of infectivity. It has been suggested that as a result of low levels of circulating HCV, transmission is more likely if exposure is repetitive and extended over a long period of time (Akahane et al., 1994). It is known that levels of viraemia fluctuate widely and, therefore, transmission may occur only at times of high levels of viraemia. Indirect evidence for this comes from studies of partners of patients infected with both HCV and HIV. Anti-HCV positivity is significantly associated with the presence of anti-HIV in all studies. HIV may be a cofactor for transmission. Similar reports identify a sexual partner belonging to a 'high-risk' group for HIV infection as a risk factor for HCV (Van Doornum et al.,1991). The assumption is that HCV concentrations are higher in the immunocompromised host. A number of studies, however, have been unable to confirm that HIV acts as a cofactor for sexual transmission of HCV (Hallam et al., 1993; Scaraggi et al., 1993).

As mentioned before, early studies based on first generation serological tests may be inaccurate due to the lack of sensitivity and specificity. In addition, other risk factors for HCV infection have often not been excluded. In particular, reports of previous episodes of parenteral exposure are often inaccurate, especially in studies of homosexual men, prostitutes and STD clients where histories of past intravenous drug use may not be

available. Later studies have employed nucleotide sequence analysis within dually infected couples (Akahane et al., 1994; Healey et al., 1995; Kao et al., 1993; Rice et al., 1993). However, the finding of conserved HCV subtypes among two infected spouses at best only indicates that both spouses may have been infected with the same isolate, but this is not proof of sexual transmission. Nonetheless, on the evidence of the published material there is general agreement that if sexual transmission does occur, then it is at a significantly lower frequency than HIV, HBV, and other STDs.

ii) Vertical (mother-to-child) Transmission

With the initial tests, vertical transmission of HCV was difficult to assess. The advent of PCR has provided the ability to detect, genotype and compare virus sequences to determine the similarity of HCV strains isolated in mother-child pairs (Inoue et al ., 1992; Novati et al ., 1992; Ohto et al., 1994; Weiner et al., 1993). The incidence of vertical transmission of HCV has also been shown to be increased in cases of HIV co-infection in the mother (Kao et al., 1992; Weintrub et al., 1990; Zanetti et al., 1995). It is likely that an anti-HIV positive woman would have a higher rate of HCV replication because of immunodeficiency. Thus, the risk of vertical transmission of HCV may be dose related, occurring only when the mother has high levels of viraemia. Ohto and associates reported that in their study no woman whose titre of HCV RNA was below 10^6 per milliliter transmitted the virus to her infant (Ohto et al., 1994). Exactly how and when the virus is transferred has not yet been studied, but transplacental transmission, transmission at birth, and post-partum transmission have all to be considered.

iii) Intrafamilial (household) Spread

Person-to-person spread by nonsexual household contacts is very rare. The Centre for Disease Control could only associate 3% of patients with household exposure (Alter MJ et al ., 1990). However, intrafamilial transmission does appear to occur. Reports of anti-HCV seroprevalence in family members of HCV positive patients vary from 8-23% (Bellobuono et al., 1991; Honda et al., 1993; Ho et al., 1994; Nishiguchi et al., 1992; Oshita et al., 1993). The inaccuracy of reporting episodes of intravenous drug use or covert percutaneous exposure for example sharing razors or toothbrushes serves to confound many of these studies and the actual route of transmission in such situations remains to be established.

With all the controversial data on the transmission patterns of community acquired HCV, experimental methods using animal models and studies of body fluids other than blood have been used increasingly as a means for evaluating possible nonparenteral routes of transmission. Transmission of HCV has been demonstrated in chimpanzee experiments (Abe and Inchauspe., 1991; Abe et al., 1987). A recent study, however, failed to show conclusive evidence of sexual or mother-to-infant transmission in a colony of chimpanzees (Suzuki et al., 1993). Acute HCV infection following a human bite has been documented (Dusheiko et al., 1990; Figueiredo et al ., 1994). Although this indicates that saliva is a potential source of infection, one should consider that in the quoted studies, saliva was inoculated into the recipient rather than ingested. Whether ingestion results in the same level of infection has not been considered. Using PCR, other investigators have also reported the presence of HCV RNA in saliva and salivary glands of patients with hepatitis C (Chen et al ., 1995; Couzigou et al., 1993; Liou et al., 1992; Mariette et al ., 1995; Numato et al ., 1993; Punchammer-Stockl et al., 1993; Takamatsu et al., 1991; 1992;

Thieme et al ., 1992; Wang et al., 1991; 1992a;). The source of HCV RNA in saliva is unclear. Apart from contamination of saliva with blood, transudation of fluid containing virus from the general circulation and active viral replication at the site of secretion must all be considered.

If sexual or personal contact were an important mode of transmission, one would expect HCV to be present in not only saliva but other body secretions too. Indeed, PCR has been successful in detecting HCV RNA in various body fluids including urine and semen (Liou et al., 1992; Kotwal et al., 1992; Numato et al., 1993; Punchammer-Stochl et al., 1994). However, in 19 patients with chronic HCV infection and who tested positive for HCV RNA in serum, viral RNA was not detected in any sample of saliva, semen, urine, stool or vaginal secretions (Hsu et al., 1991). In another study, HCV RNA was also absent from the semen of patients with chronic hepatitis C, even though it was present in serum (Fried et al., 1992). One must remember, however, the problems associated with PCR and consider these when interpreting the data.

vi) Natural Course of Hepatitis C Virus Infection

Few patients with acute hepatitis C infection develop clinical illness with jaundice. The majority of infections remain undetected as a result of most having subclinical or mild features. Although clinically indistinguishable from other forms of viral hepatitis, HCV has an incubation period shorter than that seen with HBV (8-24 weeks) and longer than HAV (2-6 weeks). Despite its relatively mild nature, acute hepatitis C infection has a propensity to become chronic. Early post-transfusion studies using serum alanine aminotransferase (ALT) levels as an indicator for persistence estimated progression to chronicity in ~50% of cases (Berman et al., 1979). Later studies monitoring HCV RNA

estimate that persistent infection occurs in the majority of infected individuals (Cordoba et al., 1994; Sherlock, 1994)

Although few studies have examined chronicity in sporadic HCV infection, progression to cirrhosis and possibly hepatocellular carcinoma (HCC) can occur regardless of how the disease was initially contracted. As yet the mechanism for this high chronicity rate in HCV infections is not well defined, although host and viral factors such as the size of the infectious inoculum at the initial infection, infecting HCV genotypes and the ability of the virus to evade the host's immunosurveillance system have all been considered (Kiyosawa et al., 1994). Chronic persistent hepatitis (CPH), will eventually progress to chronic active hepatitis (CAH) and cirrhosis over 10-20 years. Liver biopsies often reveal CAH in ~60% of chronically infected patients and cirrhosis in ~20% (Di Bisceglie et al., 1991; Mattsson et al., 1993). Most patients are asymptomatic until they develop advanced liver disease. Symptoms such as fatigue and liver tenderness are rarely observed. More commonly, infection is noticed as a biochemical disease, with fluctuating ALT levels, appearing normal at times, and slowly progressive histopathological findings. The progression from CPH to CAH to cirrhosis and, finally, to fatal hepatocellular carcinoma in some individuals with HCV infection is well established from several studies, although the actual role of HCV in the development of HCC remains unknown. HCV has neither reverse transcriptase activity nor a viral oncogene. It is believed that HCV most likely influences the development of carcinogenesis through the activation of proto-oncogenes and inactivation of suppressor genes during the regeneration of continuously damaged cells in CAH (Kiyosawa and Furuta, 1994)

vii) Treatment of Hepatitis C Virus with Interferon

Interferons (IFNs) are natural proteins with anti-viral, anti-tumour, and immunomodulatory functions. IFNs consist of three families of proteins (alpha, beta and gamma) (Bresters, 1994; Dianzini, 1993; Finter et al., 1994). So far alpha, and to a lesser extent beta, IFNs have been used successfully for the treatment of chronic HCV infection. IFN is believed to work by its antiviral activity on the primary cytopathic HCV rather than through an immune modulatory mechanism (unlike its effect on HBV) (Rubin et al., 1994). Since the original pilot study in 1986 (Hoofnagle et al., 1986), numerous trials have confirmed the suppressive effect of interferon on disease activity and its ability to induce remission. Prolonged therapy with interferon leads to improvement in approximately 50% of patients. However, a sustained response is only maintained in 10-25% (Bresters, 1994).

Although IFNα is now considered the therapy of choice for chronic hepatitis C, there are several unanswered questions concerning its use, as follows

a) Who to Treat

At present, IFNα cannot be recommended for all patients. IFNα is licensed for the treatment of those who have serological evidence of disease, elevated serum aminotransferase activity, chronic hepatitis C by liver biopsy, and no other serious complicating liver illness (Hoofnagle et al., 1993; Mutimer et al., 1993). Those criteria for selection eliminate some patients who may deserve treatment, such as patients with acute hepatitis C, those with advanced cirrhosis, those with normal transaminase levels, those who are immunosuppressed and patients who are anti-HCV negative.

i) Patients with Acute Hepatitis C

Although HCV causes very little symptomatic disease, progression to chronic hepatitis C with persistence of viraemia is common. For this reason, treatment to prevent chronicity and the associated liver injury of chronic hepatitis would be justified. Few studies have examined the role of IFN for acute hepatitis C (Esteban, 1993; Hwang et al., 1994; Lampertico et al ., 1994; Omata et al., 1991). All have been small trials, examining different types and doses of interferon. A recent study by Tassopoulos and colleagues reported the failure of IFN-α2b to prevent progression to chronic hepatitis C in anti-HCV positive cases (Tassopoulos et al., 1993). Colombo and associates also report short term improvement that fades with time after IFN withdrawal (Colombo et al., 1993). Studies suggest the benefit of IFN therapy, but in practice few cases are treated. Arguments against treatment include the unnecessary expense and side effects of treatment in a group of patients where recovery may occur spontaneously. However, where it is clear that the disease is not resolving normally, therapy should be considered.

ii) Patients with Decompensated Liver Disease

Patients with early or mildly decompensated liver cirrhosis can respond to therapy (Jouet et al ., 1994). However, these patients do suffer troublesome side effects and often therapy does not reverse the established cirrhosis and end stage liver disease.

iii) Children with Chronic Hepatitis C

Chronic hepatitis C is rare in childhood. However, it has been indicated that children will respond and tolerate IFN in the same way as adults and recommendations for treatment are the same (Ruiz-Moreno et al., 1992).

iv) Patients with Atypical Serology

Treatment of patients with atypical serology raises another problem. The sensitivity and specificity of serological investigation has improved with the development of second and third generation anti-HCV tests. However, as mentioned earlier, some patients with chronic hepatitis C can test negative for anti-HCV (false negatives). On the other hand, some other forms of liver disease (autoimmune hepatitis or alchoholic liver disease) will test positive by ELISA due to a non-specific reaction (McFarlane et al., 1990). It is important to exclude patients with autoimmune disease, as treatment with IFN will cause the disease to worsen (Lenzi et al ., 1990). The availability of PCR has enabled direct identification of viraemic individuals. However, this expensive technique is not available in all areas and as mentioned earlier, is itself not without problems.

v) Immunosuppressed Patients

An important group of patients are those who have HIV, have received an organ transplant, or who are receiving immunosuppressive therapy. Few studies report treatment of these patients. Results indicate that HCV disease progresses more rapidly in the presence of HIV co-infection, suggesting that IFN may be an appropriate therapy in such cases. Indeed, studies have shown IFN to be beneficial in patients who have both HCV and HIV (Boyer et al., 1992; Marriott et al., 1993). This is in contrast to a poor response observed in immunocompromised patients with chronic hepatitis B infection. This may be explained by the different pathological mechanisms of hepatitis B and C. The stage of HIV disease may also be an important factor in the response to IFN, an aspect which requires clarification.

Reports suggest that recurrent hepatitis C following organ transplantation can be suppressed by therapy with alpha interferon even in the presence of large doses of immunosuppressive medication (Wright et al., 1992)

b) Dose and Duration of Treatment

The dose and duration of therapy remain undecided and are likely to vary with different patients. Currently, 3MU subcutaneously thrice weekly for 6 months is recommended. However, this dose has been shown to be inadequate in inducing a sustained response, or even a response at all. The use of a higher dose can increase response rates during treatment. Introduction of IFN at a higher dose, followed by a lower dose regime has also been reported as beneficial (Bresters, 1994).

There is also uncertainty on how long therapy should be continued in order to minimise relapse following treatment cessation. Some studies have shown that prolonged treatment courses improve sustained response rates (Bresters, 1994).

c) Side Effects

Interferon has many side effects, but most are self-limiting and tolerable, and almost all are rapidly and fully reversible when the drug is stopped (Hoofnagle, 1993). Side effects are dose-dependent and may vary with different patients. The typical dose of 3MU three times weekly is tolerated well by most patients. In general, early side effects are flu-like symptoms which develop 6-8 hours after IFN is given. These features all tend to disappear 2-4 weeks into the course of treatment. More troublesome are the long-term effects like fatigue, depression, insomnia, anorexia, nausea, irritability, lack of energy and alopecia. Although numerous side-effects have been noted, it is rare that all are

encountered during therapy. Serious side effects are uncommon. These include induction of autoimmunity and autoimmune diseases, increased susceptibility to infections and severe psychiatric syndromes (Hoofnagle, 1993).

d) Monitoring Treatment

IFN is an effective treatment. However, a long term response is observed in only 10-25% of patients. Coupled to this, there are difficulties of administration, expense and dose-limiting side effects. These problems underline the need for reliable markers to assess and monitor therapy. Several potential means for monitoring therapy have been examined.

i) Serum Aminotransferase Levels and Liver Histology

Serum aminotransferase levels and liver histology are the commonest features monitored. Although good predictors of initial response, they do not predict a sustained response (Hoofnagle, 1993). Discrepancies between biochemical and virological responses to IFN are also common (Kakumu et al ., 1993; Liang et al., 1993; Pawlotsky et al., 1994; Picciotto et al., 1994).

ii) Serum Antibodies to Hepatitis C Virus

Early reports using titres of anti-HCV as a marker indicated that these did not change in a consistent manner during treatment (Di Biscegli, 1991; Hoofnagle et al., 1993). More recently, preliminary reports have suggested that IgM anti-HCV decreases and often disappears in patients with a sustained response but not in those without a response or undergoing a transient response (Brillanti et al., 1992; Nakano et al., 1993).

Similar reports suggested that IgG anti-HCV, in particular IgG anti-HCV core, decreased in those with sustained clearance (Yuki et al., 1993; Urushihara et al., 1994). These findings have yet to be confirmed.

iii) HCV RNA Qualitatively and Quantitatively in Serum

The advent of PCR has enabled the presence and the quantity of HCV RNA to be monitored during therapy. Retrospective analysis of stored serum from patients receiving treatment has shown levels of RNA to decrease and become undetectable in those with a good response (Aiyama et al., 1994 ; Balart et al., 1993; Yamada et al., 1992). However, loss of HCV RNA in the serum does not predict a sustained response. Results have shown that a negative result in PCR does not rule out the presence of low level replication of HCV, below the level of sensitivity of the PCR procedure. The introduction of a quantitative branched DNA (bDNA) assay may help this problem. This assay can quantify HCV RNA to a similar limit of detection as PCR (Davies et al ., 1992; Li et al., 1993; Lau et al., 1993). However, it does lack sensitivity with low viral titres (Bresters et al ., 1994).

iv) HCV RNA Qualitatively and Quantitatively in the Liver and Peripheral Mononuclear Cells

Studies have suggested that the presence of HCV RNA in the liver and lymphoid cells is a more sensitive marker to determine a sustained response following treatment cessation (Balart et al., 1993; Di Bisceglie et al., 1993; Gil et al., 1993; Muller, et al ., 1993; Qian et al., 1992; Saleh et al., 1994). It is possible that lymphoid cells represent extrahepatic sites of HCV replication where the antiviral effects of IFN are

limited. If so, the presence of HCV in such sites will need to be monitored to ensure complete clearance of the virus. Utilization of liver HCV RNA would provide the most specific marker of a sustained response. However, tissue assays are impractical as they require a biopsy or serial biopsies, are expensive and unacceptable to the majority of patients.

e) Predictors of Response

It would be advantageous if one could identify those patients who are likely to have a beneficial response, and those who are likely to sustain this improvement when therapy is stopped. Previous studies have been unable to establish clearly if there are any pre-treatment variables that might predict response. Shorter disease duration prior to treatment, female gender, obesity, less pronounced liver inflammation and absence of cirrhosis have been claimed to predict a good response (Reichard et al., 1994; Hoofnagle et al., 1986). However, with the exception of cirrhosis, none have been proven indisputably to be of importance (Camps et al., 1993). Recently, the genotype of HCV and the initial viral load have been reported as being good predictors of response (Tsubota et al ., 1994; Chemello et al ., 1994; Kobayashi et al., 1992; Yamada et al., 1992; Lau et al., 1993; Hino et al., 1994; Yoshioka et al., 1992). Patients infected with genotype II (type 1b), appear to respond less well than patients infected with other genotypes (Chemello et al ., 1994; Yoshioka et al., 1992; Mita et al., 1994; Hino et al., 1994). These patients also appear to have higher HCV RNA titres (Lau et al ., 1994; Yoshioka et al., 1992; Hino et al., 1994). Reports have also suggested a better rate of response to IFN in those infected by a single HCV type. The quasi-species nature of HCV may allow IFN to exert a selective pressure inducing HCV to undergo changes in its population diversity (Taniguchi et al., 1993;

Camps et al., 1993a). This may manifest itself clinically in a relapse following treatment.The production of anti-interferon neutralising antibodies may also influence response. Occasionally, specific antibodies against recombinant IFN (anti-IFN), are demonstrated in patients who do not respond or relapse after an initial response (Antonelli et al., 1991; Inglada et al., 1987). The proportion of treated patients who develop anti-IFN is variable and likely to be due to variations in IFN preparation or dose schedule. Recombinant interferon alpha-2a (ROFERON, Roche) appears to induce neutralising antibodies more often than interferon alpha-2b (INTRON, Schering-Plough), or lymphoblastoid-derived interferon alpha (Milella et al., 1993; Camps et al., 1993a; Antonella et al., 1991). The clinical relevance of these antibodies remains to be established. Some patients appear unaffected by their production. In others, a breakthrough response has been described as a consequence of anti-IFN formation (Milella et al., 1993; Camps et al., 1993a).

The mechanism for relapse following treatment cessation remains unknown. No biochemical, serological or histological feature will predict a sustained response (Lau et al., 1994; Shindo et al., 1991).

viii) Other Antiviral Therapies

At the moment IFN is the only recommended therapy. However, other antiviral agents have been considered.

a) Ribavirin

Although costly, ribavirin is absorbed orally, is well tolerated and has known activity against many RNA viruses including some flaviviruses. However, studies report a

similar level of response to ribavirin as to IFN (Di Biscegli, 1992; Reichard et al., 1993; Camps et al., 1993b).

b) Inosine

Inosine has some direct antiviral activity as well as being able to enhance the cellular immune system by influencing Interleukin-2 (IL-2) production and receptor expression. One study reported the treatment of chronic liver disease with inosine, resulting in the reduction of transaminase levels and absence of HCV RNA, but anti-HCV remained (Prohaska et al., 1991).

c) Corticosteroid

Corticosteroid therapy was given to patients with NANB hepatitis prior to the discovery of HCV markers. Response was variable, but no controlled trials were documented.

Further studies are needed to assess new and old antiviral agents alone or in combination with IFN.

ix) HCV Vaccines

Without an effective and reliable cell-culture system for HCV, an inactivated, whole-virus vaccine is not yet possible. Likewise, the scarcity of suitable primate hosts and the lack of markers of virulence and attenuation limits the prospect for a live attenuated vaccine. Therefore, a vaccine based on recombinant DNA technology appears to be the way forward. Techniques based on approaches used for preparing vaccines

against flaviviruses and pestiviruses should be employed (Zuckerman and Zuckerman, 1995). Any effective HCV vaccine will need to induce immune responses against multiple strains, whether through recognition of conserved regions among isolates, the inclusion of multiple epitopes in a single vaccine or both. Initial studies are hopeful (Choo et al., 1994). Many problems remain to be overcome and important questions still to be resolved. For example, if a vaccine becomes available, who should be vaccinated and when?.

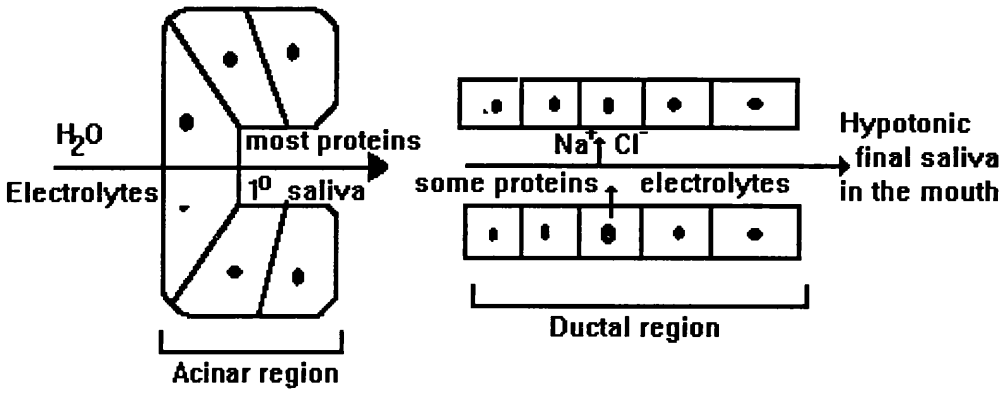
SALIVA

Saliva is a combination of the secretions of three pairs of major glands, the parotid, the submandibular and the sublingual glands, together with the secretions of the minor salivary glands, exogenous elements like serous transudate, cells, micro-organisms and food particles (Levine, 1993; Haeckel, 1989).

i) Salivary Secretion

Salivary glands are composed of two general regions, the acinar and ductal (Figure 1.3). Saliva formation occurs in two stages. The first stage takes place in the acinar region and generates an isotonic primary fluid, rich in exocrine proteins. All secretion occurs in response to neurotransmitter stimulation. There is no spontaneous salivary secretion (Baum, 1993). The second stage of salivation is fluid modification during passage through the ductal region. This includes electrolyte flux, as well as secretion of additional proteins. The saliva which finally enters the mouth is markedly hypotonic containing, on average, ~25mEq/L of NaCl and ~2-5 mg/ml protein (Kaplan and Baum, 1993).

FIGURE 1.3



Schematic depiction of saliva formation in the two general regions of the salivary gland.

Stimulation also affects many other acinar cell functions such as intermediary metabolism, protein synthesis, secretory protein N-glycosylation and membrane transport protein activity. Such broad responsiveness can influence the movement of many compounds from the blood into saliva.

Saliva has several specific, essential functions including protection of dentition and mucosa, antimicrobial action, facilitation of digestion, physical cleansing and buffering. These properties are generally mediated by specific proteins. However, most proteins are multifunctional, enabling large variations in the concentration of different components without affecting the overall protective qualities of saliva.

Salivary constituents (fluid, electrolytes and proteins) are found in saliva as a result of specific, tightly regulated processes. Very little appears accidentally or by leakage, although this is the case for low molecular weight serum constituents found in trace levels in secretions from healthy glands. These levels increase several fold with disease and in particular with inflammatory conditions.

ii) Testing for Salivary Antibodies

Mixed saliva contains primarily secretory IgA (sIgA) and low levels of IgG and IgM. Most of this IgG and IgM is derived from gingival crevicular fluid via transudation from local blood capillaries (Challacombe et al., 1978), and as such, for the purpose of diagnosis may be regarded as a dilution of a subject's plasma.

Interest in salivary antiviral antibodies was initiated by Archibald et al (1986). Since then, salivary IgG antibodies have been detected against HAV (Parry et al., 1987, 1988; 1989; Piacentini et al., 1993), HBV (Parry et al., 1987; 1988; 1989; 1993; Piacentini et al., 1993), rubella, mumps and measles viruses (Parry et al., 1987; Parry, 1993), and

HCV (Parry, 1993; Piacentini et al., 1993; Thieme et al., 1992) at a rate comparable to that of serum testing. Despite this, salivary testing has only been considered seriously for HIV testing. Several kits developed for serum/plasma testing have been modified to allow their application to saliva. As yet, few commercial anti-HIV assay have been developed for the sole purpose of salivary testing. Tests for salivary IgM have also been developed for HAV and HBV, and for mumps, measles and rubella (Parry, 1993).

iii) Testing for Salivary Antigens

In addition to measuring antibody, there is growing interest in identifying antigens in saliva. Apart from being a more specific indicator of infection, diagnosis can often be made long before the subject has seroconverted. Application of the polymerase chain reaction (PCR) has provided a powerful tool for amplication of viral nucleic acid in blood. However, researchers have indicated the presence of a PCR interfering substance in the saliva (Warren et al., 1992; Ochert et al., 1994).

A Specimen of Convenience

Most diagnosis of disease needs some type of testing of a body fluid or tissue. At present, blood is the fluid of choice, used for a wide range of diagnostic tests. Saliva testing is limited to a few distinct clinical conditions (Mortimer and Parry, 1988; Parry, 1993). However, with the increasing recognition of the risk of disease transmission associated with needlestick injuries and the relative inconvenience of obtaining blood samples, particularly among haemophiliacs, serological testing has become unattractive. Saliva testing has emerged as the forerunner in the search for a reliable, non-invasive specimen. The main advantages of saliva over blood are:

1) Greater subject acceptability - less painful and traumatic

2) Convenience - rapid, simple and inexpensive. Collection does not necessarily have to be in a clinical environment.

3) Collection is less hazardous to both the subject and the investigator.

4) Easier access to hard-to-reach populations (eg IVDU, prostitutes) and large populations (eg clinic attenders).

Lack of knowledge about the normal content of saliva, as compared with serum, low target concentration and inadequate sample volume have all delayed widespread acceptance of saliva testing. Saliva is certainly not sterile and contains many degradative enzymes. As a result, organic components are susceptible to rapid degradation unless the saliva is sterilized or the degradative enzymes inactivated (Dawes, 1993; Kaplan and Baum, 1993; Goodman, 1993). Of course, such treatment may affect the component of interest. However, with current advances in technology, highly sensitive tests have been developed such that low concentration and small sample volume are no longer limiting factors. Methods for saliva collection giving stabilized specimens of consistent quality are also being continually improved. As a result of such progress, saliva is now being considered as a useful test fluid.

Salivary Testing for Hepatitis C Virus

In recent years, there has been increasing interest in the shedding of viruses in saliva. Such shedding has obvious implications for the spread of infection and understanding of the epidemiology of infectious diseases. More recently, the value of saliva as a diagnostic fluid has become increasingly apparent. Both of these issues are currently of interest in relation to HCV, because of our poor understanding of the epidemiology of

infection with this organism. Despite our knowledge of the transmission of HCV, up to 40% of HCV-seropositive patients have become infected by an unknown route. One potential mode of transmission is via body secretions such as saliva. A number of studies have evaluated saliva, amongst other body fluids, for HCV testing. By making minor modifications to the commercially available serological test kits, some researchers have been able to demonstrate anti-HCV in saliva (Sherman et al., 1994; Thieme et al., 1992). Problems with low concentrations of HCV in saliva and small sample volume have been resolved by the development of PCR. Using PCR, the successful detection of HCV RNA in saliva has been reported (Chen et al., 1995; Couzigou et al., 1993; Liou et al., 1992; Mariette et al., 1995; Numato et al., 1993; Punchammer-Stockl et al., 1994; Takamatsu et al., 1991; Thieme et al., 1992; Wang et al., 1991; 1992a; Young KC et al., 1993). However, similar studies by other workers report the absence of HCV RNA in saliva (Hsu et al., 1991; Fried et al., 1992). These discrepant results obtained by previous studies may be a reflection of the methods used to collect and handle the samples.

