Epidemiology, Molecular Characterisation and Tropism of the Hepatitis G Virus / GBV-C

by

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Table of Contents

Figures and Tables

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Abstract

Epidemiology, Molecular Characterisation and Tropism of the Hepatitis G Virus / GBV-C

The hepatitis G virus and GBV-C are recently discovered variants of the same virus belonging to the family *Flavivirus* (HGV/GBV-C). Although initially thought to be a hepatitis virus, it has been shown to have no association with liver disease. No work has been performed on the prevalence or molecular characteristics of HGV/GBV-C in southern Africa. In addition, although it is clear that the liver is not the primary site of replication, there is no data on the sites of HGV/GBV-C replication in normal subjects. Thus, this study aimed to assess the prevalence of HGV/GBV-C carriage in the urban and rural adult Black communities of the Western and Eastern Cape Provinces of South Africa, and compare it to the prevalence of serological markers of the hepatitis viruses A-E. In addition, this study aimed to assess the molecular features of South African HGV/GBV-C isolates and demonstrate the organs where viral replication was present.

The mean prevalences of antibodies to hepatitis A IgG, hepatitis B surface antigen and antibodies to hepatitis B surface antigen were 98%, 4.3% and 61.1% respectively. The mean prevalence of antibodies to hepatitis C was 1.8%. No significant differences in prevalence were shown between the urban and rural regions for these viruses. The mean anti-hepatitis E prevalence varied from

5.8% to 19.1% in the different regions. Those living in mud houses without access to chlorinated tap water had a significantly higher prevalence of anti-hepatitis E. No anti-hepatitis D positive samples were isolated. The overall prevalence of HGV/GBV-C was 26.9%, with rural communities having a significantly lower prevalence than urban communities. A significant relationship was observed between HGV/GBV-C infection with the use of illicit drugs, female gender, younger age and past blood transfusions.

Phylogenetic analysis demonstrated a novel fourth South African HGV/GBV-C genotype, distinct from the previously described genotypes 1-3. In addition, certain isolates showed a major deletion in the highly conserved 5' non-coding region of HGV/GBV-C. Analysis of 23 tissue biopsies from infected cadavers suggested that the spleen and bone marrow were the primary sites of HGV/GBV-C replication.

List of Abbreviations

A	Adenine
AIDS	Acquired immune deficiency syndrome
ATP	Adenosine triphosphate
bp	Base pair (s)
C	Cytosine
°C	Degrees Celsius
conc	Concentration
DepC	Diethyl pyrocarbonate
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
dUTP	Deoxyuridine triphosphate
E. coli	Escherichia coli
G	Guanine
g	Gram(s)
g	Gravitational acceleration
GBV-C	GB virus C

GSH	Groote Schuur Hospital
HIV	Human Immunodeficiency virus
HAV	Hepatitis A virus
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HDV	Hepatitis D virus (Delta agent)
HEV	Hepatitis E virus
HGV	Hepatitis G virus
IHC	Immunohistochemistry
IPTG	Isopropyl -β-D-thiogalactosidase
IRES	Internal ribosomal entry site
kb	Kilobase
L	Litre
M	Molar
mg	Milligram(s)
$MgCl_2$	Magnesium chloride
ml	Millilitre(s)
mM	Millimolar
NaCl	Sodium chloride
$NaCl0_4$	Sodium perchlorate

NaOH	Sodium hydroxide
5'NCR	5'-non coding region
nm	Nanometer(s)
OD	Optical density
OPD	Out-patients department
ORF	Open reading frame
PCR	Polymerase chain reaction
pmol	Picomole
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Reverse transcription
SDS	Sodium dodecyl sulphate
SS	Strand-specific
SSC	Standard sodium citrate
T	Thymine
U	unit(s)
UCT	University of Cape Town
µl	Microlitre(s)
UV	Ultra violet

V	Volts
v/v	Volume per volume
w/v	Weight per volume
X-gal	5-bromo-4-chloro-3-indoylyl-β-galactoside
YT	Yeast-Tryptone

Preface

The history of the hepatitis G virus and GB virus C is integrally linked to liver disease as a consequence of the clinical settings within which they were isolated, as well as the global investigations that followed their isolation within the disciplines of hepatology and virology.

The final chapter of this thesis contains data that joins other international researchers who argue that these viruses have at best, a tenuous link to the liver and liver pathology. However, as they have their origins firmly within the world of hepatovirology, the data on the hepatitis G virus and GB virus C is preceded by an assessment of the prevalences of hepatitis A-E in the same communities.

A literature review relating to GB virus C and the hepatitis G virus is found in chapters 2, 3 and 4 of this thesis. In keeping with the work to be presented, the review is divided by chapter into the following components:

- General introduction and epidemiology;
- Molecular characterisation; and
- Tropism of the hepatitis G virus and GB virus C.

CHAPTER 1

Background prevalence of hepatitis A, B, C, D and E in the Western- and Eastern Cape Provinces of South Africa

1.1 Summary

The hepatitis viruses isolated to date are hepatitis A, B, C, D and E. There is no data on the prevalences of these viruses in the Western and Eastern Cape Provinces of South Africa. Thus, this study aimed to assess exposure to hepatitis A, B, C and E in adult Black South Africans, as well as the rate of HDV co-infection amongst HBV carriers.

Volunteers were recruited for the hepatitis A, B, C and E study from the out-patient department queues of state clinics in two urban and two rural communities. For comparison, further volunteers were recruited for the same study in a community sampling procedure. A standard questionnaire was administered to each person for demographic data and potential risk factors for hepatitis virus infection. For the HDV study, anti-HDV was assessed in a separate group of HBV positive individuals. Testing for markers of hepatitis A-E infection was performed with commercial assays.

A group of 767 people were studied for hepatitis A, B, C and E markers of whom 407 (53%) were from the two urban areas and 360 (47%) from the two rural districts. The mean prevalences of anti-HAV IgG, HBsAg and HBsAb were 98%, 4.3% and 61.1% respectively. The mean prevalence of anti-HCV was 1.8%. No significant differences in prevalence were shown between the urban and rural regions for these viruses and none of the questionnaire variables were significantly associated with infection.

The mean anti-HEV prevalence was 10.7% and varied from 5.8% to 19.1% in the different regions. The mean urban and rural rates of 6.6% and 15.3% respectively were significantly different (p= 0.0001). Further analysis of the rural group showed that those living in mud houses without access to chlorinated tap water had a significantly higher prevalence of anti-HEV than those living in the rural villages where drinking water was chlorinated (17.4% vs. 5.3%). The use of pit latrines was shown to be associated with anti-HEV seropositivity (p=0.0013), while those using toilets with water borne sewerage were at a lower risk of HEV infection (p=0.0017). None of the 247 sequential HBsAg positive serum samples were anti-HDV positive.