This study sought to answer the following questions:

1. Is hepatitis C virus present in the saliva of all HCV-seropositive patients ?
2. What is the most appropriate method for collection of saliva to provide stable specimens of consistent quality ?
3. Would it be feasible to introduce PCR testing of saliva for HCV into a routine diagnostic laboratory ?

Chapter 2

General Materials and Methods

The materials and methods described in this Chapter are those routinely used throughout the studies in this thesis. Modifications and methods specifically related to work in individual chapters are described in detail in the Materials and Methods section of the relevant chapter.

MATERIALS

Consumables used during the course of the studies presented in this thesis were obtained from the sources listed below:

DNA

Oligonucleotide primers :- Oswel DNA Services, University of Edinburgh, Scotland; Cruachem Ltd, Todd Campus, West of Scotland Science Park, Acre Road, Glasgow, Scotland

Modifying Enzymes

Taq polymerase, Tth polymerase :- Promega Corporation, Southampton , UK.

Molony Murine Leukaemia Virus (MMLV) reverse transcriptase:- GIBCO-BRL, Life Technologies Ltd, Paisley, Scotland.

Dynazyme:- Flowgen Instruments Ltd, Kent, UK.

Chemicals

β-mercaptoethanol, N-lauryl sarcosine, isoamyl alcohol, potassium chloride, magnesium chloride, tris base, ethidium bromide, boric acid, EDTA, sodium hydroxide, sodium

chloride, lithium chloride, taurine, sodium citrate, sodium dodecyl sulphhate, maleic acid, diethylpyrocarbonate (DEPC), light mineral oil :- SIGMA, Dorset, UK.

Guanidine isothiocyanate solution, yeast tRNA, agarose,:- GIBCO-BRL, Life Technologies Ltd, Paisley Scotland.

Water saturated phenol :- Rathburn Chemicals Ltd, Walkerburn, UK.

Nusieve® GTG® Agarose, dynawax :- Flowgen Instruments Ltd, Kent, UK.

Acetic acid, chloroform, ethanol, methanol, replicote VS, sdH_2O :- BDH Ltd, Poole, UK.

Acrylamide, ammonium persulphate, bind silane, methylenebis-acrylamide, urea, RNAguard® Rnase inhibitor, temed, ultrapure dNTP set 2'-deoxynucleotide 5'-triphosphate :-Pharmacia Biotech Ltd, Milton Keynes,UK.

Radioisotopes

[α-^{35} S] dATP :- Amersham International plc, Buckinghamshire, UK

Miscellaneous Items

Polaroid film type 667 :- SIGMA, Dorset, UK.

Salivette™ :- Sarstedt, Leicester, UK.

Omnisal™ :- SDS International Ltd, Sovereign Court, London, UK

Digoxigenin DNA labelling and detection kit, positively charged nylon membrane:- Boerhinger Mannheim UK Ltd, East Sussex, UK

Precut filter and blotting pack :- Anachem, Anachem House, Charles Street, Luton, Beds, UK

Sequenase PCR product sequencing kit, Hyperfilm™- MP :- Amersham International plc, Buckinghamshire, UK

Nucleic Acid Purification Systems

QIAamp® Blood Kit :- QIAGEN Ltd, Surrey, UK

GLASSMAX RNA microisolation Spin Cartridge System:- GIBCO-BRL, Life Technologies Ltd, Paisley, Scotland

METHODS

Sample collection

For the purpose of this research, the nomenclature used from here on is in accordance with that agreed at the New York Academy of Sciences 1992 conference on Saliva as a Diagnostic Fluid (Atkinson et al ., 1993).

i) Whole saliva : The fluid obtained from the mouth by expectoration. Whole unstimulated saliva was collected by the spitting method. Saliva was allowed to accumulate in the floor of the mouth and the subject spat it out into a sterile plastic Universal container. The whole saliva was centrifuged (4000rpm, 5 minutes) the supernate collected and the pellet resuspended in 1ml sterile distilled water (sdH_2O).

ii) Oral fluid : The fluid obtained by insertion of absorptive collectors into the mouth and subsequent removal of the fluid by pressure or centrifugation. Saliva was obtained from a Salivette™ by centrifugation (4000rpm, 5 min).

All samples were aliquoted and frozen (-20°C) within 3 hours of collection. Examples of these devices are given in Figure 2.1

iii) Blood specimens A clotted blood sample was initially transported to Ruchill Regional Virus Laboratory, separated, and the serum stored at -20°C.

Reverse transcription

On ice, a master mix containing all the reagents less the RNA was set up as shown in Table 2.2. Ten microliters of master mix were aliquoted into each reaction tube and 10µl of template RNA was added using a positive displacement pipette. Reverse transcription was carried out at 37°C for 30 minutes in a Hybaid Omnigene System (Hybaid). The RNA template was destroyed by a further incubation for 10 minutes at 95°C. Samples were next plunged into ice and the reaction product (cDNA), collected by brief centrifugation. The cDNA obtained was then used as a template for PCR amplification.

Nested-set polymerase chain reaction

Samples were amplified using two pairs of nested primers from the highly conserved 5' NCR of the genome (Chan et al 1992). Details of the primers are given in Table 2.3.

I) First round: PCR was performed over 25 cycles, each consisting of 36 seconds at 94°C, 42 seconds at 52°C and 3 minutes at 68°C. On ice, a master mix containing all the reagents less the cDNA was set up as shown in Table 2.4. The master mix was dispensed in 90µl aliquots into each reaction tube and 10µl of template cDNA was added using a dedicated positive displacement pipette. 65µl of light mineral oil was layered on top to prevent evaporation during the reaction.

ii) Second round: One microliter of the first round reaction mixture was amplified for a further 25 cycles with the same cycling programme using the inner pair of nested primers. On ice, the same master mix as before was supplemented with 200µM each of dATP,

dCTP, dGTP, dTTP (Table 2.4), and dispensed in 99µl aliquots into each reaction tube. Amplified DNA (252 bp) was detected by agarose gel electrophoresis and ethidium bromide staining.

Agarose gel electrophoresis

Agarose gel electrophoresis was carried out on horizontal agarose gel slabs submerged in 1x TBE electrophoresis buffer (Table 2.1). Agarose (1g) and Nusieve® GTG® Agarose (3g) was dissolved to a final concentration of 4% in 1xTBE buffer (100ml) by heating in a microwave oven. To the molten agarose, ethidium bromide was added to a final concentration of 1µg/ml. After cooling, the molten agarose was poured into the gel box. After the gel had solidified, the gel was submerged in the electrophoresis buffer. Gels were run at a constant voltage of 76V for a minimum of 2 hours. DNA was visualised on a UV TM-40 transilluminator (UVP inc, USA) at 302nm and photographed using polaroid type 667 film. Unless otherwise stated, all gels shown in this thesis were 4% and run in 1x TBE at 76V.

Contamination controls

Control experiments were carried out with every PCR experiment. One HCV RNA positive control was included from the start of every procedure to ensure the efficiency and specificity of RNA extraction, RT and PCR. Three negative controls were included to check the RT and the first and second round PCR reagents for contamination.

To avoid contamination of the RT and PCR, all experiments were set up in a laminar flow cabinet. All equipment was UV irradiated for 10 min with 1200µJmin^{-1} at 254nm in a UV crosslinker prior to use. Equipment and reagents used in setting up PCRs

were prepared in batches and aliquoted for storage in convenient-sized lots to prevent contamination of stock solution. All liquids were handled using plugged tips to prevent aerosols reaching the inside of the pipette itself. All RNA, cDNA and first round product were handled using dedicated positive-displacement pipettes.

TABLE 2.1

General Stock Solutions and Buffers

10x Reverse Transcription Buffer

1M KCl	5ml
1M $MgCl_2$	0.25ml
1M TrisHCl (pH 8.0)	0.25ml

Store at -20°C in 100µl aliquots. Use at a final concentration of 1x

10x TBE

Tris base	108g
Boric acid	55g
0.5M EDTA (pH 8.0)	40ml
dH_2O	to 1 litre

Use at a final concentration of 1x

10mM dNTPs (2' deoxynucleotide 5'triphosphate)

dATP	100µl
dTTP	100µl
dGTP	100µl
dCTP	100µl
dH_2O	to 1ml

Store at -20°C in 50µl aliquots. Use at a final concentration of 1mM in reverse transcription and 0.2mM in PCR.

1M KCl

KCl	74.5g
dH_2O	to 1 litre

Sterilise by filtration

1M $MgCl_2$

$MgCl_2$	203.3g
dH_2O	to 1 litre

Sterilise by filtration

1M Tris (pH 8.0)

Tris base	121.1g
dH_2O	900ml

Adjust to pH 8.4 with 84ml concentrated HCl. Make up volume to 1litre with dH_2O. Sterilise by autoclaving.

Ethidium Bromide (1 mg/ml)

Ethidium bromide	1g
dH_2O	to 1litre

Store away from light. Use at a final concentration of 1µg/ml

0.5M EDTA (pH 8.0)

EDTA	186.1g
dH_2O	900ml

Adjust to pH 8.0 with NaOH. Make up volume to 1litre with dH_2O. Sterilise by autoclaving.

TABLE 2.2

Reagent	Stock	Final	Vol per 20µl
RT Buffer	10mM	1mM	2µl
dNTP	10mM	1mM	2µl
$pd(N)_6$	50 A_{260} units	100pmol/µl	1µl
MMLV RT'ase	200 units/µl	/	1µl
sdH_2O	/	/	4µl
RNA guard	Add 1µl to the master mix for every 20 samples		

Reverse transcription master mix. Ten microlitres of master mix aliquoted into each reaction tube and 10µl of RNA template added.

TABLE 2.3

Name	Region	Position of 5'	Polarity	Sequence 5'-3'
209	5' NCR	8	-	ATACTCGAGGTGCACG GTCTACGAGACCT
211	5' NCR	-29	-	CACTCTCGAGCACCCT ATCAGGCAGT
939	5' NCR	-297	+	CTGTGAGGAACTACTG TCTT
940	5' NCR	-279	+	TTCACGCAGAAACGTC TAG

Sequences, sources, positions and polarity of primers used in PCR reaction for the amplification of HCV. Primers 209 and 939 are used in the first round of PCR and primers 211 and 940 in the second round.

TABLE 2.4

1)

Reagent	Stock	Final	Vol per 100µl
Taq pol buffer	10mM	1mM	10µl
P 209	/	0.5µM	1µl *
P 939	/	0.5µM	1µl *
sdH_2O	/	/	77.5µl
Taq pol	5 units/µl	2.5 units	0.5µl

2)

Reagent	Stock	Final	Vol per 100µl
Taq pol buffer	10mM	1mM	10µl
dNTPs	10mM	0.2mM	2µl
P 211	/	0.5µM	1µl *
P 940	/	0.5µM	1µl *
sdH_2O	/	/	87.5µl
Taq pol	5 units/µl	2.5 units	0.5µl

Master mixes used for 1) first, and 2) second round PCR reactions.

* Volume of primer used depends on concentration of stock solution.

FIGURE 2.1

Saliva collection devices for whole saliva and oral fluid used in the detection of HCV RNA by PCR. Left: Salivette™ (Sarstedt Ltd). Right: a sterile plastic Universal for the collection of unstimulated whole saliva.

Chapter 3

Application of the Polymerase Chain Reaction for the Diagnosis of Hepatitis C Virus in a Routine Clinical Laboratory

INTRODUCTION

Following the successful cloning and characterisation of HCV, diagnosis of infection has been facilitated by the development of antibody detection assays. However, as mentioned earlier, antibody detection methods are of restricted use because of the long seroconversion period following infection (mean 16 weeks). In addition, knowledge of antibody status does not provide a means to differentiate between current active infection, chronic infection, and past resolved infection. By contrast, although presence of HCV RNA is not absolute proof of HCV viraemia, it strongly suggests active virus replication and the potential for infectivity.

The principle of the PCR is relatively simple. It involves the chemical rather than biological amplification of a specific segment of DNA, and is based on the annealing and extension of two synthetic oligonucleotide primers that flank the target DNA. After heat-induced denaturation of the DNA, each primer hybridises to its specific corresponding region on one of the two separated strands. Annealed primers are then extended on the template strand under the influence of a DNA polymerase enzyme. The three steps of denaturation, primer binding and DNA synthesis represent a single PCR cycle and the resulting product serves as template for the next cycle. Repeated cycles result in the exponential accumulation of the target sequence (Saiki et al., 1988; Ehrlich and Sirko, 1994).

PCR is widely accepted as a powerful research tool. The technology provides direct analysis at the molecular genetic level, overcomes many of the constraints imposed by serological data and is independent of the host immune response. However, to date the use of PCR has been limited mainly to research applications and to use in a few

sophisticated clinical reference laboratories. The operation of a high-volume clinical diagnostic laboratory stipulates that the methodologies employed must be rapid, reproducible, and amenable to batch formatting. Thus, the extension of PCR technology to routine clinical use would necessitate simplification of the entire process from beginning to end. The technique as currently practised in most research laboratories is labour-intensive with a limit on the number of samples that can be analysed due to cumbersome detection methods.

The work reported in this thesis has examined PCR for detection of HCV RNA in serum, a technique currently recognised as the 'gold standard' for confirmation of infection. In addition, the role of PCR in detection of HCV in saliva has been studied, since saliva would be a convenient alternative non-invasive specimen for diagnosis of this infection. Examination of saliva by PCR raises additional problems, and these will be alluded to, as appropriate, in the text.

The development of PCR for clinical use should be considered in three separate parts. First, it is necessary to develop a specimen protocol that will facilitate the rapid release of the target DNA/RNA from clinical samples, with a minimum number of steps, manipulations, and container changes. This aspect is often the most time consuming part of the processing. Secondly, amplification should be carried out in a manner which reduces the possibility of false positives resulting from amplicon carry-over. With regard to HCV, amplification by PCR must be preceded by a step which generates cDNA. This step is the crucial and often limiting step of RT-PCR assays. There are numerous protocols for the cDNA reaction and PCR amplification (Cuypers, 1994). The amount of false positivity due to contamination is strongly dependent on the number of steps and manipulations performed during the processing of the samples in a given protocol. Finally, a non-

laborious method of detection, suitable for adaption to a clinical laboratory remains to be established. When PCR was first described, radioactively labelled probes were used to detect products. As amplification technology improved it became possible to visualise the amplified DNA using ethidium bromide stained gels. Detection methods range from procedures that are relatively insensitive and non-specific to those that are very sensitive and highly specific. The ideal method for detection should allow for an accurate determination of the desired product regardless of the number of non-specific bands produced.

At the onset of this study, the PCR protocol employed for both serum and saliva was one used routinely by the Scottish National Blood Transfusion Service (SNBTS), Microbiology Reference Unit, Regional Virus Laboratory, Ruchill Hospital, Glasgow. As such it took over two days to perform requiring dedicated personnel and equipment. At this point, PCR was unavailable to the National Health Service as a routine clinical laboratory method partly due to the problems mentioned above.

Therefore, the work presented in this chapter addressed the mechanics of the whole technique and sought to determine the appropriate procedures suitable for use in a clinical laboratory for the diagnosis of infection with HCV.

MATERIALS & METHODS

a) Comparison of RNA extraction protocols

HCV RNA was isolated from 200µl of HCV positive serum undiluted, or diluted 1:10, 1:100, or 1:1000 in HCV negative saliva using the following RNA extraction procedures : guanidinium isothiocyanate/ acid-phenol method, QIAamp blood kit, and the GLASSMAX RNA micro-isolation Spin Cartridge System. Reverse transcription and PCR were performed as described earlier in Chapter 2. Following amplification, products were subjected to agarose gel electrophoresis, and visualized by UV illumination as described in Chapter 2.

i) RNA extraction with guanidinium isothiocyanate / acid-phenol

HCV RNA was isolated by a modification of the procedure described by Chomczynski and Sacchi (1987). To 200µl of serum or saliva, 500µl of the denaturing solution, 4M guanidine isothiocyanate (4MG) (Table 3.1) was added on ice. This was supplemented with carrier RNA to a final concentration of 1µg/ml and 40 units of RNAguard. The solution was mixed and left on ice for 15 minutes. An equal volume of phenol (water saturated), and chloroform-isoamyl alcohol mixture (24:1) were added with thorough mixing and left for a further 15 minutes on ice. Samples were then centrifuged at 12,500rpm for 5 minutes using an Eppendorf centrifuge (model 5415 C). After centrifugation, RNA was present in the aqueous phase while DNA and proteins were present in the interphase and phenol phase. The aqueous phase was transferred to a fresh tube and mixed with an equal volume of chloroform-isoamyl alcohol mixture (24:1). The samples were centrifuged as before. The aqueous phase was again transferred to a fresh

tube and RNA was precipitated with an equal volume of ethanol (100%) at -70°C for a minimum of 2 hours. Following sedimentation in the Eppendorf centrifuge (12,500rpm, 20 minutes), RNA was washed twice in 70% ethanol. The resulting RNA pellet was air dried and dissolved in 20µl sdH_2O. At this point, the RNA preparation could be used as a template for cDNA production (reverse transcription).

ii) RNA extraction with QIAamp blood kit

This was performed according to the manufacturer's instructions as summarised in Table 3.2. The extraction mixture was supplemented with carrier RNA to a final concentration of 1µg/ml and 40 units of RNAguard.

iii) RNA extraction with GLASSMAX RNA micro-isolation spin cartridge system

RNA was extracted using the GLASSMAX kit by a modification of the manufacturer's protocol for isolating total RNA from cells or tissues (Table 3.3).

b) Comparison of PCR amplification protocols

HCV RNA was isolated from 200µl of HCV positive serum using the QIAamp blood kit. Extracted RNA undiluted, or diluted 1:10, 1:100, or 1:1000 in HCV negative saliva underwent amplification either by nested set PCR as described earlier, hot start PCR or a combined one-step RT-PCR.

i) Hot start PCR

Hot start PCR followed the same procedure as nested PCR with the exception of using two master mixes, upper and lower (Tables 3.4 and 3.5), separated by 65µl of pre-

warmed wax. Template DNA was added using a dedicated positive displacement pipette to the upper master mix. No mineral oil was added in this procedure. The primers and cycling parameters used in this procedure were the same as those employed for nested PCR.

ii) Combined one-step RT-PCR

On ice, a master mix containing all the reagents less the RNA was set up as shown in Table 3.6. Eighteen microlitres of master mix were aliquoted into each reaction tube and 2µl of template RNA were added using a dedicated positive displacement pipette. Twenty microliters of prewarmed light mineral oil were layered on top to prevent evaporation during reverse transcription and amplification. Reverse transcription was performed at 70°C for 15 min, followed by 1 min at 95°C to facilitate denaturation of RNA-DNA heteroduplexes. PCR amplification was performed using the outer set of primers (209 and 939) over 40 cycles, 2 consisting of 15s at 95°C and 20s at 60°C, followed by 38 of 15s at 90°C and 20s at 60°C. A final extension step of 4min at 60°C was also included. Ten microlitres of the RT-PCR reaction mixture were amplified using the inner pair of nested primers (211 and 940) for a further 25 cycles, each consisting of 15sec at 90°C and 20 sec at 60°C. A master mix containing all reagents less the first round product was set up as shown (Table 3.7) and dispensed in 90µl aliquots into each reaction tube. No further enzyme was added to the mix.

c) Comparison of protocols used to detect amplified product

A number of saliva samples obtained from an HCV seropositive blood donor were shown by agarose electrophoresis and Ethidium Bromide (EtBr) staining to vary significantly in intensity from very strong to barely visible. These samples were probed using a non-

radioactive format to determine firstly the specificity of the weakly positive products and secondly, whether the method of detection could be improved by using a more sensitive procedure.Amplified product was detected using the traditional method of gel electrophoresis and EtBR staining, with a subsequent hybridisation assay format using a digoxigenin labelled probe (5'-CCGGGAGAGCCATAGTGGTCTGCGGAACCGGTG-3').

i) Preparation of DNA probe

a) 3' Endlabelling with Digoxigenin-11-ddUTP

The preparation of the probe was carried out by Dr K. Takahashi, Periodontology Unit, Department of Adult Dental Care, Glasgow Dental Hospital and School. The reaction mixture was set-up on ice and contained 4µl 5× stock tailing buffer (GIBCO-BRL), 0.05mM Dig-11dUTP (Boehrinhger Mannheim), 40 units Tdt enzyme (GIBCO-BRL), 1-2µl DNA probe, and sdH_20 to a total volume of 20µl. The contents were mixed and spun briefly and the reaction was incubated overnight at 37°C. Unincorporated label, spare probe and reaction components were removed from incorporated label by separation through a Sephadex G-25 NAP-5™ column (Pharmacia). The column was prepared by removing the transport medium and flushing the column three times with 0.1% SSC/ 0.1% SDS in DEPC/ sdH_20. The reaction mixture was added to the column, washed through with 200µl of buffer and collected in an eppendorf tube. This was repeated a further nine times until 10 fractions were obtained. The fraction containing the labelled probe was identified by dot-blot.

b) Random primed DNA labelling with Digoxigenin-11-dUTP

DNA marker (100bp ladder) was labelled using a DIG DNA Labelling and Detection kit (Boehringer Mannheim) according to the manufacturer's instructions. Briefly, the reaction mixture was set up on ice and contained1μg of DNA template (100bp marker), 2μl of hexanucleotide mixture, 2μl of dNTP labelling mixture and sdH_20 to a final volume of 19μl. Finally, 100units of Klenow enzyme were added and the reaction left overnight at 37°C. Prior to use, the DNA template was heat-denatured in a boiling water bath for 10min and immediately chilled on ice for 30sec. The reaction was terminated by the addition of 2μl of 2mM EDTA, pH 8.0. Labelled DNA was precipitated with a mixture of 4M LiCI (Table 3.1)/ 100% ethanol (0.1/3) at -70°C for 30 min. The reaction mixture was then centrifuged at 13,000g for 15min using an Eppendorf centrifuge (model 5415 C). After centrifugation, the ethanol was decanted and the pellet washed with 100μl of chilled (70%) ethanol. The reaction mixture was centrifuged again at 13,000g for 5min. The resulting pellet was freeze-dried and resuspended in 50μl of sdH_20. The labelled marker was stored at -20°C until required. Labelled DNA was denatured by boiling for 10 min prior to use in hybridisation experiments.

ii) Dot blot analysis

Identification of the labelled probe using a dot blot analysis was carried out by Dr K. Takahashi, Periodontology Unit, Department of Adult Dental Care, Glasgow Dental Hospital and School. Ten squares were marked on a nitrocellulose filter (Gelman Sciences, MI). The filter was soaked in 20× SSC for 3min, blotted between 3MM Whatman paper and allowed to air dry. One microliter from each fraction was dotted onto the appropriate square and allowed to absorb. The DNA label was bound to the filter by exposure to UV

for 2min in a UVC-508 ultraviolet crosslinker (Anachem). The fraction containing the labelled probe was identified using anti-DIG-alkaline phosphatase antibody (Anti-dig-AP, Boehringer Mannheim).

iii) Southern blot analysis

PCR product was electrophoresed in 4% agarose gel in 1× TBE buffer, stained with ethidium bromide and photographed under UV light as previously described. The gel was placed in a glass baking dish and any unused areas of the gel were removed with a sharp scalpel. The DNA was denatured by submerging the gel in 200ml of 0.5M NaOH/ 1.5M NaCl (Table 3.1), for 2× 15min at room temperature with gentle shaking. The gel was rinsed with sdH_20 and next neutralised by soaking in 200ml of 0.5M Tris-HCl pH7.5/ 3M NaCl (Table 3.1), for 2× 15min at room temperature with gentle shaking. The gel was now ready for Southern blotting to a hybridisation membrane. Transfer was carried out on a commercially available blotting unit (Anachem). Twenty fold SSC buffer was added to the unit until the level was just below the support platform. The gel was placed onto the saturated platform. A piece of positively charged nylon membrane, cut to the required size, was briefly soaked in 20× SSC and placed in position on top of the gel. All air bubbles between the gel and the membrane were removed. A stack of pre-cut absorbent towelling and filter paper (Anachem) was placed on top and the floating lid of the blotting unit was put on top of the stack. A light weight was applied to the stack to ensure even contact during blotting. Transfer was allowed to proceed overnight, following which the membrane was carefully removed and briefly air dried at room temperature. The membrane was placed on a sheet of Whatman 3MM paper and the DNA bound to the nylon membrane in a

UVC-508 ultraviolet crosslinker (Anachem) for 3min. After UV cross-linking, the membrane was rinsed briefly in sdH_20 and allowed to air dry.

v) Hybridisation of immobilised DNA to labelled probe and marker

Membranes containing immobilised DNA were hybridised to DIG labelled DNA probe and marker using a Midi Dual 14 Hybridization Oven (Hybaid). Hybridisation was carried out in bottles as follows. In a plastic box containing 2×SSC, the membrane (DNA side up) was placed on top of a nylon mesh (Hybaid). Another piece of mesh was layered on top and the 'sandwich' was rolled together, ensuring no air bubbles were trapped. The roll was placed in the hybridisation bottle and 15ml of 2×SSC added. The 'sandwich' was carefully unrolled, again ensuring no air bubbles were trapped. SSC was then decanted and replaced with 6ml hybridisation buffer less probe (Table 3.1). The bottle was clipped onto the rotisserie such that it rotated in the oven in the same direction as the membrane was unrolled. Pre-hybridisation was carried out at 65°C for 1hour. Denatured probe and marker were added to the pre-hybridised membrane at a concentration of 5ng/ml in hybridisation buffer. This was accomplished by mixing the appropriate volume of probe and marker with 6ml hybridisation buffer. The pre-hybridisation buffer was discarded and the hybridisation buffer added. Hybridisation was carried out overnight in the oven at 65°C. Following hybridisation, buffer was removed and frozen for later use. Unbound probe was removed by washing in 2×SSC for 2×5 min in the oven at 65°C. Finally, membranes were rinsed in 0.1×SSC for 2×15min and allowed to air dry.

vi) Colorimetric detection of DIG-labelled probe and marker

This was performed using a DIG DNA labelling and detection kit (Boehringer Mannheim). Following hybridisation and post-hybridisation, the membrane was equilibrated in

buffer 1 (Table 3.1) for 1 min. In a freshly washed dish, the membrane was blocked in buffer 2 (Table 3.1) for 30min with gentle shaking. Anti-DIG alkaline phosphatase was added to the membrane at a concentration of 150MU/ml of Buffer 2. This was accomplished by adding 6µl of anti-DIG alkaline phosphatase to 30ml of Buffer 2. The membrane was incubated for 30 min in the prepared antibody solution. The membrane was next transferred to a new dish, washed in Buffer 1 for 2×15min, and equilibrated for 2min with 20ml Buffer 3 (Table 3.1). Bound antibody was detected with colour substrate solution freshly prepared. This was achieved by mixing 45µl NBT solution and 35µl X-Phosphate in 10ml of Buffer 3. The membrane was incubated in the dark until the desired bands had been detected. Finally the reaction was stopped by washing the membrane with 50ml of Buffer 1 for 5min.

TABLE 3.1

Stock Solutions and Buffers

4M Guanidine isothiocyanate (4MG)

4M Guanidine solution	60ml
β-mercaptoethanol	300µl
20% N-lauryl sarcosine	600µl

Stored at -20°C in 5ml aliquots

20% N-lauryl sarcosine

N-lauryl sarcosine	200g
dH_2O	to 1 litre

Sterilise by filtration

QIAgen Protease

Lyophilized QIAGEN protease	25mg
sdH_2O	1.3ml

Store at -20°C in 200µl aliquots

Buffer AW

Reagent AL_2	40parts
Reagent AL_1	160parts

Store at room temperature in 5ml aliquots

Buffer AL

Buffer AW concentrate	84ml
Ethanol (100%)	196ml

DEPC-treated water

Diethylpyrocarbonate	0.1µl
dH_20	to 1 litre

Leave to stand overnight. Sterilise by autoclaving

GUSCN/ME

GUSCN	2.3ml
β-mercaptoethanol	200µl

Store at 4°C in 200µl

Wash Buffer

Wash buffer concentrate	8ml
DEPC-treated H_20	142ml
Ethanol (100%)	170ml

Store at 4°C in 10ml aliquots

0.5M NaOH

NaOH	20g
d H_20	to 1 litre

Sterilise by filtration

0.5M Tris-HCl (pH 7.5)

Tris base	60.55g
dH_2O	900ml

Adjust pH to 7.5 with 65ml concentrated HCl. Make up volume to 1litre with sdH_2O

3M NaCl

NaCl	175.33g
dH_2O	to 1 litre

Sterilise by autoclaving

20×SSC pH 7.0

NaCl	175.3g
Sodium citrate	88.2g
dH_2O	800ml

Adjust to pH 7.0 with NaOH. Make up volume to 1 litre. Sterilise by autoclaving

10% SDS

Sodium dodecyl sulphate	100g
dH_2O	900ml

Heat to 68°C. Adjust to pH 7.2 with HCl. Make up volume to 1 litre.

0.2M EDTA

EDTA	74.4g
dH_2O	800ml

Adjust to pH8.0 with NaOH. Make up volume to 1 litre with dH_2O. Sterilise by autoclaving

4M LiCl

LiCl	169.6g
dH_2O	to 1litre

Sterilise by autoclaving

10% Blocking reagent

Blocking reagent*	10g
Buffer 1	100ml

Dissolve reagent by several 30 second heat pulses in a microwave (3 to 4 min total). Sterilise by autoclaving and store at 4°C

Hybridisation buffer

20 × SSC	125ml
10% Blocking reagent	50ml
10% N-Laurylsarcosine	5ml
SDS	1ml
dH_2O	to 500ml

Store at -20°C

*Proteolytic fragments of casein in powder form.

Buffer 1

NaCl	87.66g
Maleic acid	11.61g
dH_2O	to 900ml

Adjust to pH 7.5 with NaOH. Sterilise by autoclaving.

Buffer 2

10% Blocking reagent	50ml
Hybridisation buffer	450ml

Sterilise by autoclaving

Buffer 3

Tris-HCl pH 9.3	50ml
NaCl	5.84g
$MgCl_2$	4.76g
dH_2O	to 500ml

Sterilise by autoclaving

TABLE 3.2

Viral Nucleic Acid Extraction using QIAamp ® Blood Kit.

1. Pipette 200µl of sample into 1.5ml microfuge tube.

2. Add 25µl QIAGEN Protease stock solution, 200µl Buffer AL, 4.25µl carrier RNA and 1µl RNA guard to the sample. Mix immediately by vortexing.

3. Incubate at 70°C for 10 min

4. Add 210µl of isopropanol or ethanol (96-100%) to the sample, and mix again by vortexing

5. Place a QIAamp spin column in a 2ml collection microtube (provided). Carefully apply the mixture from step 4 to the QIAamp spin column without moistening the rim, close the cap, and centrifuge at 8000 rpm (6000xg) for 1min. Place the QIAamp spin column in a clean 2ml collection microtube (provided), and discard the tube containing the filtrate.

6. Carefully open the QIAamp spin colum and add 500µl of Buffer AW (provided). Centrifuge at 8000rpm (6000xg) for 1min. Remove the QIAamp spin column from the collection microtube, discard the filtrate, and place the QIAamp spin column in the same collection microtube

7. Carefully open the QIAmp spin column and add another 500µl of Buffer AW. Centrifuge at 8000rpm (6000xg) for 1 min, and at full speed for a further 10 sec.

8. Place the QIAamp spin column in a clean 1.5ml microfuge tube (not provided), and discard the collection microtube containing the filtrate.

9. Carefully open the QIAamp spin column. Elute the nucleic acid with 50µl of RNAase-free water (DEPC treated) preheated to 70°C.

10. Incubate at 70°C for 5 min. Centrifuge at 8000rpm (6000xg) for 1 min.

TABLE 3.3

Viral Nucleic Acid Extraction using GLASSMAX™ RNA Microisolation Spin Cartridge System

1. On ice, pipette 200µl of sample into 1.5ml microfuge tube.

2. Add 200µl GUSCN / ME solution and 1µl RNA guard to the sample. Mix immediately by vortexing.

3. Add 280µl of absolute ethanol. Mix thoroughly. Centrifuge the suspension at 13000xg for 5 min at room temperature.

4. After centrifugation , remove the supernate with a sterile pastette. Remove the liquid starting from the top to the bottom of the tube, as thoroughly as possible.

5. To the pellet add 450µl of Binding solution (provided), followed by 40µl of 3M NaOAc, pH 5.5. Vortex to resuspend pellet.

6. Add the solution from step 5 to the GlassMAX spin cartridge and cap it. Centrifuge at 13,000g for 20 sec. Empty the tube.

7. Add 0.5ml of cold (4°C) 1X wash buffer to the spin cartridge. Centrifuge at 13,000g for 20sec. Empty the tube.

8. Repeat step 7 twice.

9. Add 0.5ml of cold (4°C) 80%(v/v) ethanol to the spin cartridge. Centrifuge at 13,000g for 20 sec. Empty the tube.

10. Repeat step 9.

11. Centrifuge at 13,000g for 1 min.

12. Put cartridge into fresh sample recovery tube. Add 40µl of DEPC-treated water, preheated to 65°C, to the spin cartridge.

13. Centrifuge at 13,000g for 20 sec to elute the RNA.

TABLE 3.4

1)

Reaction Component	Stock	Final	Vol per 100µl (per reaction)
Primer 939	/	0.5µM	0.5µl*
Primer 209	/	0.5µM	0.5µl*
sdH_20	/	/	49µl

2)

Reaction Component	Stock	Final	Vol per 100µl (per reaction)
PCR Buffer	10X	1X	10µl
Taq Polymerase	/	2.5 units	0.5µl
sdH_20	/	/	30µl

Upper and lower master mixes employed in first round of nested 'hot start' PCR. (1) : Upper master mix. (2) : Lower master mix. * Volume of primer used depends on stock concentration.

TABLE 3.5

1)

Reaction Component	Stock	Final	Vol per 100µl (per reaction)
Primer 940	/	0.5µM	0.5µl*
Primer 211	/	0.5µM	0.5µl*
sdH_20	/	/	49µl

2)

Reaction Component	Stock	Final	Vol per 100µl (per reaction)
PCR Buffer	10X	1X	10µl
dNTP	10X	0.2X	2µl
Taq Polymerase	/	2.5 units	0.5µl
sdH_20	/	/	36µl

Upper and lower master mixes employed in second round of nested 'hot start' PCR.

(1) Upper master mix. (2) Lower master mix. * Volume of primer used depends on stock concentration.

TABLE 3.6

Reagent	Stock	Final	Vol per 20µl
Tth RT-Buffer	10mM	1mM	2µl
dNTP	10mM	0.2mM	0.4µl
$MnCL_2$	10mM	1mM	2µl
Primer 939	/	0.15µM	0.15µl*
Primer 209	/	0.15µM	0.15µl*
Tth	6 units/µl	/	1µl
sdH_2O	/	/	11.2µl
RNA guard	Add 1µl to the master mix for every 20 samples		

Combined RT-PCR master mix.

* Volume of primer used depends on stock concentration.

TABLE 3.7

Reagent	Stock	Final	Vol per 100µl
Tth pol Buffer	10×	1×	10µl
$MgCl_2$	25mM	2.5mM	10µl
Primer 940	/	0.5µM	0.5µl*
Primer 211	/	0.5µM	0.5µl*
dNTP	10mM	0.2mM	2µl
sdH_2O	/	/	67µl

Second round PCR master mix used with combined RT-PCR.

* Volume of primer used depends on stock concentration.

RESULTS

a) Comparison of RNA extraction protocols

Two hundred microliters of HCV positive serum undiluted, or diluted 1:10, 1:100 or 1:1000 in HCV negative saliva, were subjected to PCR following extraction with guanidinium isothiocyanate/ acid-phenol and two commercially available kits. Both the guanidinium isothiocyanate/ acid-phenol and the QIAamp blood kit extraction protocols had comparable sensitivity and specificity. Both yielded positive results and appeared to have removed any potential salivary inhibitors. The GLASSMAX RNA Microisolation Spin Cartridge System failed to provide amplifiable RNA (Figure 3.1).

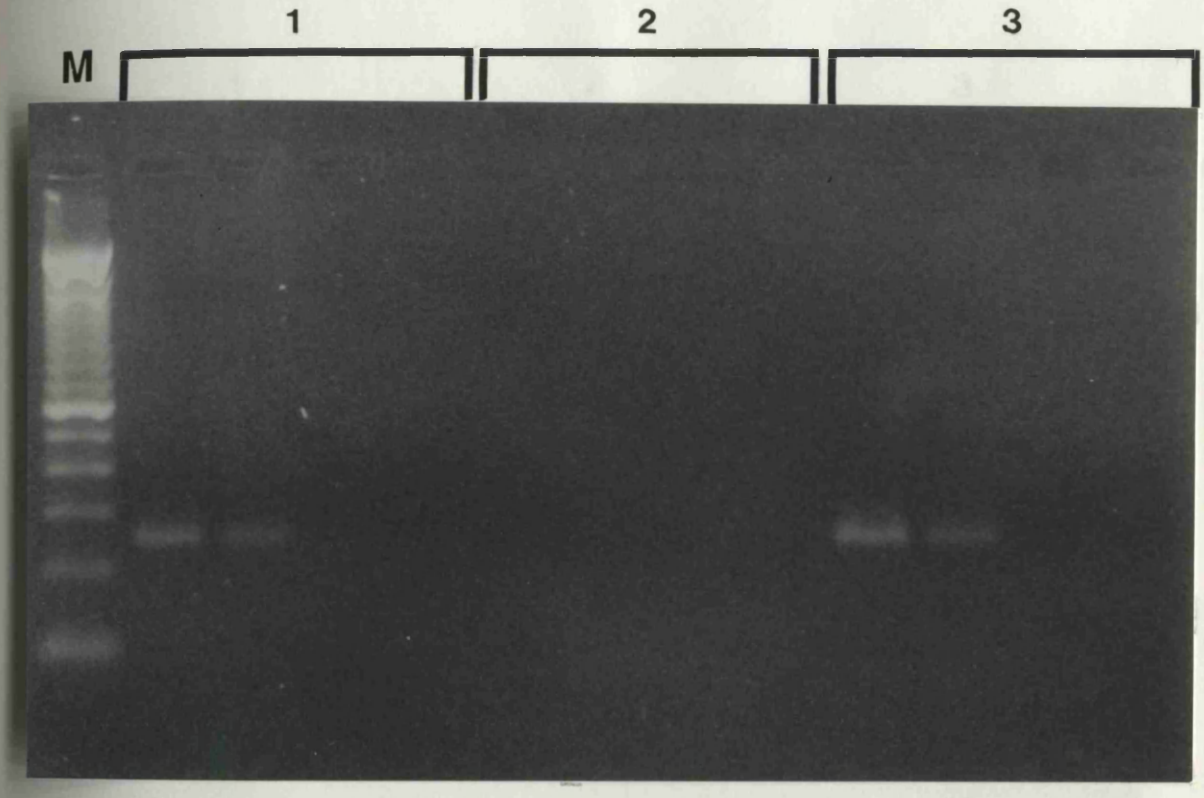
b) Comparison of PCR amplification protocols

RNA was extracted using the QIAamp blood kit, reverse transcribed undiluted, or diluted 1:10, 1:100 or 1:1000 in HCV negative saliva and amplified by PCR. Three procedures were compared by visualisation with gel electrophoresis and EtBr staining. Reverse transcription using MMLV-RT, and amplification with Taq pol was carried out using two variations of a nested PCR procedure. Hot-start PCR gave a better result detecting 10x less HCV RNA than the basic nested PCR. Samples processed by the basic nested PCR produced a greater level of background (Figure 3.2). One step RT-PCR employing Tth as both the reverse transcriptase and polymerase performed well producing the same results as the basic nested and hot-start procedures.

c) Comparison of methods of detection

Following HCV RNA amplification, a number of saliva samples obtained from HCV seropositive blood donors presented faint bands, weak positives barely visible by ethidium bromide staining. Use of a digoxigenin labelled probe in a southern blot hybridisation format provided no greater sensitivity of detection. Indeed, ethidium bromide staining of the amplified products following electrophoresis produced a clearer picture. Hybridisation with a digoxigenin labelled probe caused difficulties in differentiating between an actual weak positive signal and simple background staining (Figure 3.3).

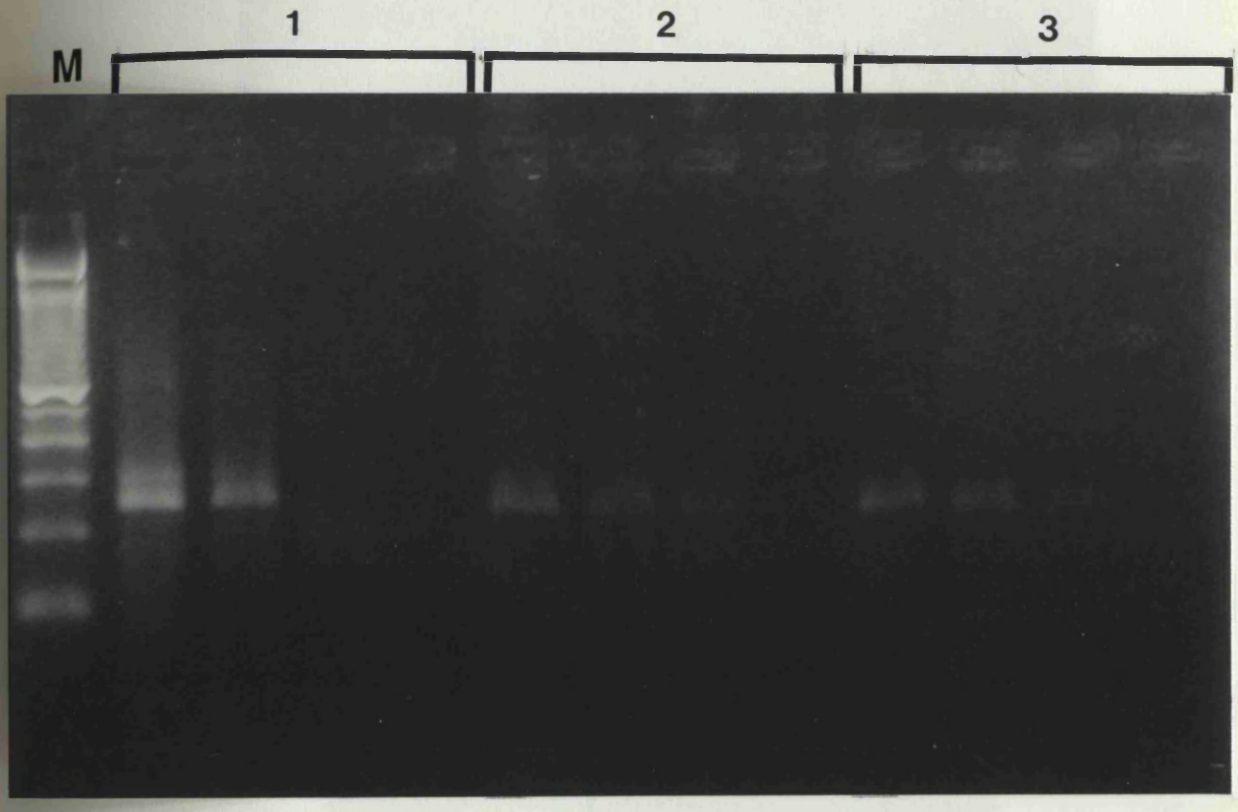
FIGURE 3.1



RNA extraction from HCV positive serum serially diluted ten fold by

1) guanidinium isothiocyanate/ acid-phenol; 2) GLASSMAX RNA Microisolation Spin Cartridge System and 3) QIAamp Blood Kit; M) 100bp DNA marker.

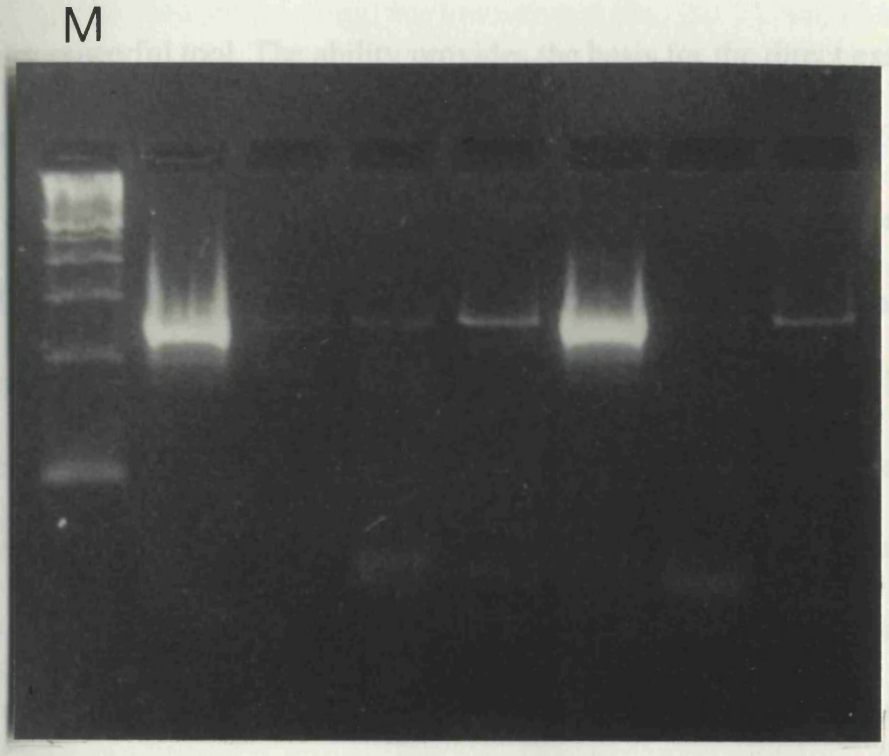
FIGURE 3.2



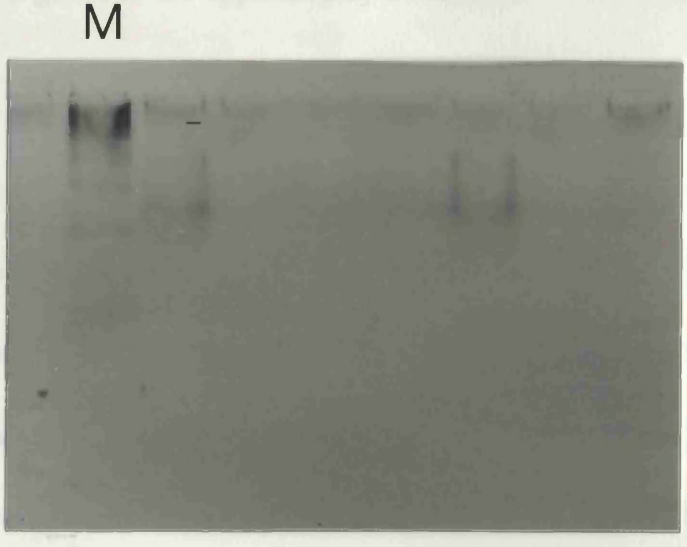
Extracted HCV RNA serially diluted ten fold and amplified by 1) reverse transcription with MMLV-RT and nested PCR; 2) reverse transcription with MMLV-RT and hot start PCR and 3) One step RT-PCR using Tth pol; M) 100bp DNA marker.

FIGURE 3.3

1)



2)



Comparison of detection methods: 1) gel electrophoresis and ethidium bromide staining; 2) Southern blot analysis of 1) using Dig-labelled probe; M) 100bp DNA marker.

DISCUSSION

The extraordinary ability of PCR to exponentially replicate a target DNA sequence has made it a very powerful tool. The ability provides the basis for the direct examination of a clinical sample for evidence of infection. It is possible to obtain unheard-of specificities by careful choice of the oligonucleotides which serve as primers for the amplification. These superior characteristics have propelled the field of PCR-based molecular diagnostics into the area of applied diagnostics for infectious agents. However, a number of issues remain to be considered before PCR becomes sufficiently reliable for routine use. These include standardisation of reagents, the ability to reduce sophisticated technology into a reproducible technique that can be performed in a standard clinical lab, and to increase the number of samples that can be easily processed in one day. In this chapter, consideration is given to the applicability of different protocols for each stage of the procedure, namely sample processing, amplification and detection.

Successful cDNA synthesis begins with the isolation of high quality RNA free of contaminants and inhibitors. RNA can be isolated for use in reverse transcription (RT)-PCR by various methods (Cuypers, 1994). These can be divided into three essentially different types of extraction. The first is digestion of the specimen with proteinase K in the presence of sodium dodecyl sulphate followed by organic extraction with phenol/chloroform and ethanol precipitation (Choo et al., 1991). Secondly, RNA may be extracted by lysis of specimen in a chaotrophic agent followed by organic extraction with phenol/chloroform and precipitation with ethanol or isopropanol (Chomzynski and Sacchi, 1987). The final method is incubation of the specimen with guanidinium isothiocyanate

and absorption of the released nucleic acid to silica hydroxide particles (Boom et al., 1990). A modification of the second method based on the phenol/chloroform extraction was the procedure employed initially in this study. We compared the efficiency of HCV RNA purification with this method to that using two commercial kits, the GLASSMAX RNA Microisolation Spin Cartridge System based on the third method and the QIAamp Blood and Tissue Kit which combines the principles of the first and third methods. In our hands, both the guanidine isothiocyanate/acid-phenol method and the QIAamp Blood kit gave comparable results. However, the QIAamp kit gave pure, ready-to-use RNA in minutes without the need for extraction with hazardous or toxic organic solvents. Using the GLASSMAX system we failed to obtain amplifiable RNA. This may be a result of the adaptations made to facilitate extraction from serum and saliva. Extraction using a guanidinium thiocyanate procedure has also previously been shown to be less successful at removing PCR inhibitory factors in saliva than other methods of extraction based on phenol-chloroform or proteinase K (Ochert et al., 1994). It is possible to further increase the specificity of HCV RNA detection by concentrating large volumes of samples by ultracentrifugation. However, ultracentrifugation also increases the risk of cross-contamination and in our studies, obtaining large volumes of saliva would have been impractical.

As the genome of HCV is a single-stranded RNA molecule, amplification by PCR must be preceded by a step to generate cDNA. Numerous protocols for reverse transcription and PCR have been published (Cuypers, 1994). Amplification of HCV RNA by RT-PCR is, in most protocols, a two-step procedure requiring separate enzymes and buffer conditions for the cDNA and PCR reactions. A variety of reverse transcriptase enzymes are available including Molony Murine Leukemia Virus reverse transcriptase

(MMLV-RT) and Tth. In both the nested and hot-start PCR, first strand synthesis using MMLV-RT is primed using random hexamers. This is a highly non-specific method and is likely to influence the amount and variety of cDNA obtained. The most specific method of priming is to use a primer containing sequence information complementary to target RNA. This is the method used in one-step RT-PCR and has the advantage of producing a specific cDNA, resulting in a more specific PCR amplification.

In nearly all the protocols a part of or the complete cDNA reaction is subsequently amplified by PCR. This is performed after a change of buffer conditions to one suitable for amplification by the DNA polymerase. The need to make a buffer change is cumbersome and increases the likelihood of contamination. To avoid this risky step a number of procedures have been developed that combine the cDNA reaction and PCR (Cuypers, 1994). The most promising of these employs the thermostable DNA polymerase from Thermus thermophilus (Tth). Using Tth-polymerase, RNA can be converted to DNA at 70°C in the presence of manganese. The addition of EGTA to the reaction switches the template specificity of Tth-polymerase from RNA to DNA. Thus a single enzyme can convert RNA to cDNA and then amplify the cDNA by PCR. There are several advantages to using Tth-polymerase in a combined one-step RT-PCR. First, the ability of Tth-polymerase to reverse transcribe at 70°C circumvents the problems associated with the use of reverse transcriptase enzymes. Retroviral reverse transcriptases are thermally labile and cannot be used at temperatures above 42°C. The lower temperature necessary for enzyme activity also increases the potential for single-stranded RNA templates to form stable secondary structures. This results in widely varying efficiencies of conversion of RNA to cDNA. Indeed the higher temperature at which Tth-polymerase is used, increases the specificity of primer extension in the RT-step. Secondly, Tth-polymerase does not produce primer-dimers. These structures are characterised by the

elongation of one primer with a sequence complementary to the other. Once such a product is formed, it can serve as an efficient template for primer binding and extension, and can act as a reagent sink thereby reducing the efficiency of amplification of the target sequence (Ehrlich and Sirko, 1994). Finally, Tth-polymerase as a reverse transcriptase is only suitable for use with sequence specific primers and not oligo(dT) or short random primers. As mentioned earlier the use of such primers produces a more specific cDNA.

Initially, HCV RNA was amplified using a two-step nested PCR. Using this protocol, the first round products were subjected to a second round of PCR using primers internal to the first primer pair. Nested amplification offers both advantages and disadvantages. The sensitivity of nested PCR is extremely high enabling target to be detected by ethidium bromide staining. This circumvents the need for laborious detection methods such as hybridisation with labelled probes. Reamplification with a second set of internal primers also confirms the specificity of the first round as any non-specific products are unlikely to be sufficiently complementary to the nested primers to be able to serve as template for any further amplification. Furthermore, the transfer of the first round product effectively serves to dilute out inhibitors that might be present in the sample initially. This is of importance with regards to amplifying HCV RNA from saliva specimens (Ochert et al., 1994).