1.2 Background on viral hepatitis

The number of known hepatitis viruses is increasing as more sophisticated molecular techniques permit the detection, isolation and characterisation of these agents. The viruses that cause hepatitis can be divided into those that are primary hepatitis viruses, which have their primary site of replication in the liver, and other viruses that have the capacity to replicate in the liver, but are generally more prominent in other organ systems e.g. cytomegalovirus. The primary hepatitis viruses isolated to date are known as hepatitis A, B, C, D and E. Each is distinct from the others and from a different virus family. Although hepatitis C infection normally causes a mild hepatitis, all five viruses have the capacity to induce a spectrum of disease severity from subclinical to fulminant. As a consequence, it is not possible to distinguish between them on the basis of the clinical picture at the bedside. A final aetiological diagnosis is thus reliant on serological analysis of patients' serum (1,2).

The hepatitis viruses may be divided according to their mode of transmission: enterically- or parentally transmitted.

1.2.1 The enterically-transmitted hepatitis viruses

1.2.1.1 The hepatitis A virus:

The hepatitis A virus (HAV) was initially identified by electron microscopy in 1973 (3) and was shown to be a small, non-enveloped spherical virus with a single stranded RNA genome. HAV was initially thought to be a member of the genus

Enterovirus within the family P*icornaviridae*. However, molecular analysis has allowed for HAV to be placed in a genus of its own, *Hepatovirus* (4).

HAV is transmitted by the fecal-oral route and is highly infectious (5,6). As a consequence, the epidemiology of HAV infection in developing countries differs markedly from more developed nations, on account of differences in living conditions. Developing country communities are infected at an early age due to persistent circulation of HAV between household members within poor communities. As a result almost all individuals in these communities show immunological evidence of past exposure to HAV by early childhood, as demonstrated by the presence of serum anti-HAV IgG (7). As the vast majority of young children tend to have a mild (or subclinical) HAV disease, the burden of HAV in these communities is paradoxically low and epidemics virtually non-existent due to the high herd immunity. In contrast, developed countries have a far lower percentage of the community infected with HAV, but have a higher disease burden, as the majority of infections occur in adulthood, where the disease is more serious (8). HAV epidemics are not infrequent in developed countries (8).

A vaccine containing purified inactivated HAV is produced commercially for those at high risk of acquiring the disease due to work, travel or other reason that increases the risk of exposure to HAV. In addition, HAV transmission to household contacts can be prevented by the administration of pooled gammaglobulin to susceptible individuals (9).

1.2.1.2 The hepatitis E virus

The hepatitis E virus (HEV), or enterically transmitted non-A non-B hepatitis, is the second of the enteric hepatitis viruses. It is a spherical, non-enveloped single stranded RNA virus that is placed within the family *Caliciviridae* and genus *Hepatitis E-like Virus* (4,10-12).

HEV is the leading aetiological agent implicated in outbreaks of waterborne hepatitis in developing countries where sanitation is poor (13-15). However, HEV has also been found to be endemic in these regions and of late has also been shown to circulate in more developed countries (14-16). Although less infectious than HAV, HEV is the leading cause of epidemic hepatitis in many under developed countries due to the high levels of HAV herd immunity, thereby preventing HAV epidemics (13).

The hepatitis E virus has been responsible for massive outbreaks of hepatitis in Asia and Africa (17-23). Travelers to these under-developed countries (24) and groups of people who reside together temporally, e.g. military personnel and refugees, are at increased risk of contracting HEV (23,25). Medical staff attending patients with HEV infection have also been infected (26). Sporadic hepatitis due to HEV infection has been described in a variety of countries, where both adults and children are susceptible (27-30). HEV has been documented in South Africa (26,31), but no data exists on the prevalence of anti-HEV in a representative sample of South African communities who are at greatest risk of

acquiring the disease, due to poor socioeconomic living conditions. As yet, there have been no documented epidemics of acute HEV in southern Africa.

In most patients, HEV infection is a mild, self-limiting illness although fulminant hepatic failure has been reported (12,32). The disease is more severe in pregnancy, and particularly so when infection occurs in the third trimester, where the incidence of fulminant hepatic failure is as high as 20% (32). In the Algerian outbreak of 1980/1, 100% of pregnant women with acute HEV died (19). This is significantly higher than the usual mortality quoted, but may be on account of the smaller numbers reported. The risk of intra-familial spread of HEV is significantly higher than for the general community (33), although substantially less than for HAV (34).

HEV can be visualised during acute infection by electron microscopy in serum, faeces (and bile) (10,35), and is also detectable over a longer period using a polymerase chain reaction (PCR) assay (36). Serum is positive for IgM anti-HEV at the onset of acute illness, while IgG levels become detectable 6 to 10 days into the illness and last for many years (11,36). Liver biopsies taken years after the illness show no signs of chronic disease (37), and no long term carriers of HEV have been documented.

There are currently no commercial vaccines available for HEV, although success with experimental vaccines has been reported (38). There is little justification for the use of pooled immunoglobulin preparations for family contacts of patients, as the donated serum is frequently from first world countries where most have not

been exposed to HEV. In addition, immunoglobulin preparations from endemic regions are of doubtful benefit (12,39).

1.2.2 The parentally- transmitted hepatitis viruses

1.2.2.1 The hepatitis B virus

The hepatitis B virus (HBV) is a member of the family *Hepadnaviridae* and genus *Orthohepadnavirus.* It is a small (42nm) spherical virus that contains a partially double stranded, circular DNA genome that is surrounded by the highly immunogenic core protein, which in turn is encapsulated by the surface protein (40). The virus was initially described as "Australia antigen", but was later found to have a global distribution and renamed HBV (41). Three types of HBV particles can be seen by electron microscopy: the "Dane particle" which is the complete virus, while the other two forms (spheres and tubules) are circulating excess surface antigen (HBsAg).

The initial laboratory diagnosis of HBV is normally made by way of detecting circulating HBsAg. These assays are extremely sensitive, as there is excess surface protein that is detectable in almost all cases of infection (42). In a small proportion of acute infections, the diagnosis is made by detecting the anti-core IgM response which precedes the detectable surface antigen by a few days (43). Past exposure to HBV can be assessed by detecting antibodies to the surface antigen (HBsAb) or core protein (HBc) or both. The hosts' immune responses are sufficient to prevent re-infection and is life-long (44). Mutant HBV has been

documented, with surface antigen mutants and pre-core mutants the most common (42,45). Of note, HBsAg mutants have been documented that do not react with adequately with certain commercial assays for the detection of HBsAg (42). The soluble portion of the core protein is the e-antigen (eAg). The presence of eAg in the serum of an HBV infected individual is indicative of high levels of viral replication. The e-antibody (eAb) is most frequently absent in the high replicative state. Seroconversion to an eAg negative, and eAb positive state is associated with lower replicative states of HBV (41).

The clinical manifestation of HBV may be sub-clinical, mild, moderate or severe resulting in fulminant liver failure. The hepatitis severity seen in HBV infection is largely attributable to the magnitude of the immunological response of cytotoxic T-cells that destroy the infected hepatocytes (46). Mutant HBV (particularly pre-core mutants) have been shown to be associated with a more severe illness (47). Chronic infection occurs in approximately 80-90% of children infected in the neonatal period or early childhood, and decreases to approximately 10-15% in adulthood (48). In both children and adults, chronic HBV infection may result in cirrhosis and/or liver carcinoma (49). HBV-associated liver carcinomas are most frequently found to contain integrated HBV sequence within the host genome (50). Children suffer the long-term consequences of cirrhosis and hepatocellular carcinoma proportionately more commonly that those who acquire HBV in adulthood due to the increased rates of chronicity seen after infection in early childhood (50,51).