Despite its advantages, the high risk of cross contamination during the preparation of the second round PCR is a major drawback. Some researchers believe that nested PCR is totally inappropriate for the clinical setting where diagnostic accuracy is of paramount importance. A further disadvantage with this method is the ability of Taq polymerase to produce primer-dimers and non-specific products, as mentioned earlier. During PCR, primers may bind to non-target DNA as well as to themselves. This may induce Taq

polymerase to synthesise unwanted DNA. At the end of the initial denaturing step, most DNA will be single stranded and incapable of hybridising with other single stranded DNA (e.g. primers). However, while the temperature is being ramped to 94°C, there will be some single stranded DNA available for primer annealing and spurious DNA synthesis. Hot start PCR prevents the primers from having the opportunity to bind single stranded DNA until a critical temperature is reached that strongly disfavours the non-specific pathways. This can be accomplished by physically separating the primers from the reaction mixture and Taq polymerase by the use of a wax. The wax melts at 80°C allowing mixing of the primers and other reagents and then serves as an evaporation barrier during the actual cycling.

All of the methods employed were equally sensitive in detecting HCV RNA in blood and saliva. The use of hot-start nested PCR should be advocated as a more specific method than the basic nested PCR. However, using one-step RT-PCR may reduce the risk of contamination. For some applications, RT-PCR alone may be sensitive enough to allow direct detection. However, with regard to HCV, the use of a further round of amplification cannot be avoided unless a more cumbersome method of detection is used and as we will discuss later this is not a preferred option. Therefore, the choice remains between hot-start nested PCR involving the transfer of DNA three times or RT-PCR (nested) requiring the transfer of DNA twice. Since the problem of product carry-over remains a major obstacle to the acceptance of PCR as a routine diagnostic tool it would perhaps be more prudent to employ the one-step RT-PCR protocol.

In procedures using nested PCR, amplified products can be analysed by visualisation of DNA band(s) of expected sizes on agarose gels stained with ethidium bromide (Cuypers, 1994). The major disadvantage to using ethidium bromide as a sole method for product detection is that it will only detect bands that contain ~5ng or more of

DNA. Hybridisation with labelled probes can improve this sensitivity, detecting DNA bands with a concentration <1ng (Jenkins, 1994). Both these methods detect all PCR products, both specific and non-specific. Specificity can be controlled for by Southern blot hybridisation using a labelled oligonucleotide probe, which is internal to the primer sequences employed in the PCR reaction. Use of radiolabelled probes is a common method of detection in many research laboratories. However, the use of radiolabels in a diagnostic laboratory would not be encouraged. The development of sensitive non-radioisotopic detection methods using probes labelled with detectable moieties such as digoxigenin has, however, made such detection formats more amenable to a diagnostic laboratory.

In our hands, visualising PCR products using ethidium bromide was sufficiently sensitive when amplifying HCV RNA from serum samples. However, for saliva samples ethidium bromide staining revealed amplified products of the expected length, fluorescing at very low intensities under UV light. Identification of the nested PCR product with southern blot hybridisation using a digoxigenin labelled probe did not, however, increase the sensitivity of detection. It is likely that further work optimising the conditions used would increase the sensitivity of this method. However, taking into account the time needed to perform the procedure, we felt justified in remaining with visualising the PCR products by EtBr staining. Southern blot hybridisation protocols have been developed for HCV detection though these are employed following a single round of PCR as an alternative to nested PCR (Cuypers, 1994). The use of such hybridisation assays is not itself without problems. Sensitivity can sometimes be compromised by inefficient transfer of amplification products onto the filter, while specificity of detection with labelled probes requires stringent hybridisation conditions

between probe and the membrane-bound PCR product. Such a procedure also suffers from low signal/noise ratio problems. With regard to a clinical laboratory, the main reason for avoiding such a procedure is that it is time-consuming and labour-intensive. For HCV diagnosis, detection with ethidium bromide following amplification with two sets of primers may be appropriate. The choice of primers is such that the PCR reaction yields a single amplified product. However, it must be remembered that the presence of a DNA band in an agarose gel whose length corresponds to the length of the expected PCR product does not necessarily provide a positive identification.

Like any new, promising technology, PCR has spawned a myriad of developmental efforts. Significant progress is being made to develop PCR kits for the clinical laboratory. These kits address all needs from sample collection and preparation, through amplification and detection. Currently there are two major companies actively developing fully integrated PCR-based diagnostic systems, as well as a host of other commercial concerns working on sample preparation kits, thermal cyclers, thermostable DNA polymerases with assorted secondary characteristics, and methods to detect rapidly the PCR amplification products. Out of the current confusion will emerge one or several diagnostic configurations which will have survived the rigours of laboratory selection and will yield clear, consistent results in a timely manner. Standardisation of HCV RNA detection is urgently needed. Quality control between and within laboratories is a major problem. The European Expert Group on Hepatitis ("Eurohep") showed only 16% of PCR laboratories operating with maximum sensitivity and specificity (Zaaijer et al., 1993). At this point there is no fully automated system available commercially through which specimens can be processed with the generation of reliable results, without significant technical effort and expertise.

Chapter 4

Detection of HCV RNA in Saliva of Haemophiliacs and Blood Donors

INTRODUCTION

The diagnosis of most diseases requires some form of testing of a body fluid or tissue. Blood is the fluid of choice for a wide range of tests. However, in areas where there are cultural barriers to collecting blood and in certain other groups (e.g. children and IVDU) the necessity for venepuncture can be ethically unacceptable or potentially difficult. In such settings, the use of non-invasive diagnostic testing would prove beneficial. In the same way, with the emergence of AIDS, haemophobia has fuelled the search for less hazardous yet reliable specimen types.

Of all the possible alternatives to blood, saliva's popularity has suffered because it lacks "the drama of blood, the sincerity of sweat and the emotional appeal of tears" (Mandel, 1993). However, sweat and tears are difficult to obtain in sufficient quantities for routine testing, and urine will always lack the charisma of other body fluids. Saliva is easily obtained on multiple occasions with less trauma than blood sampling and without the need for the privacy required for urine collection, yet physicians and dentists rarely take such samples.

The main benefits of saliva over blood as a diagnostic fluid are greater acceptability to the subject, convenience, economy, and hazard reduction. Saliva specimens can be collected by asking the subject to dribble into a sterile pot or by asking the subject to chew a small cotton wool cylinder (e.g. Salivette ™). However, low concentration of target, small sample volume, degradation of target by proteolytic activity and inconsistent collection procedures have all conspired to delay the widespread acceptance of saliva testing. Nevertheless, with the development of collection devices, which when used correctly can yield stable saliva samples of adequate volume, and with the introduction of

new and highly sensitive laboratory techniques, the low levels of sample volume are no longer considered a limiting factor.

Diagnostic interest in viral specific antibodies in saliva was initiated by Archibald et al.in 1986 who reported the presence of anti-HIV in 87.5% of HIV-seropositive subjects (Archibald et al., 1986). Since then, the presence of IgG anti-HIV (Parry et al., 1987), IgG and IgM anti-HAV (Parry et al., 1987; Parry et al., 1988; Parry et al., 1989; Piacentini et al., 1993), anti-HBsAg and IgM anti-HBcore (Parry et al., 1987; Parry et al., 1989; Thieme et al., 1992; Piacentini et al., 1993), IgG and IgM anti-measles, mumps and rubella (Parry et al., 1993) have all been successfully demonstrated in saliva. However, despite the demonstration that salivary antibody detection could have broad applications in viral epidemiological surveillance, outbreak investigation and vaccine monitoring, it has attracted little attention except in the field of HIV where the value of saliva in screening for HIV infection is well established.

As mentioned earlier, HCV can be detected earlier by PCR than by serology, and is a better predictor of infectivity. However, detection of HCV in saliva by PCR is unreliable. A wide variation in the prevalence of HCV RNA in saliva has been reported previously (0-100%) (Chen et al., 1995; Couzigou et al., 1993; Fried et al., 1992; Hsu et al., 1991; Liou et al., 1992; Mariette et al., 1995; Numato et al ., 1993; Punchammer-Stockl et al., 1994; Takamatsu et al., 1991; Thieme et al., 1992; Wang et al., 1991; 1992a; Young KC. et al., 1993). In this chapter, we aimed to determine by PCR the prevalence of HCV in saliva of a group of haemophiliacs receiving dental treatment at the Glasgow Royal Infirmary, and in a group of blood donors referred to a hepatology clinic at Gartnavel General Hospital. The purpose of this study was to determine whether HCV was present in the saliva of these patients and at what prevalence.

METHODS AND MATERIAL

a) Sample population

Twenty one haemophiliacs aged 21-88 years (mean age 42 years) attending an oral surgery unit for the full range of oral and dental care, and 47 blood donors aged 26-70 years (mean age 37.5 years) attending a hepatology clinic were studied. All of the patients were HCV-antibody seropositive on the basis of screening by second generation enzyme immunoassay (HCV EIA II System, Abbott Laboratories, Chicago), and by confirmation with a recombinant immunoblot system (RIBA 2/3, Ortho Diagnostic Systems, Raritan). All patients had been shown previously to be PCR seropositive for HCV RNA. Six of the haemophiliac patients were also HIV-antibody seropositive.

b) Sample collection

Saliva was collected as described in Chapter 2. No samples were collected from five HIV-positive haemophiliacs. Whole saliva and separate pellet and supernate samples were however available to this study for all six HIV co-infected patients. These samples had been previously collected and stored by Ruchill Regional Virus Laboratory, Glasgow. Only one duplicate set of fresh saliva samples was obtained from an HIV-positive haemophiliac.

c) RNA extraction

Initially RNA was extracted from the samples collected by the modified protocol of Chomczynski and Sacchi (1987). This was later superceded by the commercially

available QIAamp Blood Kit. A detailed account of both these methods was given earlier in Chapter 3.

d) Reverse transcription and PCR

Reverse transcription and nested PCR on the saliva specimens were carried out as described previously in Chapter 2 with no modification to the methods.

RESULTS

Ten haemophiliacs (47.6%) with serologically proven HCV infection had HCV RNA present in their saliva (Table 4.1). HCV RNA was present in saliva with a greater frequency among patients negative (8 of 15) than positive (2 of 6) for HIV infection. From the samples provided, HCV RNA was present in 5 (23%) of the Salivette™ specimens, 5 (23%) of the whole saliva specimens, 5 (23%) of the pellet fractions and 4 (19%) of the supernates. In only one patient was HCV RNA detected in all four specimens, and for two patients, in three of four specimens. The presence of HCV RNA in the supernate and/or pellet sample was not predetermined by its presence in the whole saliva specimen.

Five full sets of samples which had been previously shown to contain HCV RNA positive saliva were tested again following a further freeze-thaw cycle. No virus could be detected in any sample following this process (Table 4.2).

Sixteen (34%) HCV RNA seropositive blood donors had HCV RNA present in their saliva (Table 4.3). From the samples provided by these blood donors, HCV RNA was detected in 8 (17%) of the whole saliva samples, 12 (25.5%) of the Salivette™ specimens, 1 (2.1%) of the pellet and 1(2.1%) of the supernate fractions. No patient had HCV RNA detectable in all four specimens. Six patients had HCV RNA present in both the whole saliva and Salivette™ samples. As before, the presence of HCV RNA in the whole saliva did not predetermine its presence in the supernate or pellet samples.

Genotyping using RFLP analysis was available for 23 blood donors. Eight donors were infected with genotype 1, three with genotype 2, and twelve with genotype 3. Two blood donors with HCV RNA present in their saliva were genotype 1 and five were

genotype 3. None of the 3 blood donors infected with genotype 2 had detectable HCV in their saliva (Table 4.4)

TABLE 4.1

Patient No	Salivette	Whole Saliva	Pellet	Supernate
1	+	+	–	–
4	–	–	+	–
6	–	–	+	–
7	–	+	–	–
10	+	–	–	–
11	+	+	–	+
13[1]	2	–	–	+
14[1]	2	+	+	+
18	+	+	+	+
19	+	–	+	–

Summary of HCV RNA positive saliva specimens from HCV seropositive haemophiliacs.

[1] HIV co-infected haemophiliacs. [2] No samples available for testing .

(+) Positive PCR result. (-) Negative PCR result.

TABLE 4.2

Patient No	Saliva	Whole saliva	Pellet	Supernate
1	+	+	–	–
*	–	–	–	–
4	–	–	+	–
*	–	–	–	–
6	–	–	+	–
*	–	–	–	–
11	+	+	+	–
*	–	–	–	–
18	+	+	+	+
*	–	–	–	–

Summary of the effect of two freeze-thaw cycles on HCV RNA positive saliva.

* After two freeze-thaw cycles.(+) Positive PCR result. (-) Negative PCR result.

TABLE 4.3

Patient No.	Salivette	Whole Saliva	Pellet	Supernate
6	+	+	–	–
9	+	+	–	–
11	+	–	–	–
12	+	+	–	–
15	+	+	–	–
18	+	–	–	–
19	–	+	–	–
22	+	–	–	–
26	+	–	–	–
28	+	–	–	–
29	+	+	–	–
36	+	+	–	–
41	–	+	–	–
42	–	–	+	–
43	+	–	–	–
44	–	–	–	+

Summary of HCV RNA positive saliva samples from HCV seropositive blood donors .

(+) Positive PCR result. (-) Negative PCR result.

TABLE 4.4

Viral genotype [1]	HCV RNA positive saliva	HCV RNA negative saliva
1	2	6
2	0	3
3	5	7

Genotypic analysis of HCV RNA seropositive patients with detectable HCV RNA in their saliva.

[1] Genotyping by RFLP carried out by SNBTS, Edinburgh.

DISCUSSION

With the emergence of AIDS, the search for less hazardous yet reliable specimens has focused on body fluids such as saliva. When the cost of conventional laboratory testing is also being heavily scrutinised and more out-of-laboratory testing (i.e. home- testing) systems are being devised, saliva has emerged as the forerunner in the search for an acceptable alternative to blood suitable for such applications, providing a safe, yet reliable specimen.

In this study, whole saliva and Salivette™ samples were collected from 21 HCV seropositive haemophiliacs and 47 blood donors whose sera were previously HCV RNA positive by nested PCR. HCV RNA was detected in the saliva of 10 (47.6%) haemophiliacs and 16 (34%) blood donors. Results of earlier studies to detect HCV RNA in saliva from patients with detectable RNA in their serum, have varied substantially (Chen et al., 1995; Couzigou et al., 1993; Fried et al., 1992; Hsu et al., 1991; Liou et al., 1992; Mariette et al., 1995; Numato et al., 1993; Punchammer-Stockl et al., 1994; Takamatsu et al., 1991; Thieme et al., 1992; Wang et al., 1991; 1992a; Young et al., 1993). This suggests that HCV is either absent or alternatively, that it is present below the level of PCR sensitivity. The complex composition of saliva and the variety of physiological and pharmacological factors may influence target concentration. Some of the constituents of saliva may also be capable of destroying an RNA template or may even interfere with the PCR procedure.

The stability of HCV in saliva also needs to be determined. Whether HCV RNA breaks down spontaneously, by the action of enzymes or by heating or freezing requires further study as such factors may reduce an already low copy number. The critical

period for degradation is probably between collection and freezing of samples and in the present study this period was limited to a maximum of three hours. Further freeze-thaw cycles may also reduce the level of virus. Such handling has been reported to reduce the PCR signal in serum (Wang et al., 1992b) and in this study freeze-thaw cycles significantly reduced the viral load in saliva to that below the limit of detectability. The low level of HCV RNA positive supernate and pellet samples obtained from blood donors compared to those taken from the haemophiliacs is notable. This may have been a result of sample storage conditions. The whole saliva and Salivette™ samples from the blood donors were tested immediately whilst the pellet and supernate samples were tested at a later date, some having been stored for up to six months. Samples obtained from the haemophiliacs were tested together soon after collection. Thus, the low level of HCV RNA detected in the blood donors' pellet and supernate samples may have resulted from degradation of the virus during storage of the samples.

In this study, two of the six HIV seropositive haemophiliacs had HCV RNA present in saliva. Results for this group were, however, obtained from stored samples which had undergone a number of freeze-thaw cycles. This may have resulted in an unrealistically low rate of detection. Only one fresh sample was obtained from this group of patients and in that specimen virus was detected in the whole saliva, pellet and supernate. The number of HIV co-infected haemophiliacs in this study is unfortunately too small to comment on any association between salivary shedding of HCV and HIV seropositivity and this point will require further investigation.

Another consideration is whether or not HCV is bound to salivary proteins or cell debris, which are often removed from whole saliva by centrifugation. In this study, both the supernate and pellet samples were tested alongside the whole saliva. No data were

recorded to indicate that HCV was associated with any particular component of saliva. However, this lack of association may be a result of any of the aforementioned factors. Some studies have indicated that specimens collected by swab or into a pot produced better results than samples from Salivettes™ for determination of viral antibody status (Mortimer & Parry, 1988). In this study a greater percentage of Salivette™ samples were positive for HCV RNA. This discrepancy may result from obtaining a purer sample from the Salivette™, containing less degradative factors. Critical analysis of this and earlier studies therefore suggests that discrepancies in results from different laboratories may relate to sample collection and handling techniques and PCR protocol variations.

With regard to salivary shedding and infection with a particular genotype, again the number of blood donors genotyped is too small to draw any conclusions. Although our results indicated that patients infected with genotype 2 failed to have detectable HCV RNA in their saliva , the small number of donors infected with this particular type makes this point invalid.

We conclude that HCV is present in the saliva of some HCV seropositive haemophiliacs and blood donors. However, more work is needed to optimise sample collection and handling methods, as well as maximising PCR protocols before a true analysis of the prevalence of HCV shedding in saliva can be carried out. Finally, HCV was detected in the saliva of fewer than 38% of HCV-seropositive patients studied. Therefore, at this time, salivary tests for HCV RNA are clearly insufficiently sensitive to replace serum assays.

Chapter 5

Serological and Salivary Markers Compared With Biochemical Markers in the Monitoring of Interferon Treatment for Hepatitis C Virus Infection

INTRODUCTION

Acute infection with HCV may either be severe or, more commonly, asymptomatic and unnoticed. The acute disease may resolve completely, but chronic hepatitis C develops in 70-80% of patients who acquire acute infection (Cordoba, 1994). Studies indicate that the natural course of HCV infection is variable. Most common though is an insidiously progressive liver disease, often clinically negligible, persisting for many years. Spontaneous remission appears extremely rare and is thought not to occur. Chronic HCV can be associated with chronic liver disease, cirrhosis, and HCC. HCV infection is a significant cause of morbidity and mortality from liver disease and as such warrants a specific and effective therapy. The main aim of treatment should therefore be to prevent the progression of chronic hepatitis to end-stage liver disease by halting inflammatory activity in the liver. A complete cure, however, would aim at eliminating the virus completely. At the moment, the only agent of proven benefit is IFNα, but the treatment of chronic hepatitis C remains problematic and unsatisfactory.

Interferons are natural proteins produced in response to stimuli such as foreign cells, bacteria and viruses. There are three main types of proteins, known as α, β, and γ interferon which have direct antiviral effects (inhibition of viral replication, protein synthesis, maturation or release from infected cell) and immunomodulatory effects (enhancement of macrophage, cytotoxic T cell and natural killer cell activity) (Dianzini, 1993; Finter, 1994).

The use of IFNα to treat chronic hepatitis C infection began before the identification of HCV (Hoofnagle et al., 1986). Nowadays there are three single subtype recombinant IFNα preparations available for clinical use, IFNα-2a (ROFERON, Roche);

IFNα-2b (INTRON, Schering-Pluogh) and IFNα-2c (BEREFOR, Boehringer-Ingelheim). All are different versions of the same gene product IFNα-2. There have been many trials of IFNα preparations in patients with chronic HCV disease providing essentially similar results. IFN has been shown to have beneficial effects with short-term and long-term response rates averaging 50-70% and 25-80% respectively, as estimated by the normalisation or decrease of ALT levels in sera (Brester, 1994). However, more recent studies report that in general, improvement in ALT values does not necessarily reflect a reduction in HCV RNA levels (Picciotto et al., 1994; Kakumu et al., 1993; Liang et al., 1993; Pawlotsky et al., 1994). This indicates the importance of monitoring HCV viraemia by PCR during treatment.

At present, the monitoring of IFN treatment requires repeated venepuncture, with the associated discomfort to patients and potential risks to health-care workers. This study was run in parallel with our previous study and aimed to assess the suitability of saliva as an alternative to blood monitoring the virological effect of IFNα on a group of HCV seropositive blood donors.

METHODS AND MATERIALS

a) Sample population

Eighteen patients (8 females, 10 males, mean age 35.8 years, range 26-52 years) were studied. All were previously blood donors, who were clinically well and whose HCV status had been detected through routine screening of donated blood. All had provided HCV-positive blood donations as determined by second generation enzyme immunoassay (HCV EIA II System, Abbott), with confirmation by recombinant immunoblot assay (RIBA-2and RIBA-3, Ortho Diagnostic Systems, Raritan) and PCR. All subjects had abnormal histology on liver biopsy, with characteristic features of chronic hepatitis C infection.

b) Interferon treatment

Ethical approval for the trial was obtained from the Western and Gartnavel General Hospital Trust Ethics Committee, Glasgow. After written consent was obtained, patients were randomly assigned to a year of treatment or observation. Those patients assigned to treatment received self-administered intramuscular injections of 4.5 MU of recombinant interferon IFNα-2a (ROFERON, Roche Products Ltd, Welwyn Garden City, UK) thrice weekly for 12 weeks. The dosage was reduced to 3 MU for the next 12 weeks, 2 MU for a further 12 weeks and 1 MU for the final 12 weeks of the course. Patients were withdrawn from treatment after the first 12 weeks if no response was observed on the basis of ALT normalisation at that stage. Observation patients received no drug or placebo over the study period.

c) Follow-up

For the nine patients receiving treatment, liver function tests, including ALT, were carried out every week during the first month. This was followed by a sample two weeks later and after a further six weeks. For the remainder of the study, samples were collected at twelve weekly intervals. Observation patients were followed at 0, 12, 24, 36 and 48 weeks. Both serum and saliva samples were collected at each of the above visits.

d) Sample collection

Saliva was collected as described previously in Chapter 2. A clotted blood sample was collected and stored at -20°C by Sister Elizabeth Spence, Department of Gastroenterology, Gartnavel General Hospital.

e) RNA extraction

RNA was extracted using the QIAamp blood kit as described in Chapter 3.

f) Reverse transcription and PCR

Reverse transcription and PCR were carried out as previously described in Chapter 2.

g) Quantitative PCR

Quantitation of HCV RNA was carried out on serum samples collected at 0 and 12 weeks by Professor Johnson Lau, University of Florida using quantitative branched DNA

(bDNA) signal amplification (Quantiplex™- HCV RNA, version 1.0, courtesy of Chiron Corporation, USA) as previously described (Lau et al., 1993).

h) Genotyping

HCV genotyping was carried out by the SNBTS, Edinburgh, using RFLP analysis (McOmish et al., 1993).

i) Definition of response

Responses to IFNα were grouped into three patterns. A complete response was defined as the normalisation of aminotransferases, accompanied by the loss of serum HCV RNA, before the third month of treatment and persisting at least until the end of the IFN course. Normalisation of aminotransferases without the loss of HCV RNA was considered a partial response. Patients whose aminotransferase activities remained high and in whom serum HCV RNA persisted were considered non-responders. These definitions permitted assessment of the capacity of IFNα to induce both biochemical and virological remission of the disease.

RESULTS

Of the eighteen patients enrolled in the study, one observation and two treatment patients were lost to follow-up during the year. Complete follow-up data were available for 14 patients (7 treated and 7 untreated) . Table 5.1 gives the demographic and clinical features of the patients from treatment and control groups. The two groups were similar with respect to age, sex, ALT, AST and alkaline phosphatase levels, risk factors and prevalence of HCV RNA. Immediate-pretreatment serum HCV RNA was found to be present in all but one patient who remained negative throughout treatment. This patient had previously been shown to be HCV RNA positive by PCR.

Serum ALT levels declined to normal in 5 of the 7 IFN treated patients, but remained elevated in two patients by the twelfth week of treatment (Figure 5.1). In the 5 patients whose ALT levels returned to normal, loss of detectable HCV RNA was observed in only 3. Reduction of interferon from 4.5 MU at the twelve weeks to 3 MU was accompanied by a return of HCV RNA in one partial responder. Further reductions to 2 MU heralded an increase in ALT in the same patient (Figure 5.2). In the remaining treatment patients, gradually decreasing the dose of interferon had no adverse effects. No significant differences were found between the responding and non-responding groups in relation to mean age and pretreatment ALT, AST or alkaline phosphatase (Table 5.2). In all observation patients, ALT levels remained high accompanied by HCV viraemia, intermittent in some, throughout the study period (Figure 5.3).

HCV RNA was detected in the serum of 12 out of 14 patients (93%). Of the 7 patients receiving treatment, the three with a sustained response to IFNα had a lower pre-treatment viraemia level (mean below cut-off), than either the partial responders (mean

10.52 x 10^5 HCV RNA equivalents/ml) or the two non-responders (mean 11.26 x 10^5 HCV RNA equivalents/ml) (Table 5.2). Both non-responders had infection with type 1 HCV, while the complete and partial responders were infected with types 2 or 3 (Table 5.3). No patient receiving IFN had HCV RNA in saliva prior to treatment. Virus was however detected in the saliva of the two patients who did not respond to IFN during their course of treatment and follow-up. HCV was shown to be present in the saliva of all 7 observation patients at some point during the follow-up period. From the saliva samples provided by these 9 patients (7 observation and 2 nonresponders), throughout the year, HCV RNA was detected in 10 of the Salivette™ specimens, 11 of the whole saliva specimens, 7 of the supernate fractions and 7 of the pellet fractions (Table 5.4). The presence of HCV RNA in the supernate and/or pellet sample was not predetermined by its presence in whole saliva. In only one patient was HCV RNA detected in all four types of specimens, and for three patients, in three of the four specimens. At no point was HCV RNA detected in saliva when the parallel serum specimen was negative. Although the presence of HCV in saliva did correspond to its presence in serum, no correlation was shown between the quantitative level of HCV RNA in serum and the presence of HCV RNA in saliva (Table 5.5). No genotype could be associated with the presence of HCV RNA in saliva (Table 5.6). The raw data for this study is shown in appendices 2-4.

TABLE 5.1

	Treatment (n = 7)	Observation (n = 7)
Age[1]	41.6 ± 6.8	33.7 ± 7.1
Sex (M/F)	3/4	4/3
Liver Function Tests[1]		
ALT[2]	114 ± 71.2	94.7 ± 59
AST[3]	61.6 ± 36.0	57.0 ± 13.8
Alkaline Phosphatase[4]	181.0 ± 64.4	142.8 ± 54.5
Risk Factors		
Transfusion	5	3
IV drugs	2	2
Ear Pierced	3	4
Tattoo	2	2
Unknown	1	0
Genotype[5]		
1	2	2
2	2	0
3	2	5
Not Known	1	0
HCV RNA		
Serum	6	7
Saliva	0	3

Demographic characteristics of treatment and observation patients at enrollment on interferon drug trial.

[1] Data expressed as mean ± S.D. [2] Normal ALT range 10-50 IU/litre.

[3] Normal AST range 10-35 IU/litre. [4] Normal Alkaline Phosphatase range 70-260 IU/litre. [5] Genotyping by RFLP.

TABLE 5.2

	CR (n=3)	PR (n=2)	NR (n=2)
Age	42 ± 10.5	44.5 ± 3.5	38 ± 1.4
Sex (M/F)	0/3	2/0	1/1
Liver function tests[1]			
ALT[2]	91.7 ± 65	63.5 ± 3.5	199 ± 29.7
AST[3]	51.7 ±30.1	36.5 ± 0.7	101.5 ± 34.6
Alkaline Phosphatase[4]	158.7 ± 91.6	191.5 ± 27.6	204.0 ± 67.2
bDNA levels[5]	< 3.5	10.5 ± 8.6	11.3 ± 1.0

Pretreatment patient characteristics according to response to interferon treatment.

[1] Data expressed as mean ± S.D. [2] Normal ALT range 10- 50/IU litre.

[3] Normal AST range 10-35 IU/litre. [4] Normal Alkaline Phosphatase range 70-260 IU/litre. [5] branched DNA levels x 10^5 HCV RNA equivalents/ml.

CR = Complete responder. PR = Partial responder. NR = Non responder.

TABLE 5.3

Genotype[1]	CR (n=3)	PR (n=2)	NR (n=2)
1	0	0	2
2	1	1	0
3	1	1	0
Not Known	1	0	0

Viral genotypes with regard to response to interferon treatment.

[1] Genotyping by RFLP. CR = Complete responder. PR = Partial responder. NR = Non responder.

TABLE 5.4

Patient	Whole saliva	Salivette	Pellet	Supernate
1[1]	1/5	2/5	0	1/5
2[1]	3/5	2/5	2/5	3/5
3[1]	3/5	4/5	2/5	1/5
4[1]	2/5	0	0	0
5[1]	1/5	0	1/5	0
6[1]	0	1/5	0	0
7[1]	0	1/5	1/5	1/5
8[2]	1/10	0	0	0
9[2]	0	0	1/10	1/10

Summary of HCV RNA positive saliva samples from 7 observation patients and 2 non-responders.

[1] Observation patients. [2] Non-responders to interferon.

TABLE 5.5

Patient	Week 0			Week 12		
	bDNA[5]	HCV RNA in		bDNA[5]	HCV RNA in	
		Serum	Saliva		Serum	Saliva
1[1]	<3.5	+	+	<3.5	+	+
2[1]	49.46	+	–	4.28	–	–
3[1]	<3.5	+	–	4.47	+	–
4[1]	12.47	+	–	10.12	+	–
5[1]	30.48	+	–	3.69	+	+
6[1]	29.47	+	+	41.47	+	+
7[1]	26.25	+	+	34.57	+	+
8[2]	11.75	+	–	6.76	+	–
9[2]	10.77	+	–	<3.5	+	–
10[3]	4.41	+	–	<3.5	+	–
11[3]	16.62	+	–	<3.5	+	–
12[4]	<3.5	+	–	<3.5	–	–
13[4]	<3.5	+	–	<3.5	–	–
14[4]	<3.5	–	–	<3.5	–	–

Level of HCV viraemia in serum in relation to the presence of HCV RNA in serum and saliva by PCR.

[1] Observation patients. [2] Non responders. [3] Partial responders. [4] Complete responders. [5] branched DNA levels x 10^5 HCV RNA equivalents/ml.

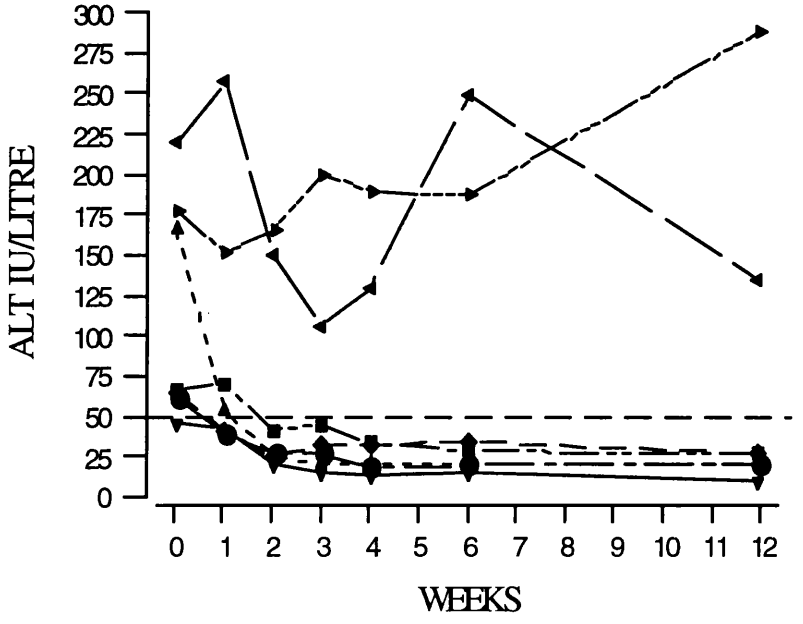
TABLE 5.6

Genotype[1]	HCV RNA present in saliva	HCV RNA absent in saliva	Total
1	4	0	4
2	0	2	2
3	5	2	7
Not Known	0	1	1

Viral genotypes of HCV in patients with relation to the presence or absence of HCV in their saliva.

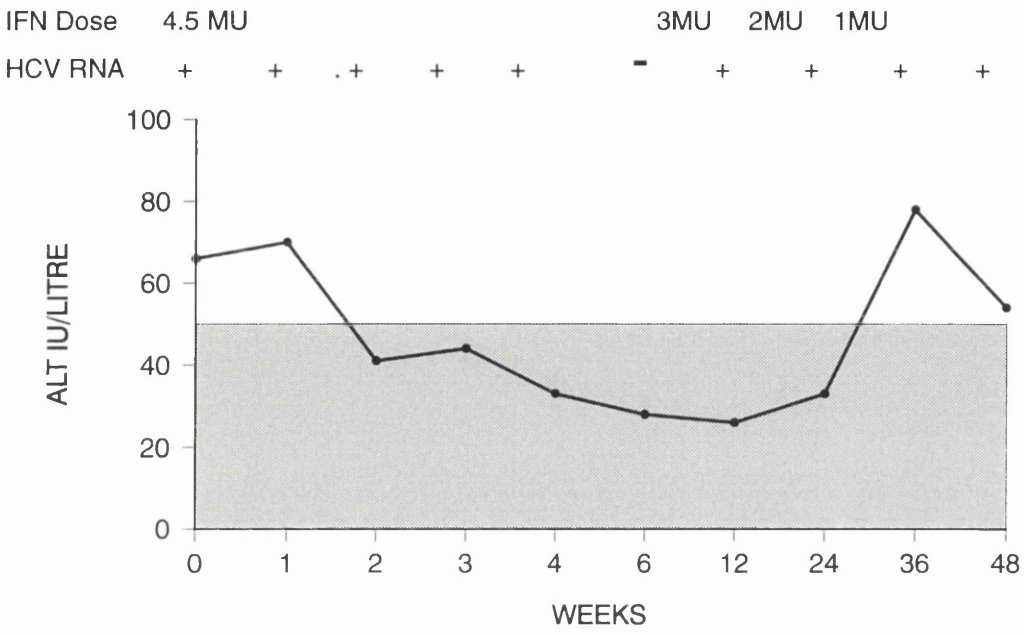
[1] Genotyping by RFLP.

FIGURE 5.1



Changes in ALT levels after 12 weeks of receiving 4.5MU IFNα-2a thrice weekly. Horizontal line (------) shows the upper limit of normal ALT level (50 IU/ litre).

FIGURE 5.2

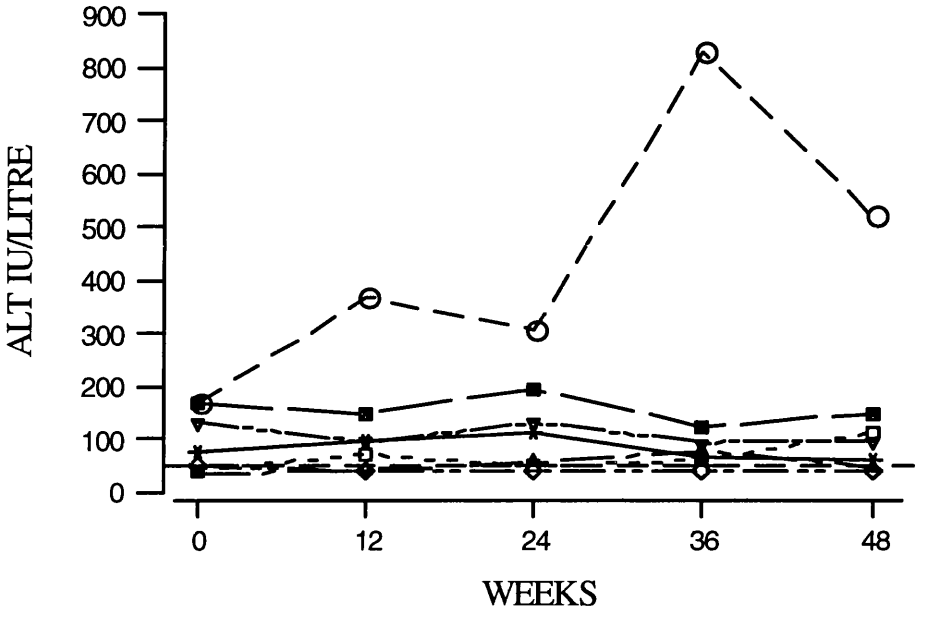


Virological relapse with biochemical relapse in Patient No. 10 after 12 weeks of IFN treatment. Biochemical relapse followed reduction in therapy

Shaded area shows the range of normal ALT level (50 IU/ litre).

(+) PCR positive. (-) PCR negative.

FIGURE 5.3



Serial changes in serum ALT levels in observation patients throughout the study. Horizontal line (------) shows the upper limit of normal ALT level (50 IU/ litre).

DISCUSSION

Therapeutic trials with IFN for patients with HCV infection were started when the diagnosis of disease was based on exclusion and known as NANBH (Hoofnagle et al., 1986). With the introduction of assays for serological diagnosis of HCV, evaluation of IFN therapy became more reliable. Beneficial effects of interferon have been reported in numerous controlled trials. The efficacy in these trials, however, varies rather widely from 40-70% with relapses occurring in 25-80% of responders (Bresters, 1994).

In the present study, the therapeutic effect of recombinant IFNα-2a in a step-down schedule when evaluated on patients with chronic hepatitis C infection was similar to that in other reports. Of the 7 patients treated, 3 showed a complete response, 2 showed only a partial response and 2 failed to respond. The observation that both non-responders were infected with Type 1 HCV concurs with earlier reports that response to IFN among patients infected by Type 1 is very low (Chemello et al., 1994, Hino et al., 1994; Mita et al., 1994). All 7 untreated observation patients remained positive for serum HCV RNA with elevated ALT values.

It should be noted that the optimal duration of therapy is currently unknown, and it is likely that different patients may require different lengths of treatment. The duration of therapy could be based upon attainment of a complete response, if a means of monitoring treatment were used which could correctly identify when HCV had been cleared. Establishment of meaningful treatment endpoints requires consideration of biochemical, virological and histologic information. Several clinical, serum biochemical, serological and histological features such as serum ALT, liver histology and HCV RNA in serum and/or liver are possibilities. Serum ALT levels and liver histology, while correlating with a

response to therapy, have been shown not to predict a sustained response. Many studies have reported persistence of viral RNA in patients with normal ALT levels (Liang et al., 1993; Kakumu et al ., 1993; Picciotto et al ., 1994; Pawlotsky et al., 1994). In our study, two patients maintained HCV RNA despite having normal ALT values. One patient did have slightly elevated ALT levels towards the end of treatment but this was attributed to the decrease in dosage of interferon (Figure 5.2). This patient later relapsed following treatment cessation. The other patient maintained normal ALT levels despite the presence of HCV RNA (Figure 5.4), and remains in the same situation to date. The mechanism that accounts for the lack of biochemical evidence of hepatic inflammation despite HCV viraemia in patients with partial responses to treatment remains to be defined.

The advent of PCR has enabled the presence and quantity of HCV RNA to be monitored during IFN therapy. Although this has been investigated in blood and levels of HCV RNA have been shown to decrease and become undetectable in patients with a good response, loss of RNA does not always predict a sustained response (Lau et al., 1993; Bresters et al., 1994; Picciotto et al., 1994; Aiyama et al., 1994; Yamada et al., 1992). Results from our study agree with this point, as three out of four partial and non-responders showed a decrease to undetectable levels of HCV RNA by bDNA assay (Figure 5.5). Very few studies have examined the presence of HCV in other tissues, fluids and organs (Balart et al., 1993; Di Bisceglie et al., 1993; Qian et al., 1992) but there are extra-hepatic sites of replication where the anti-viral effects of IFN are not documented. HCV RNA may need to be monitored in sites other than the liver and blood to confirm complete clearance of virus and to prevent later relapse. Indeed, recent studies report that a sustained response is associated with complete hepatic and extra-hepatic clearance (Gill et al., 1993; Saleh et al., 1994).

In the present study, monitoring the effect of IFN by evaluating HCV RNA in saliva has not shown a direct relationship with HCV RNA in blood. The detection of negative-stranded HCV RNA in salivary gland tissue has been reported. However, HCV RNA may also appear in saliva as part of the serum transudate in gingival crevicular fluid (Challacombe et al., 1978), in which case the amount of HCV in whole saliva would be expected to reflect the amount of virus in blood. In this study, we failed to show any correlation between the quantitative level of HCV RNA in serum and the presence of HCV in saliva (Table 5.5).

The detection of HCV RNA in the saliva of all untreated observation patients confirms that saliva has potential as a diagnostic aid. Furthermore, HCV RNA was also detected in the saliva of the two treated patients who did not respond to interferon. HCV RNA was not detected in saliva of any of the complete responders although serum HCV RNA also became undetectable within two weeks of treatment. For the partial responders, HCV RNA was detected intermittently in serum, but never in saliva. There are several reasons why HCV RNA may be undetectable in the saliva of patients with detectable virus in the serum, all of which were discussed in the previous chapter.

Although we failed to successfully monitor IFN treatment of HCV infection by detecting HCV RNA in saliva, we did confirm the shortcomings of using serum ALT alone in determining the end-point of treatment. Utilization of serum HCV RNA allows more accurate definition of response and earlier identification of relapse. Even better would be the use of liver HCV RNA as a marker. Unfortunately, tissue assays are not practical as they require a biopsy or serial biopsies.

It has not yet been established if there are reliable pretreatment variables that might predict a good response to IFNα. Previous studies have been unable to clearly

establish if there are any pretreatment variables that might predict response. A younger patient without cirrhosis and those with a shorter duration of disease appears more likely to respond to treatment (Camps et al., 1993a). Obesity has also been considered an important factor, possibly as a result of reducing the absorption of the drug when injected subcutaneously. There may be an optimal dose of IFN which is likely to vary from one patient to another. Initial low viral load has also been identified as a possible predictor of response. More recent studies report that viral genotype may be a better predictor of response (Bresters, 1994; Hoofnagle, et al., 1993). The production of anti-interferon (anti-IFN) neutralising antibodies may also influence response. Some studies have demonstrated anti-IFN in patients who do not respond or relapse after an initial response (Antonelli et al., 1991; Milella et al., 1993). Neutralising antibodies were not monitored in this study and thus we cannot comment on this point with reference to the partial and non-responders in our study.

All of the above variables may determine treatment outcome. Unfortunately most studies have identified these factors by univariate models without controlling for confounding factors which may influence outcome. As mentioned above, a number of researchers have suggested that patients with type 2 or 3 infection are more likely to respond to treatment than patients with type 1 infection. Although such a response may be entirely related to viral genotype, it could be interdependent on other factors, making it less certain that virus genotype *per se* is an independent predictive factor for response to IFN. For example in Europe, type 3a infection is more common in young individuals and a greater response rate could feasibly be as a result of younger age, and possibly shorter disease duration. In the present study, the two non-responders were both infected with HCV type 1, while the complete and partial responders were infected with types 2 and 3

(Table 5.3). Age did not appear to vary significantly with infecting genotype, therefore we cannot say whether age affected this response. Neither can we comment on disease duration as a predictive factor as this information was unavailable to the study.

The exact relationship of genotype, level of viraemia and efficacy of IFN therapy has yet to be clarified. HCV type 1b appears to replicate to a higher titre in plasma than type 2, and this difference in virus load may influence the response to IFN. Pretreatment levels in our study showed one patient infected with type 2 having a higher titre than those infected with type 1. This patient responded partially to IFN and remained a partial responder one year after treatment cessation (Figure 5.6). Both type 1 patients failed to respond to treatment. Type 3 infected patients had the lowest levels of replicating virus. Two of these patients were complete responders and have sustained that response to date. The third was classed as a partial responder at the end of treatment and has since relapsed with ALT levels returning to pretreatment values. Whether the differences in response to IFN are a result of circulating virus load (which may depend on genotype), remains unclear. There are no standards for quantifying HCV, and differences in methodology make comparison between studies difficult. The development of the quantitative bDNA signal amplification assay (Quantiplex™-HCV RNA) may improve the situation. However, the assay lacks sensitivity in patients with low viral titres (Bresters et al., 1994). Our study agrees with this point as a number of patients were shown to have HCV RNA below the level of detection with the bDNA assay, yet were PCR positive (Table 5.4).

It is important to establish the aims of IFN treatment and to determine if the result of treatment outweighs the side-effects. IFN treatment is accompanied by a number of side-effects, with no way of predicting who will suffer. All patients receiving treatment did suffer an initial 'flu-like' syndrome, common to IFN treatment. This improved or resolved

completely after the first few doses. Fatigue and lethargy were common complaints from all patients throughout treatment. Mild alopecia was also suffered by two female patients. No serious side-effects were recorded.

One of the main aims of treatment should be the prevention of progression of chronic hepatitis to liver cirrhosis and possibly HCC. These complications are usually not seen until 10-20 years after infection. As such we cannot comment on the efficacy of IFN in this respect. A true cure of HCV should aim for complete elimination of HCV. However, as we have discussed, current methodologies for monitoring HCV RNA are still relatively insensitive and a negative PCR result is no guarantee that the virus has indeed been eliminated.

The results of this drug trial confirmed those of earlier studies. An initial response was obtained in 70%, but this decreased to 42% in the follow-up. Although these results may seem disappointing, the proportion of patients responding to therapy justifies the need for frequent injections and the cost of treatment despite the possible side-effects. Although the complex interplay between viral genotype, replication efficacy and IFN sensitivity remains unclear, the emerging evidence implies that genotyping and quantifying virus levels will become an important part of patient assessment and selection for treatment. Interferon is expensive and those patients infected with type 1 may need a different treatment strategy, for example a higher dose or longer treatment period, or a combination of IFN and Ribavirin.

From this study we conclude that HCV is present in the saliva of some of the blood donors enrolled in the drug trial. However, optimisation of methods for sample collection and handling are required before we can truly asertain whether saliva is a suitable alternative to blood for the diagnosis and monitoring of HCV infection.

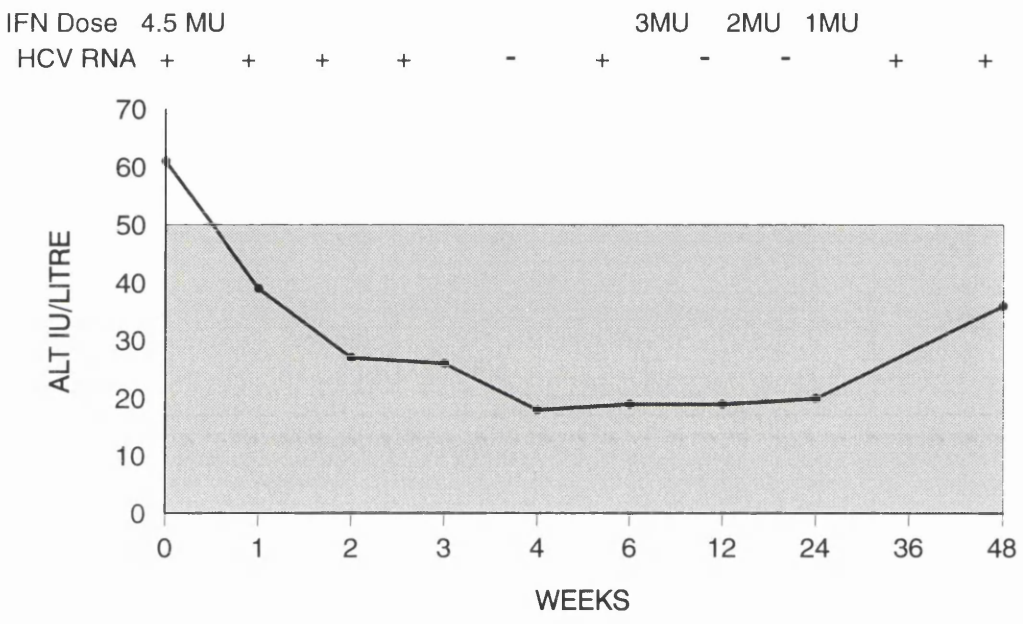
TABLE 5.7

Patient	bDNA levels in Serum[4]	
	Week 0	Week 12
8[1]	11.75	6.76
9[1]	10.77	<3.5
10[2]	4.41	<3.5
11[2]	16.62	<3.5
12[3]	<3.5	<3.5
13[3]	<3.5	<3.5
14[3]	<3.5	<3.5

Effect of interferon on viraemia levels in patients receiving treatment (4.5 MU interferon treatment thrice weekly for 12 weeks).

[1] Non-responders. [2] Partial responders. [3] Complete responders. [4] branched DNA levels x 10^5 HCV RNA equivalents/ml.

FIGURE 5.4

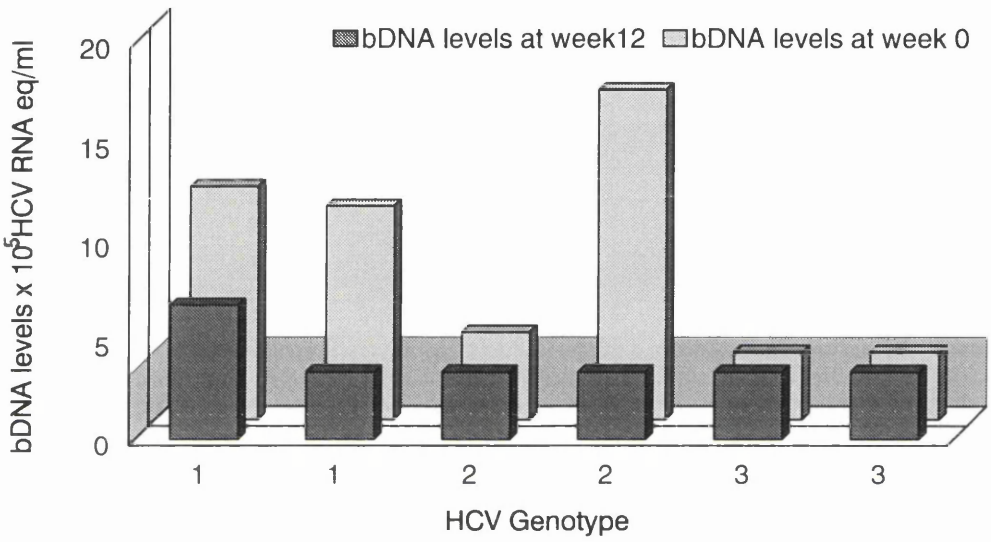


Virological relapse without biochemical relapse in Patient No. 11 after a complete response to IFN had been attained at week 12.

Shaded area shows the range of normal ALT level (50 IU/ litre).

(+) PCR positive. (-) PCR negative.

FIGURE 5.5



Relationship between genotype and the initial viral load of six patients receiving interferon. Genotype information was not available for 1 patient who had an initial viral load below the branched DNA assay cut-off. Dark grey block shows branched DNA assay cut-off at 3.5×10^5 HCV RNA equivalents/ml.

Chapter 6

Optimisation of Specimen Collection and Handling Methods for the Detection of Hepatitis C Virus in Saliva

INTRODUCTION

As explained in earlier chapters, saliva as a diagnostic fluid has many advantages over blood, especially when dealing with blood-borne pathogens. It is non-invasive for the patient eliminating the risk to healthcare workers of needlestick injuries and permits screening of large numbers of people for epidemiological surveys. However, a number of issues stand in the way of full acceptance of saliva testing. Low concentration of target (antigen or antibody), small sample volume and degradation of target by proteolytic activity are general problems associated with saliva testing.

With the emergence of AIDS, the search for a less hazardous specimen has focused on other body fluids such as urine and saliva. Although there have been many publications detailing the use of saliva to diagnose and monitor certain infections, the inconsistent specimens collection procedures that have been employed make comparisons difficult. As a result, the true benefits of saliva testing may have been under-estimated.

With regard to the use of saliva as a diagnostic fluid for HCV, critical analysis suggested that many discrepancies in our earlier attempts to detect HCV RNA in the saliva of HCV seropositive haemophiliacs (Roy et al., in press) and blood donors (Roy et al., 1995) relate to sample handling techniques. This may also explain the discrepancies between papers in the literature. There is concern that specimen processing and storage conditions might influence the stability of the target, and hence the detectability of HCV RNA. Saliva is a complex fluid, with a variety of physiological and pharmacological factors that may influence target concentration or interfere with PCR. As a result, there is a need for careful evaluation of the methods for collecting, handling and storing saliva

specimens prior to PCR analysis, thus maximizing the sensitivity and specificity of the technique.

To investigate these issues, we studied freshly collected saliva samples from patients who were previously shown to be HCV RNA seropositive. The samples were subjected to a number of handling and storage procedures that aimed to simulate conditions routinely encountered in a diagnostic laboratory. The samples were then examined for HCV RNA by PCR.

METHODS AND MATERIALS

a) Study population

Fifty-five intravenous drug users (19 females, 36 males, mean age 31 years, range 19-47 years) attending Ruchill Hospital, Glasgow, were studied. All patients were previously shown to be HCV antibody seropositive on the basis of screening by a second generation enzyme immunoassay (HCV EIA II System, Abbott Laboratories, Chicago), and by confirmation with a recombinant immunoblot system (RIBA 2/3, Ortho Diagnostic Systems, Raritan). Thirty one of these drug users were also HIV antibody positive.

A brief oral examination performed by an infectious diseases physician prior to sample collection indicated healthy oral mucosa in most of the study group, with the exception of 8 (14%) subjects who showed evidence of candidosis. Seven (87.5%) of these 8 patients were also HIV antibody seropositive. With the exception of candidosis, HIV co-infection did not correlate with the oral findings in the drug users ($P< 5\%$) (Table 6.1).

b) Sample collection

Peripheral blood (10ml) was collected by venepuncture in parallel with collection of saliva samples. Blood was transported to Ruchill Regional Virus Laboratory, where the serum was separated, and stored at -20°C until tested.