The epidemiology of HBV varies by geographical location. An estimated 300 million people are infected with HBV globally, with sub-Saharan Africa one of the most affected zones (50,52). Many developing countries have high exposure rates at an early age, where HBV transmission frequently occurs horizontally between children and vertically from mother to child (53). The Asian epidemiology of HBV infection in childhood differs from that seen in Africa, with vertical transmission common in Asia, and horizontal transmission frequently seen in Africa (53). Adequate explanation regarding these differences in spread to (and between) children has been lacking to date. Developed countries have far lower prevalences of HBV and most infections occur in high-risk groups who are exposed to the virus either sexually or by the percutaneous route, often during intravenous drug usage (53). Blood transfusions were previously associated with transmission of HBV prior to the testing of blood donations, but is now a rare occurrence (54).

There is limited data on HBV epidemiology in South Africa. Available information shows the HBV prevalence in adults to be >10% in certain communities (55), while children under the age of six years have been documented with a prevalence of approximately 10% (56). Gauteng, Kwazulu Natal and the Eastern Cape provinces are highly affected geographic regions that have been studied to date. Migrant mine workers having a prevalence of approximately 14% (56-58). It has been previously estimated that between 1.5 and 2 million individuals are HBV carriers in South Africa (58,59), although more recent data is lacking. Clustering of infections within households has been documented, although the mechanism of transmission in these settings has yet to be shown (60). Children

are frequently infected early in life, most often acquiring the infection via horizontal transmission from other children (55,56,61). Perinatal transmission is far less common than in Asia (55).

The most efficient mode of preventing HBV transmission is to vaccinate all individuals in the community (62,63). Universal vaccination for HBV was incorporated into the South African Expanded Program on Immunisation in 1995. This strategy has been shown to reduce the prevalence of HBV in endemic regions (64). Vaccination is performed using either a plasma-derived vaccine that contains HBV surface antigen, pre-S1 and pre-S2, or with a recombinant vaccine containing surface antigen that is (normally) produced in recombinant yeast expression systems. Newer trivalent recombinant vaccines are expected to be more immunogenic and replace the mono-valent vaccines currently available. Sexual transmission is prevented by way of protected intercourse between couples (53). Blood products are screened for HBV and discarded if infected. Perinatal transmission is dramatically reduced with administration of HBV immune globulin at birth to children of infected mothers, followed by active vaccination thereafter (65).

1.2.2.2 The hepatitis D virus (Delta agent)

The hepatitis D virus (HDV) is an incomplete RNA virus that is placed within the family *Arenavirus* and genus *Delta virus*. HDV is entirely dependent on prior or co-infection with HBV for survival, as it uses the surface antigen of HBV as its envelope to prevent degradation (66). The HDV infects hepatocytes and causes a chronic infection along with HBV. Super-infection of HBV with HDV often

results in a worsening of the patients condition, and co-infection with HBV and HDV may result in a more severe disease state than infection with HBV alone (67).

There is currently no evidence for HDV infection in the HBV-infected community of South Africa. The work by Dusheiko et al (1989) and S.S, Abdool Kariem et al (1991) (58,68) showed HDV infection to be extremely rare. A conference abstract report in a 1997 supplement of the South African Medical Journal gave evidence of HDV introduction into the South African HBV infected community with an 80.4% prevalence in HBV positive individuals from Pretoria and Ga-Rankuwa. However, this data was later (partially) retracted, both in the press and by way of personal communication (S. Aspinall, Medical University of South Africa) (in response to reference number 69). The above conference report encouraged a formal assessment of the situation in the Free State Province, where no HDV infection was demonstrated (70).

There is no treatment for HDV infection. HDV/HBV infected individuals progress to cirrhosis more rapidly than those infected with HBV alone (67). However, those who are co-infected appear to have better survival rates after liver transplantation than those who are infected with HBV alone (71). HDV prevention measures are the same as for HBV, as preventing HBV infection results in fewer HDV-susceptible individuals.

1.2.2.3 The hepatitis C virus

The term, "the hepatitis C virus (HCV)" was coined after the discovery of the HCV genome in 1989 (72,73). However, the existence of this agent had been presumed with the ongoing hepatitis cases, after the establishment of adequate assays for HAV and HBV (74). This "agent" was initially termed "post-transfusion hepatitis", as it was well documented that there was at least one further transfusion-transmissible agent that caused hepatitis, after the exclusion of those donations infected with HBV (and HAV).

The HCV genome was detected in serum after the screening of complementary DNA (cDNA) clones generated from patients suspected of being carriers of the "post-transfusion hepatitis virus" (72). Sequencing of overlapping clones resulted in the identification of the complete HCV genome, that was shown to be a positive strand RNA virus with close resemblance to the members of the family *Flaviviridae*. Extensive investigation of the molecular structure of HCV has resulted in its placing as a separate genus *Hepacivirus* (4,75). Current recommendations of the International Committee on Taxonomy of Viruses (ICTV) are that the older "genotype" system of phylogenetic analysis be replaced by a "clade" system comprising clades 1-6 (75). Genotypes 1,2,4 and 5 will be named clades 1, 2,4, and 5 respectively, while genotypes 3 and 10 will be placed in clade 3. Clade 6 will comprise genotypes 6, 7, 8, 9, and 11.

The hepatitis induced by HCV is generally a mild disease, and many infected individuals do not give a history of being at-risk of acquiring HCV through blood products, intravenous drug use, dialysis, etc (76-78). On account of its mild

nature, many of those infected with HCV are only found on routine screening through the blood banks. Although the infection may present as a mild disorder, most go on to chronic infection (77,79). Chronic infection is associated with cirrhosis and liver carcinomas (79). The mechanism by which HCV induces carcinomas is not fully identified. However, the HCV genome is not integrated into the host genome, as is seen with HBV (79).

Diagnosis of HCV infection is normally performed in routine laboratories by initially screening with third generation anti-HCV assays. There is no laboratory standard for testing of antibodies to HCV. Thus sensitivity and specificity measurements are not exact. However, third generation assays containing four recombinant antigens are considered to be highly sensitive and specific, based on the data from known HCV seroconvertions. The earlier generation assays that used fewer recombinant antigens gave both false positive and false negative results (80). Viraemia detection is normally only performed on anti-HCV positive samples and is detected by PCR amplification of a segment of the genome, after reverse transcription (RT) of the RNA genome into cDNA. For cases where the antibody assays give indeterminate results, a further recombinant immunoblot assay (RIBA) may be performed to assess the nature of the antibody response (81). RIBA results appear to correlate well with PCR for the purpose of screening large populations for viraemia (80).

There is limited data on HCV infection in South Africa. The community prevalence of anti-HCV in Kwazulu Natal was shown to be 0.9% and 1.7% in rural and urban communities respectively (82), and this is in keeping with data

from a smaller study of maxillofacial patients at Medunsa Dental Hospital where the prevalence was 1.1% (83). The prevalence of anti-HCV in donors of the Western Province Blood Transfusion Service (WPBTS) was shown to be 0.41%, using a second generation anti-HCV assay (78). Dialysis patients in South Africa have been shown to have a prevalence in excess of 20% in two haemodialysis units in South Africa (84). Clade 5 HCV predominates in South Africa (85). Clade 5 is implicated in the genesis of liver carcinomas, but to no greater or lesser extent than any other HCV clade (86).

1.2.3 Additional hepatitis viruses

It is presumed from epidemiological evidence that there is at least one further hepatitis virus (87-89). This is based on the finding that post transfusion hepatitis remains prevalent, even after the exclusion of the known hepatitis viruses. No other proven hepatitis viruses have been isolated to date, although the newly discovered "transfusion transmitted virus (TTV)" is currently being investigated as a possible cause of hepatitis (90). However, the high prevalences of this virus in the general community suggest that it likely to play a minor role in disease induction (91-93).

1.3 Study rationale

No systematic analysis of the epidemiology of hepatitis viruses A-E has been performed in the Western and Eastern Cape Provinces of South Africa. In

addition, at the time of setting up the study, no data was available on the prevalence of HEV in sub-Saharan Africa.

1.4 Aims

This study aimed to assess:

1. the background prevalence of hepatitis A, B, C and E in rural and urban adult Black South Africans living in the Western and Eastern Cape Provinces of South Africa. The study was designed to detect differences in hepatitis virus exposure between rural and urban groups, and sought to identify factors associated with an increased risk of infection.
2. the prevalence of HDV co-infection amongst HBV carriers of the Western Cape Province.

1.5 Materials and methods

1.5.1 Institutional approval

Both studies below were approved by the Ethics and Research Committee of the Faculty of Health Sciences of the University of Cape Town. Study 1 also had approval from the regional health authorities responsible for the clinics where the volunteers were recruited.

1.5.2 1: Hepatitis A, B, C and E study

1.5.2.1 Community support and permission for random sampling

Access to the rural villages for the random sampling in the Keiskammahoek district was negotiated with the elected community leaders, in consultation with the local Community Health Department of the S.S. Gida Hospital, Keiskammahoek.

1.5.2.2 Communities sampled

All recruiting was performed by the candidate within selected communities of the Western and Eastern Cape Provinces of South Africa with assistance from study nurses and students who administered the questionnaire.

Two urban and two rural communities were chosen for the study:

Urban communities:

- The urban suburbs of Nyanga and Guguletu were chosen in Cape Town.
- The urban region of Mdantsane was chosen in East London.

The volunteers from both cities lived in both the formal housing sector and informal squatter communities.

Rural communities:

- The rural town and surrounding villages of Keiskammahoek; and
- the rural village of Peddie, in the Eastern Cape Province were designated as a suitable comparison sites for rural sampling.

This sample group included people living in formal housing within the towns, as well as those living as subsistence farmers in isolated clusters of traditional mud house villages.

1.5.2.3 Sampling methods

The recruitment of volunteers willing to participate and donate blood samples was performed in two phases.

Phase 1: Out-patient department sample group:

The first phase was performed identically in the outpatient department (OPD) clinics of the:

- S.S. Gida Hospital of Keiskammahoek;
- Nompumelelo Hospital of Peddie;
- Cecelia Makiwane Hospital in Mdantsane, East London;
- Nyanga and Guguletu Day Hospitals of Cape Town.

Those recruited in these four health centres were sequential, consenting adults attending non-specialist OPD clinics of state-run health facilities. The patients were attending the clinic for routine management of their medical problems, and not for the purposes of the study.

Phase 2: Random community sample:

The second phase of sampling was performed to assess any differences between the OPD group and the general community for HEV (and HGV/GBV-C).

In this separate, random, community-based study, a sample population of 160 persons was recruited in Keiskammahoek. The random collection of volunteer samples was performed in the following manner:

- Keiskammahoek is divided into 45 regions by the local health authority (as shown in appendix 1). Four of these regions were selected as sampling areas, using a random number chart after assigning numbers to the regions.
- Within these chosen regions, plots of approximately half an acre in size (independent of the number of houses on each plot) were considered to be a sampling unit.
- An aerial photograph was obtained of the relevant villages and plots were numbered on the photo.
- The starting plot for sampling was chosen with the aid of a random number chart and every second unit was sampled until a total of 40 people were included i.e. a total of 160 individuals in the four districts.
- Eligible, consenting occupants of each unit were numbered and a maximum of two volunteers were chosen per unit (by random number chart).

1.5.2.4 Volunteer consent

It was predicted that many of those that we aimed to recruit to the study would be either illiterate, or have poor literacy levels. Thus, the university ethics committee and the local authorities granted permission for the volunteers to give verbal consent at the time of entry into the study. The interviews were performed by Xhosa-speaking medical students and nurses dedicated to the study. The persons interviewing the volunteers standardized the questionnaire and confirmed that each person was asked the questions in an identical manner.

The nature of the study was explained to potential OPD volunteers in a private room adjacent to the queues of the clinics. The same informed consent process

was performed during the community sampling, with as much privacy as possible in the individuals' homes. No person was coerced into participating, and all volunteers were informed that they would not receive the results individually. However, the community leaders were given the assurance that epidemiology results would be fed back to the local hospital authorities.

1.5.2.5 Inclusion and Exclusion Criteria

Volunteers were included in the study if they were resident in the relevant sample area for a minimum of five years prior to inclusion. "Residence" was defined as living in the region for a minimum of nine months in each of the five years. All volunteers were 18 years or older, and ambulant.

1.5.2.6 Questionnaire

A standard questionnaire was administered to each person recruited to the study. This elicited information regarding age, gender, house type (brick, corrugated iron or mud), source of household water (tap or river), mode of sewerage disposal, employment, a history of hepatitis or any form of liver disease, past transfusions, scarification (i.e. ritual scarring) and a history of significant trauma requiring hospitalisation.

Patients attending the OPD were also questioned about the quantity of alcohol consumed and the use of illicit drugs. For the purposes of this study habitual consumption of >60g of alcohol per day or >360g alcohol per weekend was considered excessive [Grønbæk et al., 1994; Becker et al., 1996]. Data on alcohol consumption and drug use was not collected during the door-to-door

community sampling in Keiskammahoek because of resistance voiced by the community leaders when negotiating access to the villages. The community leaders stated that, as the houses of the region were almost invariably single-roomed, the privacy of the individual was not guaranteed during the interview and thus a) the answers to the questions may be incorrect and, b) the process may compromise the person being interviewed.