Whole saliva and oral fluid obtained from the Salivette™ were collected as described earlier (Chapter 2). A third device called the Omnisal™ (Saliva Diagnostic Systems, Inc., Oregon, USA), was also employed to collect oral fluid (Figure 6.1). Subjects were asked to allow saliva to collect in their mouths and the pad of the collector was placed

under their tongue. No chewing or sucking was necessary. Once adequate saliva had been collected, the indicator on the stem of the collector turned blue. At this point, the collection device was removed from the mouth and placed into the transport tube supplied, forcefully pushing the cap downwards until the cap 'snapped' level with the top of the tube. On arrival in the laboratory, the pad was separated from the stem with a series of 'flicks' of the wrist, flicking the tube against the palm of the operator's hand. Once separated, the cap and stem were removed and discarded. The saliva sample could now be separated from the pad by inserting the separator into the tube and pressing down firmly. The eluate was aspirated directly from the separator for testing or storage (Figure 6.2).

c) Sample processing

On arrival in the laboratory, 200µl of each specimen underwent immediate RNA extraction followed by reverse transcription and nested 'hot-start' PCR. A second aliquot (200µl) went through the same procedure after storage overnight at room temperature. This manoeuvre was included to determine the effect of delayed processing on target stability. The residual saliva was further divided into two aliquots. The first was heat-treated (60°C; 15 min) prior to storage at -20°C for one month. This aimed to denature salivary enzymes that may affect target stability or interfere with the PCR procedure. The second (unheated) aliquot was stored immediately at -20°C and subsequently examined for HCV RNA in parallel with the heated specimen after one month of storage. This procedure was to determine the effect of storage on target stability. HCV RNA positive specimens stored at -20°C were re-examined. This was to determine the effect of repeat freeze-thaw cycles on target stability.

d) RNA extraction and reverse transcription

RNA was extracted using the QIAamp blood kit and reverse transcription was carried out as previously described (Chapters 2 and 3).

e) Polymerase chain reaction

For this study 'hot-start' nested PCR was employed to eliminate primer-dimers and non-specific products. This method was described in Chapter 3. Appropriate controls, as discussed in Chapter 2, were included in all runs. HCV negative saliva spiked with HCV RNA positive serum was employed as a positive control to determine any adverse effects of saliva on the PCR procedure.

f) DNA sequencing

DNA was sequenced by the dideoxychain termination method (Sanger et al , 1977), using the Sequenase PCR Product Sequencing Kit (Amersham Life Sciences). All reagents used in the protocol, with the exception of the label and primer, were supplied in the kit. As the expected sequence is G-C rich, 7-deaza-dGTP-containing reaction mixtures were employed to eliminate gel compressions which result when the DNA does not remain fully denatured during electrophoresis. PCR products were reamplified using the same protocol as described for second round PCR to attain sufficient quantities of DNA suitable for sequencing.

i) Radioactive label

The radioactive label used for all the sequencing reactions was deoxyadenosine 5-[α-^{35} S] thiotriphosphate triethylammonium salt (dATP, Sp isomer) (Amersham Life Sciences). The specific activity of the [α-^{35} S] dATP label was >1200Ci/mmol and the concentration was 10mCi/ml.

ii) Enzymatic pretreatment

PCR products were enzymatically pretreated to remove residual single-stranded extraneous DNA and primers, and remaining dNTPs from the PCR mixture which would interfere with the labelling step of the sequencing process. To 0.2 - 0.5 pmol of PCR product, 10 units of Exonuclease I and 2 units of Shrimp Alkaline Phosphatase were added, and the reaction incubated for 15 min at 37°C. Both enzymes were inactivated by heating to 80°C for a further 15 min.

iii) Denaturing reaction

To 0.5 pmol of treated PCR product, 5-10 pmol of primer 211 was added and dH_2O to a final volume of 10µl. Denaturation was carried out at 100°C for 2 -3 min. Samples were immediately placed on ice and allowed to cool for 5 mins. This ice-cold DNA template was used immediately in the annealing reaction.

iv) Annealing reaction

The annealing mixture comprised 10µl DNA template, 2µl 5 x reaction buffer, 1µl 0.1M dithiothreitol, 2µl labelling mix (7.5µM each of 7-deaza-GTP, dCTP and dTTP), 0.5µl (5µCi) [^{35}S] dATP and 2µl of Sequenase DNA polymerase. The contents of

the annealing reaction were mixed well and incubated at room temperature for 2 -3 min. 3.5µl of the labeling reaction mixture were added to the side wall of each of four 1.5ml microcentrifuge tubes labelled A, C, G and T, which contained 2.5µl of each termination mix (A, C, G and T) respectively. The reactions were started by spinning briefly in a benchtop microcentrifuge and the samples were incubated at 37°C for 5 -10 min. Reactions were terminated by the addition of 4µl stop solution and samples were stored at -20°C until required. Samples were heated to 75°C for 2 min prior to the loading of 3µl of each sample onto the sequencing gel.

v) Polyacrylamide sequencing gel

The stock solutions and mixes used in the preparation of the gel are shown in Table 6.2 . The concentration of acrylamide used was 6%. The Macrophor gel sequencing system was used according to the manufacturers instructions (Pharmacia LKB). Wedge-shaped gels were used routinely. To allow for easier handling, gels were bound to the notched glass plate by treatment with Bind-Silane. Treatment of the thermostable plate with Repel-Silane prevented sticking of this plate to the notched glass plate containing the bound gel. After casting of the gels and insertion of the well-forming combs, they were allowed to polymerise for at least one hour at room temperature. Gels were pre-electrophoresed for 30 min at 2000V and 60°C in 1x glycerol tolerant buffer. Sample wells were flushed with running buffer prior to loading of samples, which were applied to the gels using duck-billed tips. Gels were run at 2000V and 60°C until the bromophenol blue band had reached the bottom of the gels. Gels were soaked in gel fixing solution (10% acetic acid and methanol) for 30min prior to drying onto the notched glass plate with a hair drier and then subjected to autoradiography in a light proof box for 8 to 24 hours at room temperature.

g) Analysis of DNA sequencing data

DNA sequence data were compiled using PILEUP from the University of Wisconsin Genetic Computer Group programmes. RFLP analyses were carried out using MAPSORT from this group of programmes. Cleavage of sequence data using RsaI/ HaeIII and HinfI/ MvaI produces a number of cleavage patterns (Figure 6.3). When used together, they can successfully identify all six major genotypes of HCV (Figure 6.4). Further cleavage of genotype 1 with BstU1, or genotypes 2 and 3 with ScrFI can allow the identification of subtypes 1a,1b,2a,2b,3a and 3c (Figure 6.5).

TABLE 6.1

	HCV^+, HIV^- (n = 24)	HCV^+, HIV^+ (n = 31)
Denture wearer		
Yes	6	14
No	18	17
Oral Hygiene		
Good	5	10
Moderate	8	8
Poor	11	13
Oral mucosa		
Healthy	23	24
Candidosis[1]	1	7
Ulceration	0	0
Lichen planus	0	0
Xerostomia		
Yes	10	11
No	14	20

Summary of the oral health of the subjects enrolled in the study with respect to their HIV serostatus.

[1] $P< 5\%$ using X^2

TABLE 6.2

Sequencing gel stock solutions and mixes

20 x Glycerol Tolerant Buffer

Tris base	216g
Taurine	72g
EDTA	4g
dH_2O	to 1litre
Sterilise by autoclaving	

Stock acrylamide 40%

Acrylamide	38g
Methylene-bisacrylamide	2g
dH20	to 100ml

Urea mix

Stock acrylamide	15ml
1X GTB	5ml
Urea	42g
dH2O	to 100ml

10% Ammonium Persulphate (APS)

APS	0.2g
dH_20	2ml
Prepare fresh when required	

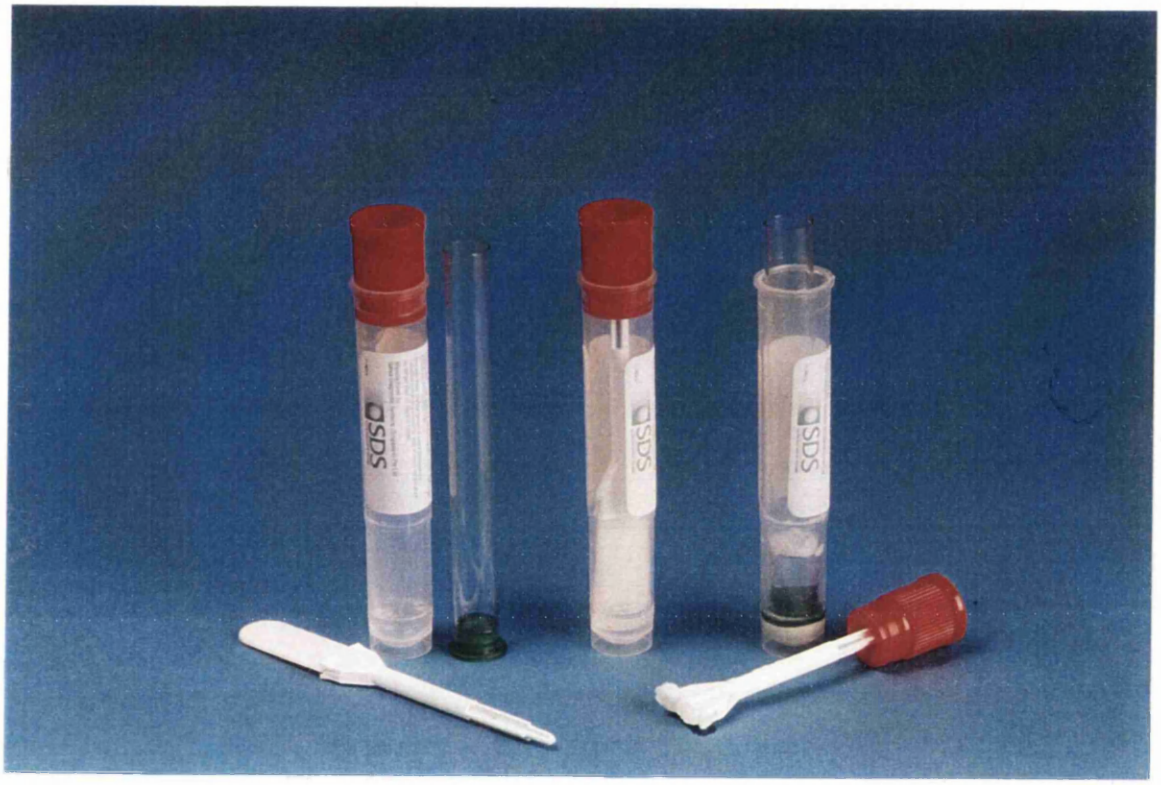
Sequencing gel mix

Urea mix	60ml
10% APS	0.4ml
TEMED	40µl

FIGURE 6.1

The Omnisal™ collection device consisting of sterile saliva collection pad with sample adequacy indicator stem, and transport tube with preservation buffer solution with snap cap. Buffer contains 0.2% sodium azide as a preservative.

FIGURE 6.2



Collection protocol for the Omnisal™. 1) Omnisal collector, separator and tube containing adequate storage buffer. Subject places pad of collection device under tongue taking care not to suck or chew. 2) Once an adequate volume of saliva is collected, the pad is placed in the transport tube. 3) Saliva is obtained by removing the pad from the stem and inserting the separator provided. Saliva can be aspirated for testing or storage.

FIGURE 6.3

1)

a	44	58		# 114/5			9	26
b	102			# 114/5			9	26
c	44	12	46	58	56		9	26
d	44	12	46	114			9	26
e	56		46	114			9	26
f	33	69		114			9	26
g	33	23	46	114			9	26
h	44	12	46	# 117			9	26

2)

A	53	63	41	94
B	53	63	# 44	94
C	53	56	# 142/3	
D	53	198		

Predicted cleavage patterns for digestion with HaeIII / Rsa1 and Hinf1 / Mva 1.

1) Cleavage with HaeIII / Rsa1. 2) Cleavage with Hinf 1 / Mva 1.

Used together with Figure 6.4 to identify HCV genotypes.

represents the position of 1 or 2 bp insertions found in some 5'NCR sequences.

FIGURE 6.4

	A	B	C	D
a b	1		4	5
c d e				2
f g			3	
h		6		

Predicted combinations of RFLP patterns obtained from cleavage with Hae III/ Rsa 1 and Hinf1/ Mva 1, associated with sequences of HCV types 1 to 6.

FIGURE 6.5

1)

1a	209		42
1b	179	30	42

2)

2a	53	15	48	41	94
2b	53	15	183		
	53	15	48	135	

3)

3a	53	15	57		126	
3b	53	15	48	9	32	94

Predicted cleavage patterns for cleavage with Bst U1 or Scr F1.

1) Cleavage with Bst U1 of genotype 1 to identify HCV subtype 1a and 1b.

2) Cleavage with Scr F1of genotype 2 to identify HCV subtypes 2a and 2b.

3) Cleavage with Scr F1 of gentoype 3 to identify HCV subtypes 3a and 3b.

RESULTS

Fifty one complete sets of saliva were obtained from the 55 IVDU. HCV RNA vas detected in the saliva of 27 (52.9%). Table 6.3 gives the oral characteristics of the 51 IVDU with respect to the presence of HCV RNA in their saliva. The two groups were smilar in relation to wearing dentures, oral hygiene, and the health of their oral mucosa. A complaint of oral dryness was, however, significantly more common in those with detectable HCV RNA in their saliva (P< 5%). HCV RNA was present in the saliva with a smilar frequency among patients negative (13 of 23) or positive (14 of 28) for HIV co-infection.

From the total number of positive saliva samples provided, HCV RNA was present in 16 (57%) of the whole saliva samples, 10 (35.7%) of the Omnisal™ specimens and 10 (35.7%) of the Salivette™ specimens (Table 6.4). In only two patients was HCV RNA detected in all three specimen types, and for five patients, in two of three specimen types (Table 6.5). No collection method or device provided a sample that identified all patients with HCV RNA in their saliva.

Of the 16 patients with detectable HCV RNA in their whole saliva specimens, seven (43.8%) were positive following immediate RNA extraction and PCR on the fresh sample within three hours of collection. Four (25%) were positive following overnight incubation at room temperature, six (37.5%) were positive after incubation at 60°C for 15 min and storage at -20°C for 1 month and two (12.5%) were positive after storage at -20°C for 1 month (Table 6.6).

From the 10 patients with detectable HCV RNA in their Omnisal™ specimens, HCV RNA was present in five (50%) of the fresh samples, 6 (60%) of the samples left

overnight at room temperature, three (30%) of the samples heated and stored at -20°C and one (10%) of the samples frozen immediately (Table 6.7).

Of the 10 subjects with detectable HCV RNA in their Salivette™ specimens, five (50%) were positive following immediate RNA extraction, three (30%) were positive after incubation overnight at room temperature, and three (30%) were positive after freezing at -20°C alone (Table 6.8).

Repeat freeze-thaw cycles were performed using the six samples that were HCV RNA positive following storage at -20°C. A second round of freezing and thawing resulted in a loss of detectable HCV RNA in all but one of the samples, which itself became negative following a third round of freezing and thawing (Table 6.9).

Serum from 49 of the 51 patients, collected at the same time as the saliva samples, was available for testing by PCR. Twenty eight patients were HCV RNA seropositive. Of the 23 patients who were HCV RNA seronegative, HCV RNA was detected in the saliva of 10 (43.5%).(Figure 6.6).

Of the 10 patients with HCV RNA positive saliva, but negative serum, 5 (50%) were co-infected with HIV. Oral hygiene was good in three of these subjects, moderate in two and poor in five. In general the oral mucosa was healthy in all but one of the ten subjects, who showed signs of oral candidosis. Six of these ten patients (60%) complained of xerostomia.

Of the 15 patients with HCV RNA positive saliva and serum, 9 (60%) were co-infected with HIV. Oral hygiene was good in three subjects, moderate in six and poor in six. The oral mucosa was healthy in all but two subjects who showed signs of oral candidosis (Table 6.10).

Eleven of the 28 (39.3%) IVDU with HCV RNA positive serum samples were infected with HCV genotype 1a, four (14.3%) were infected with genotype 2a, six (21.4%) were infected with genotype 3a and one (4%) with genotype 6. Six of the 27 (22.2%) IVDU with HCV RNA positive saliva samples were infected with genotype 1a, 14 (51.8%) with genotype 2a, three (11.1%) with 3a and one (4%) with genotype 6 (Table 6.11). Genotypes are described using the proposed consensus nomenclature (Simmonds et al., 1994).

Comparison of genotypes isolated from the 15 IVDU with HCV RNA positive serum and saliva identified five patients with different genotypes in their serum and saliva: genotypes 3a and 2a in four cases and 2a and 1a in one. Two serum samples were untypable due to the small quantity of PCR product obtained even after further reamplification. Of the 10 IVDU with HCV RNA present in their saliva but not in the paired serum sample, serum collected prior to the study and which had been stored at Ruchill Hospital was available from seven for genotyping. Comparison of genotypes from these seven patients identified three with different genotypes in the paired serum and saliva samples: genotypes 3a and 2a in all three cases. Two serum samples were untypable. The raw data for this study is shown in appendices 5 and 6.

TABLE 6.3

	HCV RNA Positive Saliva (n = 27)	HCV RNA Negative Saliva (n = 24)
HIV Positive	14	14
Negative	13	10
Denture wearer		
Yes	10	8
No	17	16
Oral Hygiene		
Good	6	9
Moderate	8	7
Poor	13	8
Oral mucosa		
Healthy	24	21
Candidosis	3	3
Ulceration	0	0
Lichen Planus	0	0
Xerostomia		
Yes[1]	15	5
No	12	19

Oral characteristics of patients with respect to the presence of HCV RNA in their saliva.

[1] $P < 5\%$ using Chi square.

TABLE 6.4

	Whole saliva	Omnisal™	Salivette™
HCV^+, HIV^-	8	6	6
HCV^+, HIV^+	8	4	4
Total	16	10	10

Summary of total PCR positive saliva samples collected by the three different devices from the HCV and HIV co-infected intravenous drug users.

TABLE 6.5

Patient	Whole saliva	Omnisal™	Salivette™
5	+	+	-
6	+	+	-
7[I]	+	-	-
9[I]	+	-	-
13[I]	+	-	-
15	-	+	-
18[I]	+	-	-
19[I]	+	-	-
20	-	-	+
21[I]	+	+	+
22	+	+	+
23	-	-	+
32	-	+	-
33[I]	-	-	+
35[I]	-	+	+
39	+	-	-
40	+	+	-
41	-	-	+
42[I]	+	-	-
43	+	-	-
48	-	-	+
49[I]	-	+	-
50[I]		-	+
51[I]	-	-	+
52	+	+	-
54	+	-	-
55[I]	+	-	-

Summary of PCR on the positive saliva samples collected from HCV seropositive intravenous drug user.

[I] HIV co-infected intravenous drug users.

(+) Positive PCR result. (-) Negative PCR result.

TABLE 6.6

Patient	To	O/N	60°C	-20°C
5	-	+	+	-
6	-	-	+	-
7[1]	-	-	+	+
9[1]	+	-	-	-
13[1]	-	-	-	+
18[1]	+	-	-	-
19[1]	+	-	-	-
21[1]	-	-	+	-
22	-	-	+	-
39	+	-	+	-
40	-	+	-	-
42[1]	+	-	-	-
43	+	-	-	-
52	+	-	-	-
54	-	+	-	-
55[1]	-	+	-	-

Summary of PCR on whole saliva samples.

[1] HIV co-infected intravenous drug user. (+) Positive PCR result.

(-) Negative PCR result. (To) Samples processed immediately upon arrival in laboratory. (O/N) Samples processed following incubation overnight at room temperature. (60°C) Samples processed following incubation at 60°C for 15 min and storage for 1 month at -20°C. (-20°C) Samples frozen immediately upon arrival at laboratory and processed 1 month later.

TABLE 6.7

Patient	To	O/N	60°C	-20°C
5	-	+	-	-
6	+	+	+	-
15	+	+	-	-
21[1]	-	-	+	+
22	-	-	+	-
32	-	+	-	-
35[1]	+	+	-	-
40	+	-	-	-
49[1]	-	+	-	-
52	+	-	-	-

Summary of PCR on samples collected using the Omnisal™ device.

[1] HIV co-infected intravenous drug user. (+) Positive PCR result.

(-) Negative PCR result. (To) Samples processed immediately upon arrival in laboratory. (O/N) Samples processed following incubation overnight at room temperature. (60°C) Samples processed following incubation at 60°C for 15 min and storage for 1 month at -20°C. (-20°C) Samples frozen immediately upon arrival at laboratory and processed 1 month later.

TABLE 6.8

Patient	To	O/N	60°C	-20°C
20	+	-	-	-
21[1]	-	-	-	+
22	-	-	-	+
23	-	-	-	+
33[1]	+	-	-	-
35[1]	+	+	-	-
41	+	-	-	-
48	-	+	-	-
50[1]	+	-	-	-
51[1]	-	+	-	-

Summary of PCR on samples collected using the Salivette™ device.

[1] HIV co-infected intravenous drug user. (+) Positive PCR result.

(-) Negative PCR result. (To) Samples processed immediately upon arrival in laboratory. (O/N) Samples processed following incubation overnight at room temperature. (60°C) Samples processed following incubation at 60°C for 15 min and storage for 1 month at -20°C. (-20°C) Samples frozen immediately upon arrival at laboratory and processed 1 month later.

TABLE 6.9

Patient	Freeeze-thaw Cycles		
	1	2	3
7[1]	+	-	-
13[1]	+	-	-
21[2]	+	-	-
21[3]	+	+	-
22[3]	+	-	-
23[3]	+	-	-

Effect of repeat freeze-thaw cycles on the stability of HCV RNA.

[1] Whole Saliva specimens. [2] Omnisal ™ sample. [3] Salivette™ samples.

(+) Positive PCR result. (-) Negative PCR result.

TABLE 6.10

	Serum HCV RNA Positive (n = 15)	Serum HCV RNA Negative (n = 10)
HIV Positive	9	5
Negative	6	5
Denture wearer		
Yes	6	4
No	9	6
Oral Hygiene		
Good	3	3
Moderate	6	2
Poor	6	5
Oral mucosa		
Healthy	13	9
Candidosis	2	1
Ulceration	0	0
Lichen planus	0	0
Xerostomia		
Yes	7	6
No	8	4

Oral characteristics of patients with detectable HCV RNA in their saliva with respect to HCV serostatus.

TABLE 6.11

Genotype	Serum (n=28)	Saliva (n=27)	Total
1a	11	6	18
2a	4	14	17
3a	6	3	9
6	1	1	2
Untypable	8	3	11

Viral genotypes of HCV in the blood and saliva of IVDU. Genotypes described using the proposed consensus nomenclature (Simmonds et al., 1994).

DISCUSSION

Handling and storage conditions of blood and saliva could alter the stability of HCV RNA and hence the ability of PCR to detect it. Direct detection of HCV RNA confirms that in many a persistent, productive infection exists. Thus, it is important to establish standard conditions in the handling and storage of the samples to maximise sensitivity and specificity of PCR since it has now become a routine assay for HCV.

In general, expectorated whole saliva is most commonly used for diagnostic purposes, either unstimulated, collected by passive drooling, or stimulated using masticatory stimulation (eg rubber band) or gustatory stimulation (eg 2% citric acid applied to the tongue). When volume measurement is not needed, a variety of collection devices such as the Salivette™ and Omnisal™ can be used. Indeed, when used correctly both claim to yield adequate volume. The question remains over which method of collection is appropriate for clinical investigation, though this is likely to depend on the aim of the investigation. With regard to HCV, we compared the collection of whole saliva with oral fluid obtained using two commercially available devices (Salivette™ and Omnisal™).

From the 55 drug users sampled, inadequate sample volume was a problem for only four individuals. Three subjects provided insufficient whole saliva, three insufficient Omnisal™ samples and three provided an inadequate volume from the Salivette™. Only one subject provided insufficient samples from all collection devices. This patient did, however, complain of xerostomia.

All three methods were easy to use, and acceptable to all subjects. However, some volunteers found the provision of a whole saliva sample somewhat distasteful. Both the

Omnisal™ and the Salivette™ devices, when used correctly, provided sufficient sample volume. The incorporation of an indicator in the stem of the Omnisal™ gave visual proof of sufficient volume. The only disadvantage of using the Omnisal™ in this study relates to the preservation buffer, which while ensuring sample stability may also have served to dilute the sample and, as a consequence, the target. This would have to be taken into account if the Omnisal™ were to be used for quantitative studies.

It has been reported that specimens collected by swab or into a pot produce better results than other collection methods (Mortimer and Parry, 1988). Our results are in accordance with this finding. However, although the whole saliva samples provided more HCV RNA positive samples in total, the Omnisal™ and Salivette™ together identified an additional 11 HCV RNA positive specimens. In our earlier studies this pattern was less clear cut, yet there is agreement that whole saliva and oral fluid samples do not always identify the same subjects with HCV RNA positive saliva. There are several possible reasons. It is likely that whole saliva would contain more degradative factors, whereas oral fluid may be a 'purer' specimen. Alternatively, HCV may be associated with cell debris or even with the material of the collection device and as a result, would not be detected in the Omnisal™ and Salivette™ specimens or perhaps not released from the devices. Both these points will be expanded upon later.

Manipulation and storage conditions of clinical specimens are known to influence significantly the stability of viral nucleic acids and, consequently, their rate of detection (Busch et al., 1992; Wang, et al ., 1992b; Cuypers et al., 1992). Most previous studies have evaluated residual sera left over after routine serological testing has been completed. By this stage they have undergone varying periods of storage at room temperature and in a refrigerator as well as several freeze-thaw cycles. It is generally agreed that sera should be

frozen as soon as possible after collection and that only short periods of room temperature storage and minimal freeze thaw cycles are allowable if the sample is to maintain stability. With regard to saliva the picture remains unclear, as appropriate handling conditions have yet to be ascertained. Our study, although confirming the effect of freeze thaw cycles on reducing target positivity, has not established a sample handling protocol that is completely satisfactory.

Of the 16 subjects with positive whole saliva samples, seven were identified following testing of their samples within three hours of collection (T_o). A further four were positive following incubation of a second aliquot of sample overnight at room temperature, while seven subjects previously shown to have a positive sample at T_o , became negative after overnight storage. Heating a further aliquot of each sample, followed by a period of storage at -20°C, identified four more subjects with positive specimens but missed 10 of those previously identified. Only one subject with a positive aliquot at T_o , and one with a positive aliquot after incubation overnight at room temperature remained positive after heating and storage at -20°C. Only one further subject, previously unidentified, and one identified using a different method of sample handling, were HCV RNA positive following freezing of an aliquot of the sample within three hours of collection, and without any further manipulations, for one month (Table 6.6)

For analysis of the specimens collected by the Omnisal™, leaving the samples overnight at room temperature identified more subjects with HCV RNA positive samples than testing at T_o . However, once again, only three subjects were identified by both these methods of handling. A further two positive samples were identified using the aliquots that were heated and stored at -20°C. Simply freezing aliquots did not identify any new subjects

with HCV RNA positive oral fluid. Only one sample was positive using this method of handling and this subject had been previously identified (Table 6.7).

From the 10 subjects with HCV RNA detected in their Salivette™ specimens, half were identified using the samples tested at T_0. Only two more were picked up following testing of their samples that had been left overnight at room temperature. Unlike the whole saliva specimens, heating the sample prior to storage at -20°C did not maintain the target. Simply freezing the sample identified a further three subjects, unidentified by previous sample handling methods (Table 6.8).

In conclusion, no single method of specimen collection and handling identified all subjects with HCV RNA positive saliva. Indeed, it seems that certain methods of handling are more suited to particular types of specimen. For example, a greater number of HCV RNA positive whole saliva specimens were identified by immediate testing within three hours of collection, while the Salivette™ samples left overnight at room temperature identified more HCV RNA positive saliva specimens. HCV stability was only maintained in the longterm in whole saliva samples following heating at 60°C. However, there was agreement for all specimens that simply storing the samples at -20°C was not suitable for maintaining detectable amounts of HCV RNA (Figure 6.7). This agrees with observations in our earlier study (Chapter 4), regarding the pellet and supernate samples collected from HCV-seropositive blood donors. It should be noted that while the Omnisal™ and Salivette™ specimens (both oral fluid) identified a similar total number of subjects with HCV RNA in their saliva, a concordant result was only shown with three subjects (Table 6.5).