1.5.2.7 Sample preservation

Two 10ml tubes of blood were taken from each volunteer in a glass Vacutainer tube (Beckton Dickinson, France) containing no additives. The blood was allowed to clot and the tubes centrifuged at 3000 revolutions per minute for 10 minutes, within four hours of sample collection. The serum was transferred to separate storage tubes and placed at -20°C. On returning to the laboratory, these were then transferred to a -80°C freezer for long-term storage. The samples remained in the -80°C freezer and were not thawed until processed for this study.

1.5.3 2: Hepatitis D virus study

1.5.3.1 Sample site

The combined Virology Laboratory of the Groote Schuur Hospital and the South African Institute for Medical Research was used as a source of HBV positive samples for the study.

Sequential HBsAg positive serum samples received by the laboratory were prospectively collected for inclusion in the testing and analysis. This combined laboratory was chosen as it services an extremely wide range of clinics and hospitals. The samples were from children and adults with both acute and chronic hepatitis from all levels of the Western Cape health structure, from primary health facilities to specialist hepatology clinics.

1.5.3.2 Laboratory methods

The prevalence of hepatitis markers was determined with standard commercial assays for each virus. Each assay was performed in accordance with the manufacturers' instructions.

The following assays were used: (All assays were from Abbott Laboratories, North Chicago, USA, except where indicated).

- Anti-HAV IgG was assessed with the HAVAB-G radio-immunoassay.
- HBsAg was detected using the AUSRIA assay.
- HBsAb was detected with the AUSAB assay.
- HBeAg and HBeAb were detected with the HBeAg and anti-HBe radio-immunoassay respectively.
- Anti-HCV was determined by both Ortho and Behring second generation EIA's (Ortho Diagnostics, Raviran, NJ, USA; and Behringwerke AG, D-35001, Marburg), and only considered positive when repeatedly reactive with both assays. (Third generation anti-HCV assays only became available in South Africa after this testing was complete).
- Anti-HDV IgG was detected using the anti-Delta EIA.

- The HEV enzyme-linked immunosorbent assay was used to test all samples for anti-HEV IgG in duplicate. The test was repeated again if the result was indeterminate. Samples were only considered true positives when repeatedly reactive.

1.5.3.3 Statistical methods

Statistical analysis was performed using the Epi-Info version 6 software program (Center for Disease Control, GA, Atlanta, USA) and the Statistica software program (Statsoft, Tulsa, USA). Differences in prevalence were analysed using the Chi squared test. Discriminant analysis by stepwise method assessed factors associated with increased risk of infection. The linear model was used to estimate whether gradients differed significantly.

1.6 RESULTS

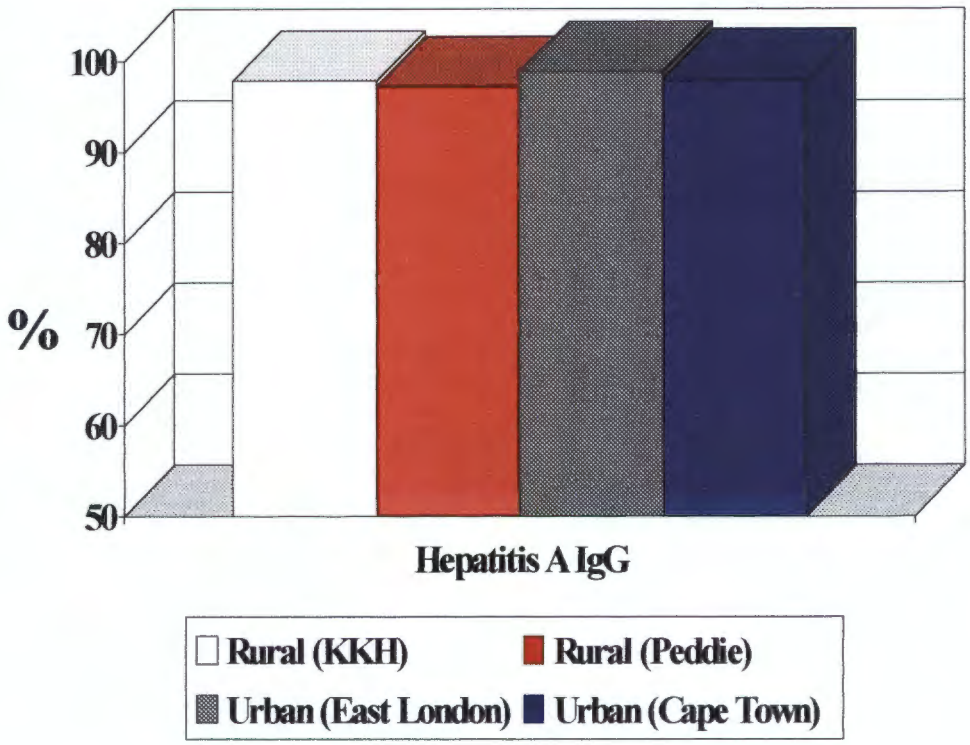
1.6.1 1: Hepatitis A, B, C and E study

A cohort of 767 people were studied, of whom 407 (53%) were from the two urban areas and 360 (47%) from the two rural districts. Two hundred and eighty (68.8%) urban dwellers lived in formal housing, while the remainder were from urban squatter settlements. The rural OPD sample size was 200, while the random community sample size was 160. Seventy percent (252) of the rural group lived in traditional mud houses, with inadequate sewerage disposal and no access to chlorinated tap water. The average age of the entire sample was 42.4 years (range 18-92), and the male:female ratio was 1:1.8.

1.6.1.1 Hepatitis A

The mean prevalence of anti-HAV IgG was 98%. The graph below (Figure 1.1) shows the anti-HAV IgG prevalences were for the four regions. No statistically significant differences were detectable between the regions. None of the questionnaire variables were significantly associated with past infection.

Figure 1.1: Prevalence of anti-HAV IgG.



1.6.1.2 Hepatitis B

The mean prevalences of HBsAg and HBsAb were 4.3% and 61.1% respectively. The following two graphs (Figures 1.2 and 1.3) demonstrate the HBsAg and HBsAb prevalences in the four communities. No differences were shown between the regions for either of these variables or for the total exposure to HBV; i.e. the summation of the two. None of the questionnaire variables were significantly associated with either current or past HBV infection.

Figure 1.2: Prevalence of HBV surface antigen.