How can these discrepancies be explained?. Perhaps whole saliva, being a far cruder specimen, contains more degradative enzymes. With storage overnight at room

temperature HCV is gradually broken down and thus fewer positive samples are detected. However, this would not explain why some samples shown to be negative at T_0 became positive following incubation overnight. Perhaps whole saliva also contains factors capable of inhibiting PCR, but which break down overnight at room temperature, thus allowing identification of more positive samples, as a result of less interference. More likely there may be a combination of virus and inhibitor breakdown. Certainly heating is known to prevent salivary inhibition of PCR (Ochert et al., 1994), and it is also capable of destroying degradatory enzymes. Heating the samples and then freezing them was possibly a mistake. Although heating may prevent degradation of HCV by enzymes and prevent PCR inhibition, it may not prevent spontaneous degradation of HCV which appears to occur with storage at -20°C. Oral fluid collected by the Omnisal™ and Salivette™ devices may contain less degradative enzymes and/or PCR inhibitory factors. Thus we may have expected to see a greater number of subjects identified as having HCV RNA positive saliva using oral fluid specimens. In the current study, HCV RNA negative saliva spiked with HCV RNA positive serum was employed as a positive control to determine if any inhibition of PCR by an unknown salivary component was occurring, but none was observed. It is unclear how and where HCV appears in the oral cavity, and oral fluid collected by the Omnisal™ and Salivette™ may not be appropriate for HCV diagnosis

HCV sequences present in the saliva and serum collected were amplified with primers corresponding to sites in the 5'NCR that are well conserved between all known HCV variants. Sequencing of the PCR product allowed approximately 190bp in the centre of the region to be compared with equivalent published sequences. A number of sites are polymorphic within most genotypes while other substitutions are strongly associated with particular virus types (Smith et al ., 1995) (Figure 6.8). Comparisons of the sequenced

PCR products indicated that PCR on saliva was specific. All saliva positive samples that could be sequenced contained isolates that were indeed HCV.

Genotyping was carried out by computerised RFLP analysis as opposed to 'hands on' RFLP analysis because of the low quantity of PCR product generated. 0.2-0.5pmol of PCR product is required for sequencing by this method. The manufacturers recommend reamplification to achieve sufficient template. However, reamplification often generates nonspecific bands capable of interfering with the seqencing protocol. Eleven samples could not be sequenced (eight serum and three saliva samples). With the exception of two serum samples, these were samples collected from those patients with either HCV RNA positive serum or saliva, but not both. This is suggestive of low level viraemia resulting in low quantities of PCR product. For these difficult samples, gel purification and concentration would be required.

In Scotland, 50% of anti-HCV positive blood donors are infected with HCV type 1, most of which carry type 1a sequences. Forty per cent are infected with type 3, all of which correspond to type 3a. Only 10% are infected with type 2, in particular with 2b, although 2a and 2c can also be found. The results obtained with the serum samples in the present study were in agreement with this distribution, though all of the IVDU infected with type 2 carried 2a sequences. Genotyping the HCV RNA positive saliva samples provided an alternative picture. Genotype 2, and in particular type 2a, was four times more common in saliva compared to serum ($P < 0.1\%$), while the relative frequencies of 1a and 3a were halved. The predominance of type 2 in saliva is of interest, because patients infected with type 2 show a lower level of liver function abnomalities which may indicate infection of non-hepatic areas such as salivary glands (P. Simmonds, personal communication). The predominance of type 2a sequences in particular is, however,

unusual. Information to date regarding genotype distribution has been obtained from studies of blood donors, and the distribution may well differ within the drug-abusing community. The high prevalence of type 2a in these specimens requires confirmation by sequencing of another region, such as the core, that shows distinct sequences specific to viral type. Interestingly, type 6 was identified in both the serum and saliva samples from one IVDU. This genotype is particularly associated with Hong Kong and the Far East (Davidson et al ., 1995).

The definitive method for deducing virus genotype is nucleotide sequence analysis. However, sequencing can only be employed if an individual is viraemic. Furthermore, sequencing cannot readily detect more than one virus type within an individual. The detection of type specific antibodies in a sample is possible using a commercially available enzyme immunoassay (Murex HCV Serotyping 1-6 Assay, Murex, UK). This assay uses synthetic peptides, representing the variable antigenic regions from the NS4 of HCV types 1-6 and is capable of identifying multiple infections. It is likely that those IVDU with different genotypes isolated from their serum and saliva specimens have a mixed infection, with a different predominant genotype in the different fluids. This can be confirmed by serotyping.

In those patients with the same genotype identified in both serum and saliva, comparisons of the nucleotide sequence were made to identify any distinct sequence variability associated with the isolates from these two body fluids. None was found. As anticipated, all patients were infected by genotypes showing 100% homology between serum and saliva isolates. However, if any difference in serum and saliva isolates exists, it is unlikely to occur in this region (5'NCR) currently used widely for genotyping and which was used in this study. The 5' NCR appears to adopt a secondary structure suggestive of an

internal ribosome entry site and overall, this untranslated region shows much less variability between HCV types than any of the coding regions (Simmonds, 1994). Sequence changes associated with particular genotypes or the presence of other polymorphic sites appear not to disturb the secondary structure (Smith et al ., 1995).

Several mechanisms may account for the ability of HCV to penetrate saliva. Virus could replicate in haematopoetic cells and/or epithelial cells frequently present in secretions. A number of studies have confirmed virus replication in peripheral blood mononuclear cells (PBMC) (Chen et al., 1995; Müller et al., 1993; Qian et al., 1992; Wang et al., 1992c; Young et al., 1993). An obvious factor that could increase the serum PBMC content of saliva and hence its HCV concentration is inflammation or bleeding of the oral tissues. All of the subjects underwent a dental examination to exclude oral mucosal lesions as a source of contamination of the saliva. Most of the subjects had healthy oral mucosa with the exception of eight who showed signs of oral candidosis (Table 6.1). This did not, however, correlate with the presence of HCV RNA in saliva (Table 6.3). Finally, a gradient may exist between blood and secretions in which virus is passively transferred into secretions depending on the concentration of virus in the blood. More often than not, it has been agreed that transudation of fluid containing HCV from the general circulation into saliva occurs, rather than active replication at the site of secretion (Liou et al., 1992; Loiseau et al., 1994; Mariette et al., 1995; Wang et al., 1992a).

Our data are not in accordance with this theory, and provide evidence that HCV may reach saliva via routes other than the gingival crevicular fluid. First, serum transudate containing virus enters the mouth primarily by the gingival crevices and thus the concentration of HCV in saliva will be influenced by the degree of periodontal disease and the number of teeth present. Of the 27 subjects with HCV RNA positive saliva, 12 (43%)

had less than half their adult teeth. Indeed four patients had no teeth at all (Table 6.12). Interestingly, these four patients had different genotypes isolated from their saliva and serum samples. Secondly, for a gradient to exist between blood and saliva, virus must be present in blood. Of the 25 subjects with HCV RNA positive saliva, whose blood was available for testing, 10 (40%) were HCV RNA seronegative (Figure 6.6).

We have also shown previously among blood donors that the amount of virus in blood does not correlate with its presence or absence in saliva (Roy et al., 1995). The most obvious question regarding this group of 10 HCV seronegative patients is whether the positive saliva results are not merely false positives. In each assay, four controls (one positive and three negative) were introduced, all of which had to provide satisfactory results before the results obtained from the specimens were accepted. While HCV isolates have shown extensive sequence diversity, the 5'NCR of HCV has been shown to be highly conserved among different isolates. Therefore, in choosing primers based on the nucleotide sequence of the 5' NCR we attempted to maximise and identify isolates that might have nucleotide sequence differences outside this region. However, as mentioned before, the presence of a DNA band whose length corresponds to the length of the expected PCR product does not provide a positive identification. Bearing this in mind, sequencing has been employed, not only to identify isolate genotype but also to confirm the amplified product as being a true HCV positive result and that the HCV RNA detected in saliva is not an artefact.

It is interesting to note that six (66%) of the seronegative patients with HCV RNA positive saliva complained of xerostomia (Table 6.13). Xerostomia was the only significant ($P<5\%$) oral characteristic that differentiated patients with or without HCV in their saliva,

although, no particular HCV genotype was associated with xerostomia. Is this an indication of salivary gland dysfunction as a result of active HCV infection ?. Certainly, replication of HCV in salivary glands has been observed (Takamatsu et al., 1992).However, this result should be interpreted with some caution. The normal unstimulated whole salivary flow rate is about 0.3ml/ min, but the range of normality is extremely large. Dry mouth can be experienced by patients not only with a salivary flow rate of zero, but also when flow rate is reduced to 40-50% of the normal for that patient. Thus, without baseline information on a patient's salivary flow rate or unless the mouth is completely dry, it is almost impossible to be certain that the flow rate is less than normal (Dawes, 1993). Indeed, asking patients to qualify dry mouth would provide wide variations of what they consider 'a dry mouth'.

The clinical value of PCR for the detection of HCV RNA does depend on the sensitivity and reproducibility of the applied procedure. It is possible that during the time between specimen collection, freezing, storage and subsequent RNA extraction, certain enzymes present in saliva (eg proteases and lipases) might disrupt the intact virus and allow ribonucleases to attack and degrade the genome, thereby destroying the template for PCR. This point cannot be stressed enough and has important consequences for the interpretation of previous studies in which investigators failed to indicate whether they processed and stored samples under uniform conditions. The observed variations in HCV RNA results may, therefore, have occurred *in vitro* rather than *in vivo.*

Unfortunately, this study has failed to identify an ideal collection and handling protocol appropriate to using saliva as a diagnostic fluid for HCV. Whole saliva processed within 3 hours of collection provided the best results but this is impractical in a routine clinical laboratory. We therefore conclude that collection of saliva for detection of HCV RNA is not yet a suitable alternative to blood as a diagnostic fluid for this infection

TABLE 6.12

Patient	No of Teeth	Dentures	Oral Mucosa
5	0	Yes	Healthy
6	26	No	Healthy
7	0	Yes	Candidosis
9	20	No	Candidosis
13	23	No	Healthy
15	24	Yes	Healthy
18	6	Yes	Healthy
19	17	No	Healthy
20	24	No	Healthy
21	14	Yes	Healthy
22	26	No	Healthy
23	26	No	Healthy
32	10	Yes	Healthy
33	25	No	Healthy
35	0	Yes	Healthy
39	20	No	Healthy
40	13	No	Healthy
41	19	No	Healthy
42	9	Yes	Healthy
43	18	Yes	Healthy
48	22	No	Healthy
49	0	No	Healthy
50	7	Yes	Candidosis
51	6	No	Healthy
52	11	No	Healthy
54	22	No	Healthy
55	25	No	Healthy

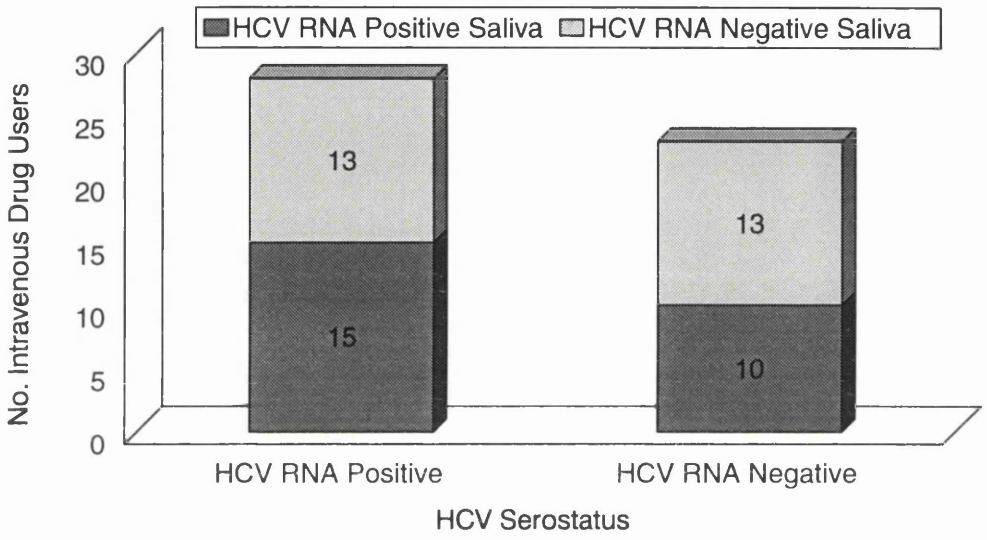
Oral characteristics of the 27 intravenous drug users with detectable HCV RNA in their saliva.

TABLE 6.13

Patient	No of Teeth	Oral Mucosa	Xerostomia
5	0	Healthy	No
20	24	Healthy	Yes
21	14	Healthy	No
22	26	Healthy	Yes
33	25	Healthy	Yes
39	20	Healthy	Yes
42	9	Healthy	Yes
48	22	Healthy	No
50	7	Candidosis	Yes
51	6	Healthy	No

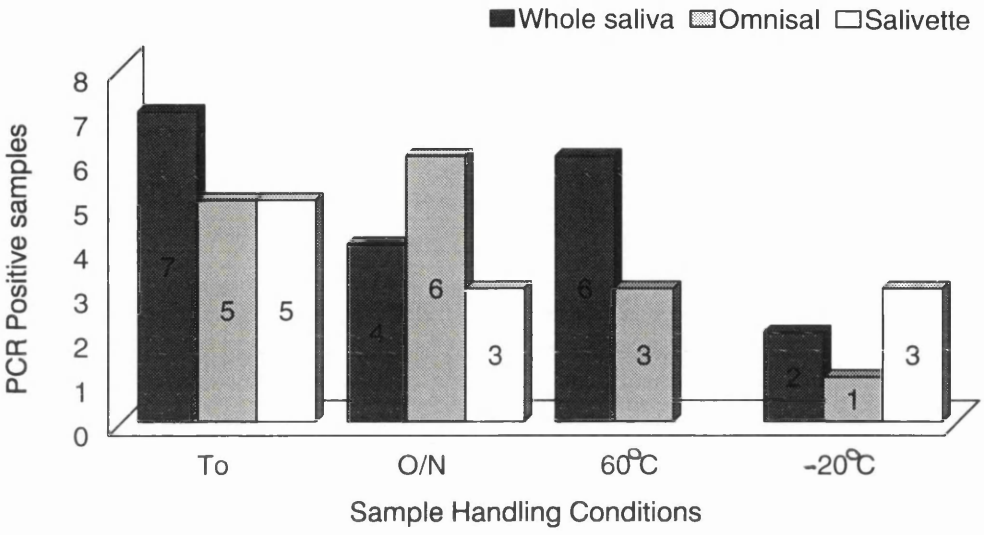
Oral characteristics of the intravenous drug users with HCV RNA positive saliva but negative serum

FIGURE 6.6



Summary of total HCV RNA positive saliva samples from the intravenous drug users with regard to HCV serostatus.

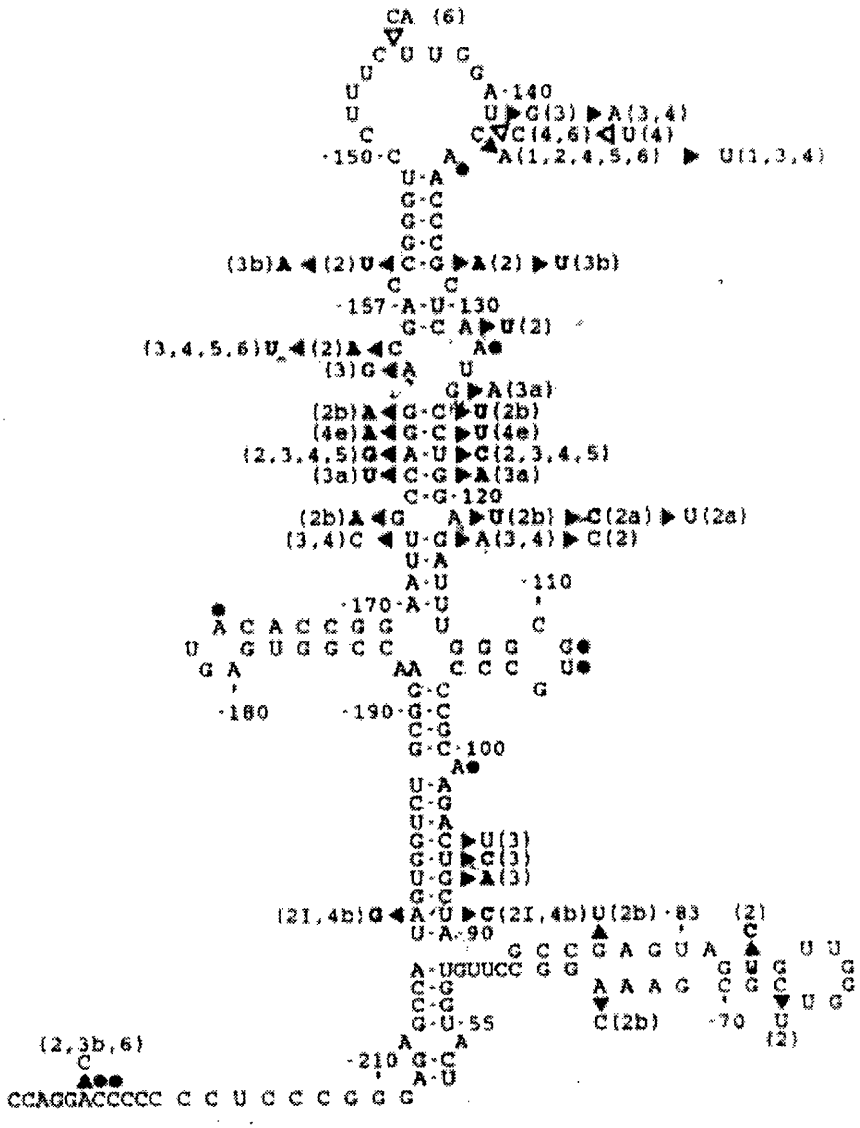
FIGURE 6.7



Effect of sample handling conditions on the PCR result with regard to collection device.

(To) Sample tested within three hours of collection. (O/N) Sample tested following incubation at room temperature overnight. (60°C) Sample tested following incubation at 60°C for 15 min. (-20°C) Sample tested following storage at -20°C for one month.

FIGURE 6.8



Association between 5'NCR polymorphisms and virus genotype within the region sequenced. Substitutions relative to the HCV-1 sequence are indicated by ▲ with the virus genotypes associated with that substitution in parentheses. Polymorphic sites are indicated by • and sites of insertion by ∇. Adapted from Smith et al., 1995

Chapter 7

General Discussion

Following HCV infection there is characteristically a prolonged period before seroconversion occurs. This limits the usefulness of currently available serological assays. Following determination of the HCV genomic sequence, RT-PCR has been used to detect viral RNA during the acute phase of illness, and in the absence of anti-HCV antibodies. The presence of viral RNA also appears to be a more accurate predictor of infectivity.

A major part of the studies presented in this thesis sought to determine the appropriateness of PCR as a routine diagnostic tool for HCV infection, and in particular its suitability with regard to use of saliva as an alternative specimen to blood. PCR is a complex technique that entails numerous manipulations. Many attempts have been made to simplify the process and thereby facilitate the application of this method in a routine clinical laboratory . However, assay simplification alone would not be sufficient to allow PCR to become a routine diagnostic tool for any organism, because a more serious problem must be solved. Owing to the exquisite sensitivity of PCR, a single molecule of target DNA can be amplified to the point of detectability. Inadvertent transfer of minute amounts of previously amplified DNA corresponding to the region of interest results in the phenomenon of PCR end product contamination dubbed "carryover". The avoidance of such contamination is critical to generating consistent, correct results. The low molecular weight amplified DNA is easily aerosolised by opening sealed test tubes or by pipetting and this can result in the contamination of all reagents, instruments and personnel. During the course of the study, the guidelines of Kwok and Higushi (1989) were adhered to strictly. Furthermore, all equipment was UV sterilised prior to each use. Every effort was made to prevent the physical transfer of DNA, but even when following these precautions, low level contamination may occasionally occur. Appropriate negative controls were included with

every run to rule out reagent contamination, although it must be realised that these cannot control for random contamination of individual samples attributed to aerosolised DNA.

The only way to be certain that carry-over contamination will not be a problem is to chemically or enzymatically inactivate the amplified DNA. There is a commercially available kit for HCV diagnosis with serum samples, based on the principles of restriction modification and excision repair systems of cells (Amplicor PCR Diagnostic, Roche Diagnostic Systems). This method allows the selective destruction of previously amplified DNA, while DNA from clinical samples is unaffected. The protocol involves substitution of deoxynucleoside 5' uridine triphosphate (dUTP) for deoxynucleoside 5'thymidine triphosphate (dTTP) in the master mix, thus ensuring that all new amplified DNA will contain uracil rather than thymidine. Any carry-over to the next reaction is destroyed by incorporation of the enzyme uracil-N-glycosylase (UNG) which catalyses the excision of uracil from single-stranded and double-stranded DNA thus destroying the contaminating templates. UNG is inactivated at most temperatures used in PCR cycling so newly synthesised amplicons will not be destroyed prior to detection. There are several further advantages to employing the Amplicor diagnostic kit, in particular the provision of all components for every aspect of the assay (sample processing, amplification, and detection) in a bid to ensure reproducibility, sensitivity, and specificity.

The issue of false negative results is an equally important problem as false positive results. Certain specimens, as well as specimen handling procedures and instruments, can contain or introduce inhibitory factors which reduce the efficiency of the PCR process (Busch et al., 1992: Cuypers et al.,1992). With regard to sera, it is now routine to collect blood samples in non-heparinized tubes, as heparin has been shown to inhibit PCR (Wang et al., 1992b). The effect of storage or freezing and thawing is less

clear, but researchers advise the aliquoting and freezing of samples as soon as possible after collection. Saliva has been shown previously to interfere PCR, but the unknown inhibitory factor appears to be removed by appropriate specimen preparation methods (Ochert et al., 1994). This situation was controlled for in the current studies by mixing a known HCV-positive serum sample with a known negative saliva sample to produce a 'spiked' specimen prior to cDNA synthesis. Identification of the correct product was illustrated by competent cDNA synthesis and PCR amplification. This in turn was indicative of the absence of inhibitors.

False negative results can also occur following the use of inappropriate primers. The selection of primers used in a particular system is based upon a number of criteria. The specificity includes both inclusivity and exclusivity. Thus, it is necessary to be able to amplify all isolates of the target of choice, while avoiding the amplification of closely related micro-organisms. The 5'NCR of the HCV is the most highly conserved region of the virus genome and as a result this region is often used as the target for detection of HCV RNA by RT-PCR. However, genotype-specific sequence polymorphisms exist throughout the 5'NCR. Consequently it is possible that a given primer pair will not support amplification due to minor differences within the primer binding site which prevent annealing or elongation by the polymerase.

Recently, Smith et al (1995) investigated the variation in the 5'NCR of HCV isolates by comparing 314 5'NCR sequences of viruses of genotypes 1 to 6. Through identifying the polymorphic sites within the 5'NCR, improved oligonucleotide primers were proposed that would be equally efficient in detecting RNA of different virus genotypes. The primers employed during the course of the present study were based on the HCV-1 sequence. This would of course result in different efficiencies of detection with

genotypes other than type 1. As such, we cannot rule out the existence of false negative results through primer mismatches when interpreting the results of our studies. However, genotypes 1, 2, 3 and 6 were amplified and sequenced from our study population.

HCV RNA was found in the saliva of all three subject groups at varying rates (34-52.9%). However, the studies also confirmed that saliva does not provide a reliable, consistent and stable specimen for detection of HCV RNA. In Chapter Six it is shown clearly that specimen type and handling conditions can affect the success of detection of HCV RNA by PCR. As shown previously for serum, multiple freeze-thaw cycles and prolonged periods of storage of saliva specimens reduce the chances of amplifying HCV RNA. Both these points were alluded to in Chapter Four and confirmed in Chapter Six, when the sample handling protocols employed were studied in more detail.

This study also failed to identify a saliva collection and handling protocol that would optimise the detection of HCV RNA by PCR. Saliva is a complex biological fluid, and much remains to be learned about its normal constituents. Factors are likely to be found that inhibit PCR, although in the studies reported here this did not appear to be a major problem. It is more likely that the inconsistent nature of PCR for detecting HCV in saliva is related to the effect of saliva on the stability of HCV RNA. RNA is susceptible to in situ degeneration at ambient temperatures and this may well occur to a lesser extent at -20°C over a period of time.

The appropriateness of PCR as a diagnostic tool for HCV in a routine clinical laboratory remains ambiguous. There is no doubt that it is sensitive and specific in confirming infected blood and blood products for the purposes of the Blood Transfusion Service. However, in this situation only those donors providing a RIBA-3 or indeterminate result are screened further by PCR. With the commercial production of the UNG system by

Roche (Amplicor PCR Diagnostics for HCV), the Regional Virus Laboratory, Ruchill Hospital, began to employ PCR as a diagnostic assay. However, the National Health Service only provides funds for anti-HCV antibody assays and as a result PCR is restricted to those patients being considered for IFN treatment, or patients suffering acute hepatitis who provide a RIBA-3 indeterminate specimen. The problems of false positive and negative results remain too great to allow HCV diagnosis by PCR alone in a low risk population using blood, let alone saliva. In its present configuration, PCR cannot replace serological assays where broad based screening is required.

Even when all of the aforementioned problems associated with PCR are taken into consideration, this study has shown that HCV RNA can be detected in the saliva of a substantial number of individuals. Indeed, consideration of the problems involved suggests that this work may even have underestimated the true prevalence of HCV RNA in saliva. This poses the question of which route HCV takes to enter the mouth. As mentioned earlier, HCV may penetrate saliva by several mechanisms including serum transudate and migration of infected PBMC.

A recent study by Chen et al.(1995) failed to detect HCV RNA in saliva obtained from the submaxillary glands. These workers also failed to detect negative stranded HCV RNA, which represents replicative intermediates of HCV. They concluded that HCV seldom replicated in the cells originating from the oral cavity and that salivary HCV RNA was a result of migration of HCV-infected mononuclear cells. This was in accordance with the general assumption that HCV enters saliva by serum transudation through the gingival crevices.

During the course of the present study, evidence has accumulated which questions this theory. First, HCV RNA was detected in both the supernate and pellet of some of the

samples collected from the haemophiliacs and blood donors. However, it is conceded that the protocol employed only determined the presence of positive stranded HCV RNA, and that this does not indicate HCV replication within the mouth. In addition, information was not obtained on the dental or periodontal status of each patient and thus an external source of HCV cannot be ruled out in these studies.