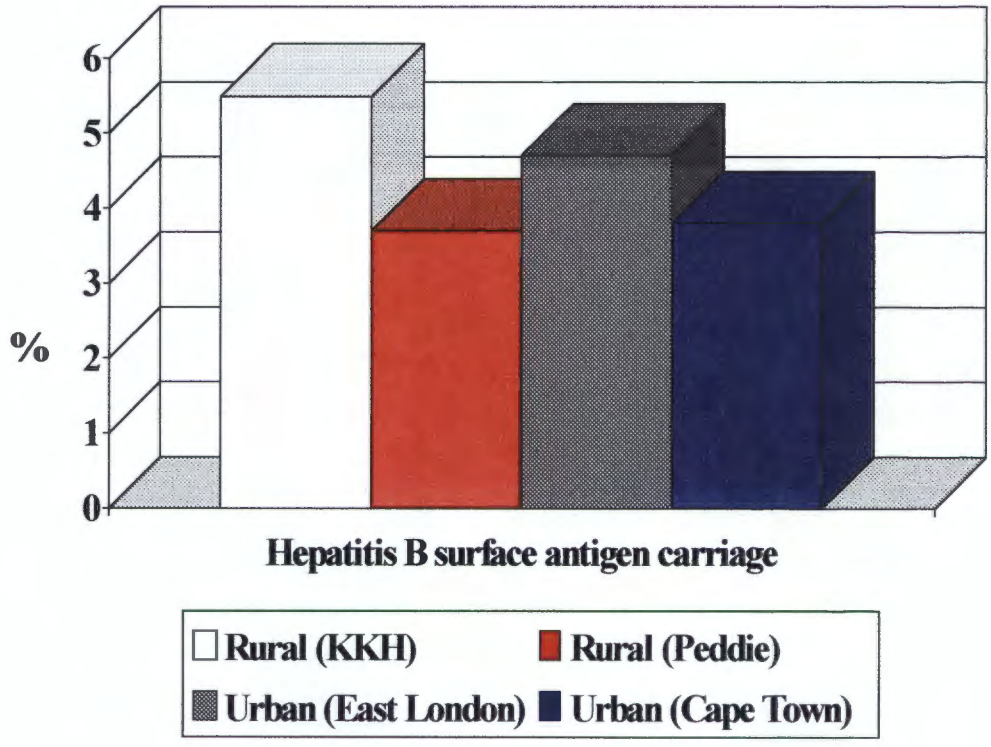
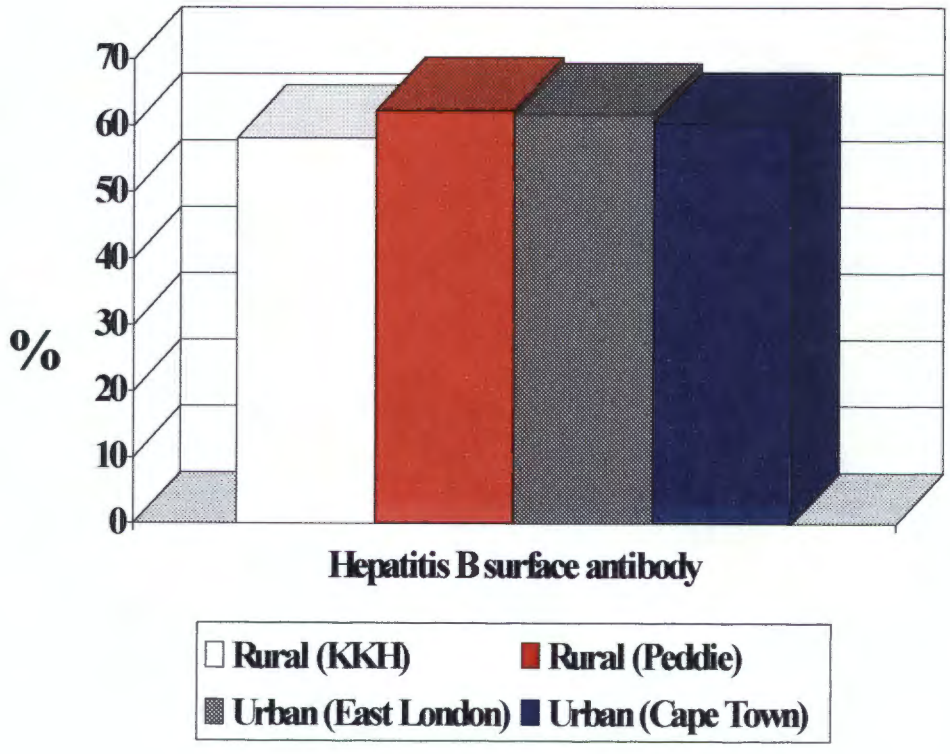


Figure 1.3: Prevalence of antibodies to HBV surface antigen.

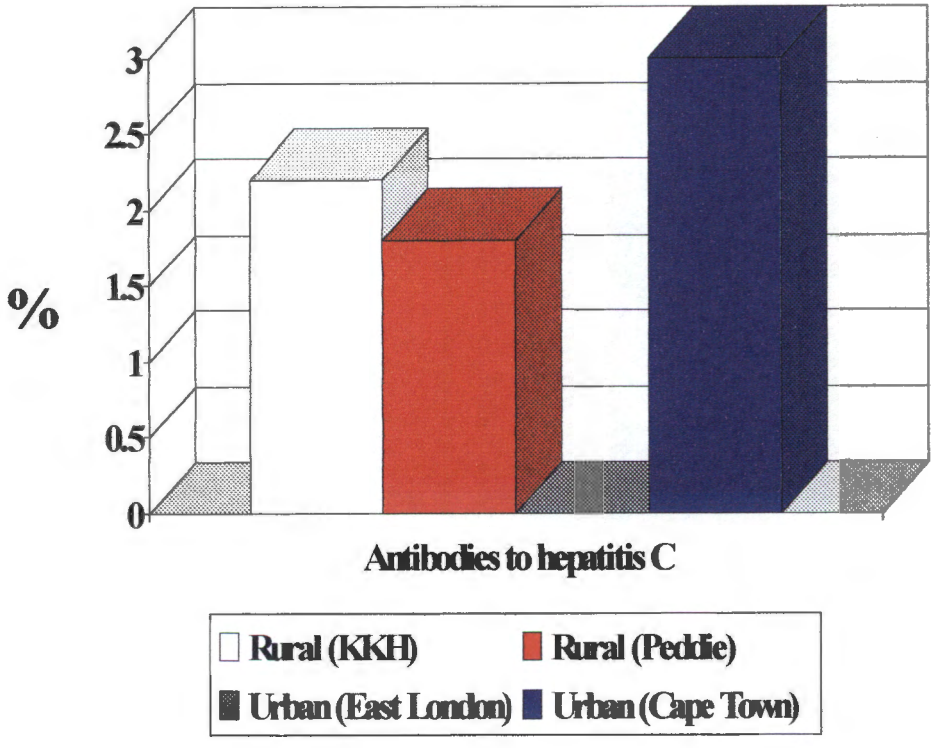


HBeAg and HBeAb status of the HBV positive samples showed 12.5% to be eAg positive and eAb negative, while 87.5% were eAg negative and eAb positive. None of the samples were positive for both eAg and eAb serum markers.

1.6.1.3 Hepatitis C

The mean prevalence of anti-HCV was 1.8%. The following graph (Figure 1.4) shows the prevalence of anti-HCV in the four communities. The number of positive samples was too small to detect statistically significant differences between the four communities, and none of the questionnaire variables were significantly associated with detectable serum anti-HCV.

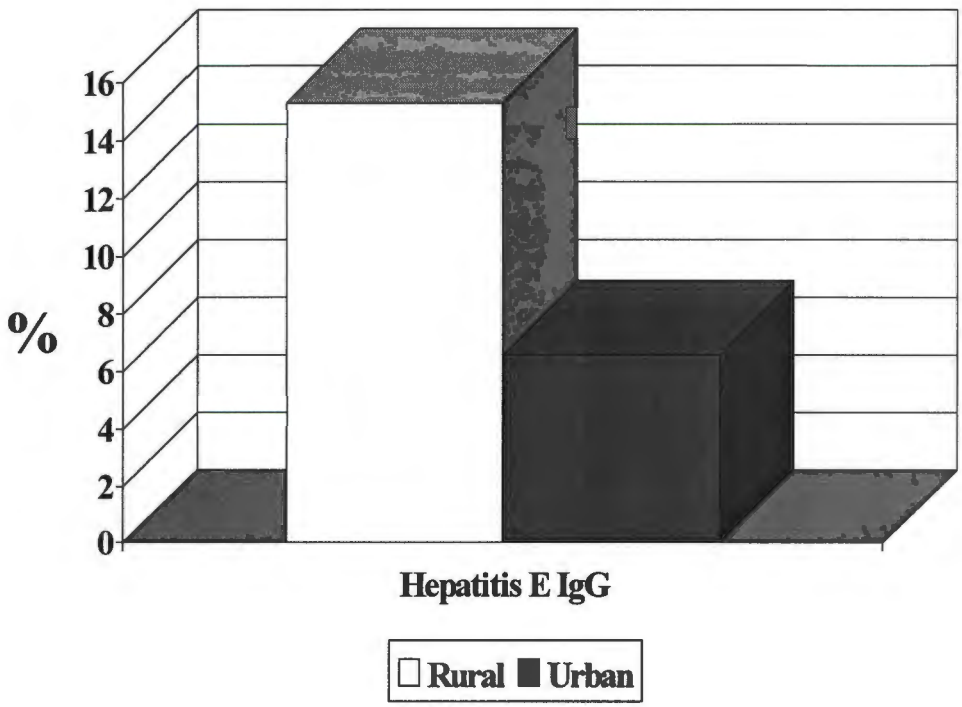
Figure 1.4: Prevalence of antibodies to HCV.



1.6.1.4 Hepatitis E

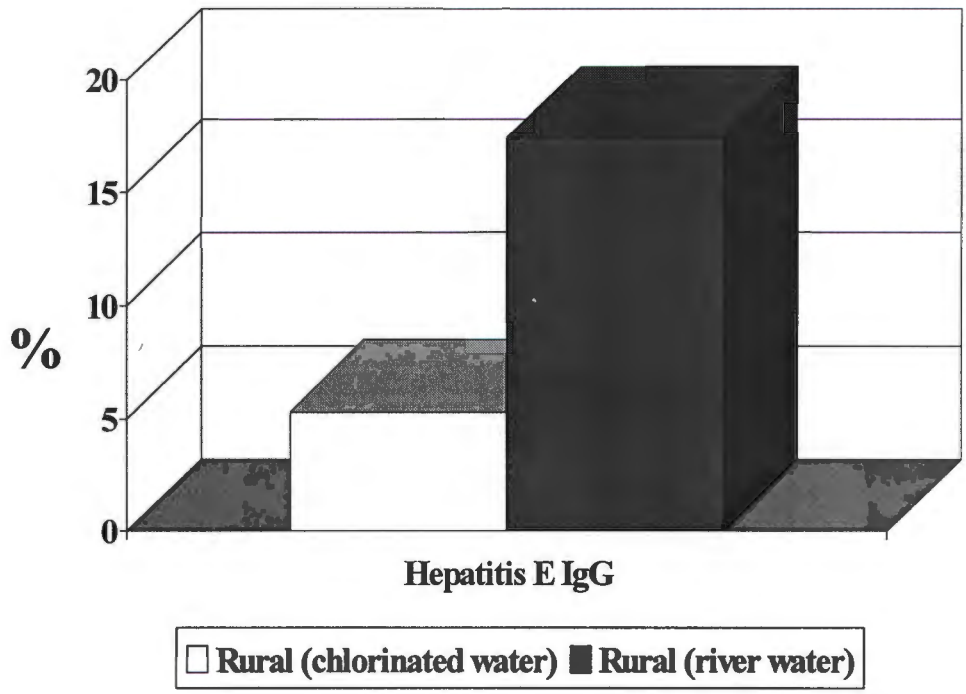
The mean anti-HEV prevalence was 10.7% and varied from 5.8% to 19.1% in the different regions. The mean urban and rural rates below (Figure 1.5) of 6.6% and 15.3% respectively were significantly different (p= 0.0001).

Figure 1.5: Urban and rural anti-HEV prevalence.



Further analysis of the two rural groups showed that rural living alone was not the true association with HEV infection. Those living in mud houses without access to chlorinated tap water had a significantly higher prevalence of anti-HEV than those living in the rural villages where drinking water was chlorinated (17.4% vs. 5.3%) (p = 0.008: odds ratio = 2.85) (Figure 1.6). Thus, those rural inhabitants with access to chlorinated tap water had an identical prevalence to the urban dwellers.

Figure 1.6: Rural prevalence of anti-HEV.



No differences in prevalence were found between the out-patient based and community based sampling. No significant differences were noted between the urban squatter and urban formal housing groups for any of the hepatitis markers ($p > 0.05$).

Univariate analysis for anti-HEV

The use of pit latrines was shown to have an association with anti-HEV seropositivity ($p=0.0013$), while those using toilets with water borne sewerage were at a lower risk of HEV infection ($p=0.0017$). Extremely few gave a history of hepatitis or any form of liver disease, suggesting a high degree of subclinical or low grade hepatitis.

Multivariate analysis for anti-HEV

Discriminant analysis of all recruits showed rural location (0.364), age (0.589), living in mud houses (0.450) and a lack of access to chlorinated tap water (0.188) to be predictors of anti-HEV positivity (standardised coefficients in brackets). Discriminant analysis of the rural group alone confirmed a greater risk for mud hut dwellers.

A linear relation was found between anti-HEV seroprevalence and age. When individual communities were examined, this relationship was maintained wherever sufficient positives allowed such analysis.

1.6.2 2: Hepatitis D virus study

The study assessed 247 sequential HBsAg positive serum samples, of which:

- 69 were eAg positive and eAb negative,
- 167 eAg negative and eAb positive,
- 11 were positive for both eAg and eAb.

None of the 247 samples were anti-HDV positive.

1.7 DISCUSSION

The hepatitis A and B results demonstrate exposure levels in keeping with the data reviewed in the introductory components of this chapter, showing that there

is almost universal (past) exposure to HAV and very high levels of HBV infection in the adult Black South African population. Most HAV infections occur in the first years of life amongst Black South African communities (94), and therefore it is likely that the anti-HAV prevalence results would have been as high even with a younger volunteer group from the same communities. HBV carriage rates appear to be lower than the figures for Gauteng, Kwazulu Natal and Namibia (57,60,94-96), but did not show significant variation between the urban and rural regions. The lack of a history of symptomatic hepatitis would suggest that the majority of infections occur in the absence of clinical hepatitis. The eAg/eAb results for the HBsAg positive individuals show the majority of HBV infected individuals to be eAg negative and eAb positive; highly suggestive of low replication rate of HBV.