Secondly, for a gradient to exist between blood and saliva to allow passive transfer of virus, HCV must be present in a greater concentration in blood. The lack of such a gradient was shown clearly in the patients enrolled in the IFN drug trial (Chapter 5). Thus, HCV RNA was detected in the saliva of some patients for whom serum HCV RNA was positive by PCR but whose HCV RNA level as measured by bDNA was below the assay cut-off, but was undetectable in others whose serum HCV RNA levels were ten fold greater than the assay cut-off. This challenges the assumption that salivary HCV RNA is always derived from serum. Likewise, the presence of HCV RNA in the saliva of 10 HCV RNA negative IVDU suggests that transudation from serum is not the only mechanism. These patients may have had serum HCV RNA levels below the limit of detection with our assay (100 genomes/ml)(Dow et al., 1993) . Unfortunately, no sera were obtained in parallel with the saliva samples taken from the haemophiliacs and blood donors in the initial prevalence study (Chapter 4), as the source of virus was not being considered at that stage. In the study monitoring interferon treatment by saliva testing (Chapter 5), HCV RNA seronegative samples were obtained only from the patients receiving therapy and therefore it was assumed that absence of virus from the mouth was a result of the treatment.

Thirdly, if serum transudate, containing virus, enters the mouth by the gingival crevices the concentration of HCV in saliva is likely to be influenced by the number of teeth and the degree of periodontal disease. Periodontal lesions are inflammatory,thus

causing exudation of serum into saliva and increasing the shedding of potentially infected mononuclear cells into the salivary pool. This source of salivary contamination was taken into consideration in the final study. Severe periodontal disease is common in intravenous drug users, but in the present study showed no correlation with the presence of HCV RNA in saliva. Similarly, salivary HCV RNA was detected in almost 50% of the patients who had less than half their adult teeth, and in some who were edentulous.

It is possible that in addition to transudation of fluid containing virus from the general circulation, there is also active replication at the site of salivary secretion in some individuals. More research is needed to identify the cells (if any) that harbour the virus. Techniques such as in situ PCR are available which would enable the assessment of gland biopsies for the presence of replicative intermediates.

Xerostomia is a prominent clinical sign associated with Sjogren's Syndrome (SS). This is an autoimmune disease which is characterised by lymphocytic infiltration and destruction of the major and minor salivary glands, leading to varying degrees of salivary hypofunction. The factors that trigger such a focal immune response remain unknown but viral infection has often been suggested (Fox, ., 1994). Recent evidence indicates that HCV should be considered as a candidate trigger in this disease.

HCV may be associated with a number of symptoms and signs involving salivary glands, including complaints of oral dryness. Reports of HCV associated with SS have been published in the recent literature (Aceti et al., 1992; Almasio et al., 1992; Haddad et al., 1992; Mariette et al., 1993). HCV infection appears to induce a complex immune response that may partly account for its propensity to cause persistent infections and autoimmune phenomena such as low titre autoantibodies. Such phenomena may explain salivary gland lymphocytic infiltration resulting in a form of sialadenitis distinct from SS.

At present, no consensus criteria exist for the diagnosis of SS and this is causing general confusion in the identification of causative factors of the disease. Evidence does, however, suggest histologically different patterns of lymphocytic infiltration associated with HCV salivary gland dysfunction and different patterns of autoantibodies.

Dry mouth is a common symptom and has a variety of causes. Symptomatic complaints of xerostomia or quantification of salivary output alone are not sufficient to establish a diagnosis of salivary gland dysfunction. Salivary secretion may be decreased by a number of factors, the most common of which is medication. Sedatives, opiates, antipsychotics, antidepressants, antihistamines and diuretics are the classes of drugs most often associated with xerostomia. All of the IVDU enrolled in the present study had been prescribed, or were abusing, at least one drug capable of reducing salivary output.

When first identified, HCV was believed to be acquired almost exclusively through percutaneous exposure. This early view evolved from the fact that the virus was first recognised in the context of transfusion studies. Subsequent investigations focused on an analysis of risk factors for viral acquisition within the community. Risk factors associated with the illness included previous receipt of blood or blood products and parenteral drug use.

In approximately 40-50% of cases of HCV infection, a history of either transfusion or injecting drug use is inapparent and the precise route of infection cannot be defined. This raises the possibility of less obvious modes of transmission. Virological studies of body fluids other than blood is of importance when evaluating possible non-parenteral routes of transmission. It is possible that infection may occur through non-sexual household contact as a result of contamination of household items such as eating utensils or toothbrushes, or through the small spillages of blood and body fluids that may

occur in the domestic situation. Transmission of HCV by body fluids is feasible and more likely to occur if exposure is repetitive and extends over a long period of time. It is also possible that transmission may only occur at times of peak viral replication and may also be dependent on viral genotype. Although such transmission may occur with low efficiency, the total proportion of new infections resulting may be significant.

Early epidemiological surveys appeared to support the concept of sexual transmission of HCV. In most cases the early studies were poorly designed with insufficient consideration being given to the exclusion of other risk factors or the general background rate of HCV infection in the study population. Furthermore, most studies were too small to give meaningful results. Infectivity is likely to be low and large numbers would be necessary to demonstrate significant results.

With the development of increasingly sensitive assays for HCV, it was anticipated that sexual transmission would be clearly established. The presence of HCV in saliva and semen has been reported (Chen et al., 1995; Couzigou et al., 1993; Liou et al., 1992; Mariette et al., 1995; Numato et al., 1993; Punchammer-Stockl et al., 1994; Takamatsu et al., 1991; Thieme et al., 1992; Wang et al., 1991; 1992a; Young et al., 1993). However, it has been difficult to prove true sexual transmission and to identify the body fluid involved. Current evidence suggests that HCV can be transmitted sexually, although the importance of the route appears low.

The presence of HCV in saliva is of concern in dental practice, where the degree of exposure of dental staff to the saliva and blood of patients is high. However, studies to date indicate that the prevalence of HCV infection among health care workers is low. Only two studies have examined anti-HCV serology in groups of dental surgeons. An American study reported that dentists, especially oral surgeons, run an occupational risk of acquiring

HCV infection (Klein et al ., 1991). In contrast, a smaller study of 94 dentists in Wales showed no such risk (Herbert et al., 1992). It is likely that the discrepancy in these studies results from differences in the patient populations treated by the two groups of dentists particularly with respect to intravenous drug users. It is argued that in general, the risk to dentists is dependent on the HCV prevalence in the patient population. However, this risk may increase substantially during oral surgery (Klein et al ., 1991) and possibly even less severe trauma causing intraoral bleeding. It is difficult in such situations to distinguish transmission through saliva or blood. To argue that transmission of HCV through saliva is inefficient as a result of the low prevalence of detectable HCV in saliva may be inaccurate. Early reports of HCV detection in saliva may have underestimated the true levels. The work carried out in Chapter 6 shows clearly the difficulties of testing saliva for HCV RNA and we cannot ignore these when interpreting the earlier prevalence studies. It is likely that HCV is more widespread in saliva than acknowledged.

Although there have been no documented cases of HCV transmission through dental handpieces, the potential risk of HCV infection to patients receiving dental treatment must also be considered. Some types of reusable dental equipment might be responsible for cross-contamination if patient material were lodged in internal mechanisms that are difficult to disinfect. Air driven high speed handpieces and prophylaxis angles take up and expel patient material and could potentially provide a vehicle for cross-infection (Lewis et al., 1992; Lewis and Arens, 1995). Depending on individual dental practice protocols, handpieces and attachments may only be externally disinfected between patients, rather than autoclaved. Infectious material present in the internal areas of the equipment may not be reached by such procedures. In addition, water repellent lubricants used inside the equipment coating the internal mechanisms may prevent contact of steam or

disinfectant solutions with infectious material. The probability of encountering this type of contamination will again depend on the HCV prevalence in the practice population and on the sterilisation and disinfection practices of the dentist. The British Dental Association (BDA) Advisory Committee recommends that all instruments and equipment must be cleaned and sterilised after use. Autoclaving at 134-138°C for 3 min is recommended for all instruments. Chemical disinfection must only be used for those items that cannot be sterilised by conventional methods.

A further source of infection to both the dentist and patient may be through direct contamination of surfaces in dental practices (Lewis et al., 1992). Consistent enviromental contamination through exhausts that leave the dental suction units has been shown in dental surgeries after treatment of anti-HCV and HCV RNA positive patients (Piazza et al., 1995). Environmental contamination through the dispersion of aerosols and/ or droplets of saliva and blood seems inevitable and unfortunately there are no definite data on HCV survival in the enviroment. The presence of HCV RNA on contaminated surfaces for at least 20 hours has been recorded (Piazza et al., 1995). Impervious disposable coverings can be used to cover surfaces liable to contamination and can be changed between patients. The BDA Advisory Committee also recommends that between sessions all work surfaces should be thoroughly cleaned and decontaminated with detergent or a suitable virucidal disinfectant. The lack of data on the ability of many chemical agents to inactivate HIV, HBV and HCV makes recommendation of disinfectants difficult. A fresh solution of sodium hypochlorite is recommended for general surface disinfection and freshly activated 2% alkaline glutaraldehyde for non-corrosive disinfection of metals.

The infectivity of saliva from HCV seropositive persons for sex partners, bite victims, and health care workers is a subject of continuing interest to epidemiologists,

clinicians and virologists. The presence of HCV in saliva has been shown regularly but studies have failed to provide conclusive evidence that saliva may act as a vehicle for transmission. Comparisons can be made with HIV and the lack of transmission from sexual, occupational or casual contact with saliva and / or the oral cavity. Much research has been carried out to determine the infectivity of saliva from HIV-seropositive patients. However, epidemiological data and intensive family studies have failed to provide any conclusive data that saliva can act as a source of infection (Archibald and Cole, 1990, Qureshi et al., 1995).

The virtual absence of oral transmission of HIV, together with reports of antiviral activity in the saliva of healthy and infected individuals suggests the presence of a factor in saliva that can inhibit transmission of the virus. It is believed that there are at least two mechanisms present in saliva that may provide antiviral activity in vivo. One of these is the physical entrapment of virus particles. Macromolecules such as mucin and other glycoproteins in saliva tend to form aggregates by binding to other salivary molecules including microbial and viral proteins. Therefore, virus can be trapped into these complexes and cleared from the mouth by swallowing or expectoration (Bergey et al., 1994; Yeh et al., 1992). Specific and non-specific aggregation could also be a major host defence mechanism against HCV infection in the mouth. With regard to HIV, the other mechanism of salivary inhibition is a serine protease inhibitor known as secretory leukocyte protease inhibitor (SLPI). Evidence suggests that SLPI interacts with a host cell molecule(s) other than the HIV receptor, CD4 (McNeely et al., 1995; Aldous, 1995). Saliva from which SLPI has been removed loses most of its ability to inhibit HIV. It is possible that a similar mechanism could exist for HCV. Saliva is known to be one of the major host defence mechanisms yet our understanding of its antiviral actions is limited.

There would appear to be several possible mechanism of antiviral activity but further studies are required to establish the role of saliva in preventing the transmission of HCV.

Given that the estimated general prevalence of HCV infection in the general population is between 0.4 and 2.2% and that there is currently neither an effective vaccine or treatment, it is clear that HCV represents a major clinical and public health problem (Viral Hepatitis Prevention Board, 1995). The control of HCV is a priority because of the frequency of occurrence of infection, its high chronicity rate, the common and serious nature of its sequelae and the high associated direct and indirect costs.

The prevalence of HCV infection in different groups of blood donors varies and because of the current policy of self-exclusion of at-risk donors, these rates cannot be considered to reflect the prevalence in the community, which is possibly several fold higher. The development of cheaper, simpler, but highly sensitive and specific tests would allow the collection of information on the distribution of HCV infection and spread through apparently non-parenteral routes. The use of saliva for broad-based community screening programmes would be advantageous. However, for the determination of HCV RNA, saliva is not appropriate. Future research should concentrate on detection of HCV salivary antibodies. The modification of commercially available HCV antibody testing kits has enabled successful detection of salivary antibodies (Piacentini et al., 1993; Thieme et al., 1992). Confirmation of HCV infection by a saliva analysis would necessitate a testing algorithm similar to that with blood, namely initial detection with a screening ELISA, followed by an alternative ELISA and a supplemental assay. Such a procedure would require the availability of a range of screening and supplemental tests suitable for testing saliva.

Research to determine the mechanisms and efficiency of transmission of infection through apparently non-parenteral routes is also needed. The presence of HCV in body fluids other than blood indicates their potential as an infectious fluid, yet little is known about the dynamics of such transmission. Research along these lines is necessary to support future development and refinement of strategies to control HCV infection and associated diseases.

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APPENDIX 1

Some of the work presented in this thesis has already been accepted for publication. The relevant references are as follows:

ROY, K.M., BAGG, J., BIRD, G.L.A., SPENCE, E., FOLLETT, E.A., MILLS, P.R. and LAU, J.Y.N. Serological and salivary markers compared with biochemical markers in the monitoring of interferon treatment for hepatitis C virus infection. J. Med. Virol., 47, 429-434.

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APPENDIX 2

Patient	Age	Sex	Liver Function Test			Risk Factors					Genotype[6]	HCV RNA	
			ALT[3]	AST[4]	Alk. Phosp.[5]	Transfusion	IV Drug	Ear Pierced	Tattoo	Unknown		Serum	Saliva
1[1]	27	F	32	25	316	+	-	-	-	-	3	+	+
2[1]	38	M	49	/	/	+	-	+	-	-	3	+	-
3[1]	37	F	133	55	173	+	-	+	-	-	1	+	-
4[1]	30	M	166	53	189	-	+	+	+	-	1	+	-
5[1]	26	M	41	/	/	-	-	+	+	-	3	+	-
6[1]	46	M	166	53	189	/	/	/	/	/	3	+	+
7[1]	32	F	76	65	150	-	+	-	-	-	3	+	+
8[2]	39	M	178	77	157	-	-	-	-	+	1	+	-
9[2]	37	F	220	126	252	+	-	+	-	-	1	+	-
10[2]	42	M	66	37	211	+	+	+	-	-	3	+	-
11[2]	47	M	61	36	172	+	-	-	+	-	2	+	-
12[2]	52	F	166	86	198	+	-	-	-	-	2	+	-
13[2]	43	F	45	30	54	+	-	-	-	-	/	+	-
14[2]	31	F	64	39	224	-	+	+	+	-	3	+	-

Demographic characteristics of treatment and observation patients at enrollment on interferon drug trial.

[1] Observation patients. [2] Non-responders to interferon. [3] Normal ALT range 10-50 IU/litre. [4] Normal AST range 10-35 IU/litre.

[5] Normal Alkaline Phosphatase range 70-260 IU/litre. [6] Genotyping by RFLP.

APPENDIX 3

Patient	Age	Sex	Genotype[4]	Liver Function Tests			bDNA[5]	Response
				ALT[1]	AST[2]	Alk. Phosph.[3]		
8	39	M	1	178	77	157	11.75	Non
9	37	F	1	220	126	252	10.77	Non
10	42	M	3	66	37	211	4.41	Partial
11	47	M	2	61	36	172	16.62	Partial
12	52	F	2	166	86	198	<3.5	Complete
13	43	F	/	45	30	54	<3.5	Complete
14	31	F	3	64	39	224	<3.5	Complete

Pretreatment patient characteristics according to response to interferon treatment.

[1] Normal ALT range 10-50 IU/litre. [2] Normal AST range 10-35 IU/litre. [3] Normal Alkaline Phosphatase range 70-260 IU/litre.

[4] Genotyping by RFLP. [5] branched DNA levels x 10^5 HCV RNA equivalents/ml.

APPENDIX 4

Patient	Week 0			Week 12			Genotype[2]	Response
	bDNA[1]	HCV RNA[1]		bDNA	HCV RNA			
		Serum	Saliva		Serum	Saliva		
1	<3.5	+	+	<3.5	+	+	3	Observation
2	49.46	+	-	4.28	-	-	3	Observation
3	<3.5	+	-	4.47	+	-	1	Observation
4	12.47	+	-	10.12	+	-	1	Observation
5	30.48	+	-	3.69	+	+	3	Observation
6	29.47	+	+	41.47	+	+	3	Observation
7	26.25	+	+	34.57	+	+	3	Observation
8	11.75	+	-	6.76	+	-	1	Non
9	10.77	+	-	<3.5	+	-	1	Non
10	4.41	+	-	<3.5	+	-	3	Partial
11	16	+	-	<3.5	+	-	2	Partial
12	62	+	-	<3.5	-	-	2	Complete
13	<3.5	+	-	<3.5	-	-	/	Complete
14	<3.5	-	-	<3.5	-	-	3	Complete

Level of HCV viraemia in serum in relation to the presence of HCV RNA in serum and saliva by PCR, the genotype and response to treatment.

[1] branched DNA levels x 10^5 HCV RNA equivalents/ml. [2] Genotyping by RFLP.

APPENDIX 5

Patient	Age	Sex	HIV	No. Teeth	Denture Wearer	Oral Hygiene	Oral Mucosa	Xerostomia	HCV RNA		Genotype		Serotype[4]
									Serum	Saliva	Serum	Saliva	
1	27	M	-	13	No	Poor	Healthy	No	-	[1]	/	/	/
2	27	M	+	19	No	Poor	Healthy	No	-	[1]	/	/	/
3	34	M	+	21	No	Poor	Healthy	No	-	-	/	/	/
4	26	F	+	26	No	Good	Healthy	No	-	-	/	/	/
5	34	F	-	0	Yes	Good	Healthy	No	-	+	3a[2]	2a	
6	27	M	-	26	No	Moderate	Healthy	No	+	+	2a	2a	UNT
7	42	M	+	0	Yes	Moderate	Candidosis	No	+	+	2a	1a	
8	25	F	+	21	Yes	Poor	Candidosis	Yes	-	[1]	/	/	/
9	31	M	+	20	No	Poor	Candidosis	No	+	+	3a	2a	
10	31	M	-	26	No	Good	Healthy	No	+	-	UNT	/	2
11	29	M	+	0	Yes	Good	Healthy	No	-	-	/	/	/
12	34	M	+	13	No	Poor	Healthy	No	+	-	1a	/	
13	26	F	+	23	No	Good	Healthy	No	+	+	3a	3a	
14	27	M	-	14	No	Poor	Healthy	No	+	-	UNT	/	
15	29	F	-	24	Yes	Moderate	Healthy	Yes	+	+	1a	1a	
16	28	M	-	15	Yes	Poor	Healthy	No	+	-	UNT	/	3
17	30	F	-	18	No	Good	Healthy	No	-	-	/	/	/
18	31	M	+	6	Yes	Poor	Healthy	No	+	+	3a	2a	
19	30	M	+	17	No	Moderate	Healthy	Yes	+	+	3a	3a	
20	42	M	-	24	No	Poor	Healthy	Yes	-	+	3a[2]	2a	

(Table Continued)

Patient	Age	Sex	HIV	No. Teeth	Denture Wearer	Oral Hygiene	Oral Mucosa	Xerostomia	HCV RNA		Genotype		Serotype[4]
									Serum	Saliva	Serum	Saliva	
21	29	M	+	14	Yes	Good	Healthy	No	-	+	2a[2]	2a	
22	27	M	-	26	No	Good	Healthy	Yes	-	+	/	2a	1
23	27	M	-	26	No	Poor	Healthy	Yes	[3]	+	/	1a	
24	31	F	+	28	No	Good	Healthy	No	+	-	2a	/	
25	41	M	+	17	Yes	Moderate	Healthy	Yes	-	-	/	/	/
26	25	F	+	26	No	Good	Healthy	No	+	-	1a	/	
27	33	F	+	23	No	Poor	Healthy	No	-	-	/	/	/
28	31	M	-	26	No	Good	Healthy	No	+	-	1a	/	
29	46	M	+	9	Yes	Poor	Candidosis	Yes	+	-	1a	/	
30	38	M	+	0	Yes	Good	Healthy	No	+	-	UNT	/	UNT
31	27	M	+	28	No	Moderate	Healthy	No	+	-	1a	/	
32	25	F	+	10	Yes	Good	Healthy	Yes	+	+	3a	2a	
33	28	M	+	25	No	Moderate	Healthy	Yes	-	+	[3]	UNT	/
34	25	F	-	15	Yes	Moderate	Healthy	Yes	-	-	/	/	/
35	31	M	+	0	Yes	Good	Healthy	No	+	+	1a	1a	
36	41	F	+	0	Yes	Good	Healthy	Yes	-	-	/	/	/
37	38	M	+	0	Yes	Moderate	Candidosis	No	-	[1]	/	/	/
38	34	M	+	20	No	Poor	Candidosis	No	+	-	1a	/	
39	24	M	-	20	No	Poor	Healthy	Yes	-	+	2a[2]	2a	1
40	35	F	-	13	No	Poor	Healthy	No	+	+	6	6	UNT

(Table Continued)

Patient	Age	Sex	HIV	No. Teeth	Denture Wearer	Oral Hygiene	Oral Mucosa	Xerostomia	HCV RNA		Genotype		Serotype[4]
									Serum	Saliva	Serum	Saliva	
41	19	F	-	19	No	Moderate	Healthy	Yes	+	+	1a	1a	UNT
42	28	F	+	9	Yes	Moderate	Healthy	Yes	-	+	UNT[2]	2a	
43	27	M	-	18	Yes	Moderate	Healthy	No	+	+	UNT	3a	1
44	27	M	-	22	No	Moderate	Healthy	Yes	-	-	/	/	/
45	25	M	-	13	No	Poor	Candidosis	No	-	-	/	/	/
46	33	M	-	12	Yes	Moderate	Healthy	No	-	-	/	/	/
47	28	F	-	23	No	Moderate	Healthy	No	+	-	2a	/	
48	31	F	-	22	No	Poor	Healthy	No	-	+	UNT[2]	UNT	1
49	27	M	+	0	No	/	Healthy	Yes	+	+	UNT	2a	
50	47	M	+	7	Yes	Poor	Candidosis	Yes	-	+	[3]	2a	
51	39	F	+	6	No	Poor	Healthy	/	-	+	3a[2]	2a	
52	41	M	-	11	No	Poor	Healthy	Yes	[3]	+	/	UNT	
53	30	F	+	21	No	Moderate	Healthy	No	+	-	1a	/	
54	38	M	-	22	No	Poor	Healthy	Yes	+	+	3a	2a	3
55	29	M	+	25	No	Poor	Healthy	Yes	+	+	1a	1a	

Summary of oral characteristics, PCR and genotyping results of IVDU.
[1] Patient provided an incomplete set of saliva samples. [2] Genotyped using previously stored sample from HIV and Hepatitis Laboratory, Ruchill Hospital. [3] No sample available. [4] Serotyped using Murex HCV Serotyping 1-6 Assay. (UNT) untypable.

APPENDIX 6

Patient	HIV	Serum	Whole Saliva				Omnisal™				Salivette™			
			To	O/N	60°C	-20°C	To	O/N	60°C	-20°C	To	O/N	60°C	-20°C
1	-	-	[1]	-	-	[1]	-	-	-	-	+	-	-	-
2	+	-	-	+	-	-	-	-	-	[1]	-	-	-	-
3	+	-	-	-	-	-	-	-	-	-	-	-	-	-
4	+	-	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-		+	+		-	+	-	-	-	-	-	-
6	-	+	-	-	+	-	+	+	+	-	-	-	-	-
7	+	+	-	-	+	+	-	-	-	-	-	-	-	-
8	+	-	[1]	[1]	[1]	[1]	-	-	[1]	[1]	-	-	[1]	[1]
9	+	+	+	-	-	-	-	-	-	-	-	-	-	-
10	-	+	-	-	-	-	-	-	-	-	-	-	-	-
11	+	-	-	-	-	-	-	-	-	-	-	-	-	-
12	+	+	-	-	-	-	-	-	-	-	-	-	-	-
13	+	+	-	-	-	+	-	-	-	-	-	-	-	-
14	-	+	-	-	-	-	-	-	-	-	-	-	-	-
15	-	+	-	-	-	-	+	+	-	-	-	-	-	-
16	-	+	-	-	-	-	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18	+	+	+	-	-	-	-	-	-	-	-	-	-	-
19	+	+	+	-	-	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-	-	+	-	-	-

(Table Continued)

Patient	HIV	Serum	Whole Saliva				Omnisal™				Salivette™			
			To	O/N	60°C	-20°C	To	O/N	60°C	-20°C	To	O/N	60°C	-20°C
21	+	-	-	-	+	-	-	-	+	+	-	-	-	+
22	-	-	-	-	+	-	-	-	+	-	-	-	-	+
23	-	[3]	-	-	-	-	-	-	-	-	-	-	-	+
24	+	+	-	-	-	-	-	-	-	-	-	-	-	-
25	+	-	-	-	-	-	-	-	-	-	-	-	-	-
26	+	+	-	-	-	-	-	-	-	-	-	-	-	-
27	+	-	-	-	-	-	-	-	-	-	-	-	-	-
28	-	+	-	-	-	-	-	-	-	-	-	-	-	-
29	+	+	-	-	-	-	-	-	-	-	-	-	-	-
30	+	+	-	-	-	-	-	-	-	-	-	-	-	-
31	+	+	-	-	-	-	-	-	-	-	-	-	-	-
32	+	+	-	-	-	-	-	+	-	-	-	-	-	-
33	+	-	-	-	-	-	-	-	-	-	+	-	-	-
34	-	-	-	-	-	-	-	-	-	-	-	-	-	-
35	+	+	-	-	-	-	+	+	-	-	+	+	-	-
36	+	-	-	-	-	-	-	-	-	-	-	-	-	-
37	+	-	[1]	-	-	-	[1]	-	-	-	[1]	-	-	-
38	+	+	-	-	-	-	-	-	-	-	-	-	-	-
39	-	-	+	-	+	-	-	-	-	-	-	-	-	-
40	-	+	-	+	-	-	+	-	-	-	-	-	-	-

(Table Continued)

Patient	HIV	Serum	Whole Saliva				Omnisal™				Salivette™			
			To	O/N	60°C	-20°C	To	O/N	60°C	-20°C	To	O/N	60°C	-20°C
41	-	+	-	-	-	-	-	-	-	-	+	-	-	-
42	+	-	+	-	-	-	-	-	-	-	-	-	-	-
43	-	+	+	-	-	-	-	-	-	-	-	-	-	-
44	-	-	-	-	-	-	-	-	-	-	-	-	-	-
45	-	-	-	-	-	-	-	-	-	-	-	-	-	-
46	-	-	-	-	-	-	-	-	-	-	-	-	-	-
47	-	+	-	-	-	-	-	-	-	-	-	-	-	-
48	-	-	-	-	-	-	-	-	-	-	-	+	-	-
49	+	+	-	-	-	-	-	+	-	-	-	-	-	-
50	+	-	-	-	-	-	-	-	-	-	+	-	-	-
51	+	-	-	-	-	-	-	-	-	-	-	+	-	-
52	-	[3]	+	-	-	-	+	-	-	-	-	-	-	-
53	+	+	-	-	-	-	-	-	-	-	-	-	-	-
54	-	+	-	+	-	-	-	-	-	-	-	-	-	-
55	+	+	-	+	-	-	-	-	-	-	-	-	-	-

Summary of PCR on serum and saliva samples.

[1] Insufficient sample volume. (+) Positive PCR result. (-) Negative PCR result. (To) Samples processed immediately upon arrival in laboratory. (O/N) Samples processed following incubation overnight at room temperature. (60°C) Samples processed following incubation at 60°C for 15 min and storage for 1 month at -20°C. (-20°C) Samples frozen immediately upon arrival at laboratory and processed 1 month later.