There is little data for comparison of HCV infections in the Western and Eastern Cape Provinces. However, it is noteworthy that the prevalence of antibodies to HCV in this community (1.8%) is significantly higher than that of the blood donors of the Western Cape Province (0.41%) (78). Although different commercial anti-HCV assays were used for these studies (Abbott versus Behring and Ortho), the laboratory methods are directly comparable, as both were performed just prior to the release of the third generation anti-HCV assays in South Africa. No HCV infections were detected in the East London group of 172. However, there was no statistically significant regional variation.

This study found none of the Western Cape HBV positive samples to be HDV co-infected. The 1997 conference report by Faber et al. alerted South African virologists to the possibility that HDV infection had become common amongst

HBV infected individuals in the Gauteng Province. The Western Cape data contained in this thesis is in keeping with the recent Free State Province data also showing no HDV infection in their group of HBV positive patients (70). The partial retraction of the initial data by Faber et al. (69,70) suggests that HDV prevalence remains at an extremely low level in the community.

The data show the prevalence of anti-HEV in adult Black South Africans to be higher than expected, given that no epidemics of HEV have been documented in South Africa. The rural prevalence was significantly higher than the urban prevalence. However, subgroup analysis of the rural group alone showed that there was a distinct difference between those with access to chlorinated water and those who collected water from nearby rivers. The prevalence amongst those with access to tap water in the rural regions was the same as the urban group. However, persons living with no access to chlorinated water had a 2.85 fold increase in the risk of being anti-HEV seropositive.

The univariate and multivariate analyses give evidence for increased transmission of HEV in the absence of adequate sewerage disposal. This is in keeping with the knowledge that HEV is transmitted by contact with faeces containing HEV. It is interesting to note that, although HAV and HEV are both transmitted by the fecal-oral route, their seroprevalence rates differed significantly in this study. The prevalence of anti-HEV increased linearly with age, strongly suggesting that there has been no recent epidemic outbreak of HEV in the communities sampled and thus that infection is sporadic.

The lack of difference in prevalence between the urban formal and urban squatter communities was surprising in view of the poor living conditions and the use of non-waterborne sewerage disposal methods within the majority of squatter areas. However, unlike their rural counterparts who use river water for consumption, urban squatter communities rely entirely on chlorinated tap water for domestic use, even if that water is brought to the house from a nearby tap in a container.

In summary, this study has shown that the adult Black communities of the Western and Eastern Cape Provinces of South Africa have a very high exposure rate to the known hepatitis viruses. HAV exposure is almost universal, while hepatitis B carriage of approximately 3% to 5% is lower than for other provinces. However, overall exposure to HBV is over 60% in all regions studied. HCV exposure is 1.8%, which is significantly higher than the figure for blood donors. The prevalence of anti-HEV is significantly higher than expected, given that there have been no epidemics in the regions. In addition, evidence is presented to show that those with inadequate access to chlorinated tap water and waterborne sewerage were at increased risk of HEV acquisition.

CHAPTER 2

Epidemiology of HGV/GBV-C viraemia in South African urban and rural communities

2.1 Summary

The discovery of HGV/GBV-C and its published associations with hepatitis resulted in extensive research activity to assess its possible contribution to liver disease. However, no hepatitis-inducing role has been found for HGV/GBV-C. Global HGV/GBV-C prevalences differ significantly. Approximately 1-3% of the developed country inhabitants and between 5% and 18% developing nations are infected with HGV/GBV-C. Prior to initiating this study, no work had been performed on HGV/GBV-C in southern Africa.

This study was performed on the same serum samples described in chapter 1. While the hepatitis A-E study evaluated four communities, the HGV/GBV-C assessment contained in this chapter investigated the samples from one urban and one rural community. A RT-PCR assay was developed for the 5'NCR of HGV/GBV-C.

One hundred and thirty of the 484 samples were HGV/GBV-C PCR positive, giving an overall prevalence of 26.9%. The rural (20%) and and urban (34%) prevalences were significantly different (p=0.0005). Analysis of the questionnaire variables showed a significant relationship between HGV/GBV-C infection and the use of illicit drugs (marijuana), female gender, younger age and past blood transfusions.

This data shows the prevalence of HGV/GBV-C in South Africa to be higher than any other published figure to date, excluding those studies assessing extremely high risk groups such as intravenous drug abusers.

2.2 Introduction

After the discovery of the hepatitis A, B, C, D and E viruses, the presence of a further viral agent causing hepatitis was supported by the finding that many patients with an episode of clinical hepatitis had no known causative agent identified, in the presence of features compatible with viral infection (97,98). Thus, the discovery of two novel viral agents, GB virus C and hepatitis G, and their published associations with cryptogenic hepatitis caused an explosion of research activity by hepatologists and hepatovirologists assessing their possible contribution to liver disease.

2.2.1 HGV and GBV-C discovery

2.2.1.1 GBV-C

The first description of GBV-C was published in 1995 by Simons et al. after an extensive search for novel viral sequences by the Virus Discovery Group of Abbott Laboratories, North Chicago (102). One of the sera investigated by this group was that of a surgeon (initials GB) who had a clinical episode of hepatitis, later shown to be non-A-E hepatitis (99). The serum was obtained from this surgeon on day 3 of his icteric illness (which occurred in the 1960s) and investigated over the following decades. The Abbott Laboratory research group inoculated this serum into tamarins and by a series of immunological and molecular processes, discovered three novel, putative viral genome sequences after 11 passages of the virus in these animals. The first of these two agents, GBV-A and GBV-B have been characterised and shown to be of primate (and not human) origin (100,101), while the third, GBV-C has been shown to infect a great

number of individuals globally (102,103). However, all three are related RNA viruses that are similar in genetic structure to previously described members of the family *Flaviviridae*, although distinct from HCV (104).

2.2.1.2 HGV

Another independent group of collaborators coordinated by Genelabs Technologies Inc., Redwood City, USA discovered similar but apparently novel viral sequences and published their results in 1996 (103). The initial isolate was extracted from the serum of a patient with community-acquired chronic cryptogenic hepatitis. This serum was inoculated to tamarins, and like the serum from the surgeon (GB) above, caused hepatitis in the primates. Similar immunological and molecular biology technologies (such as immuno-screening, anchored PCR and rapid amplification of cDNA ends) were employed to extract and expand upon the novel viral sequences, after the production of cDNA libraries (103). The virus was named "hepatitis G" in the publication.

The early molecular studies characterising GBV-C and HGV gave clarification that these were distinct from HCV. However, it was only established later that GBV-C and HGV were variants of the same novel flavivirus (105). Thus, for the purposes of this thesis, they will be named "HGV/GBV-C". Importantly, neither of the two, "hepatitis G" nor "GBV-C" have been accepted by the International Committee on Taxonomy of Viruses (ICTV).

This chapter will review the natural history after infection with HGV/GBV-C as well as the epidemiology of HGV/GBV-C in different global communities and

assess the factors associated with increased risk of acquiring the infection. The review of the genetic components and tissue tropism of HGV/GBV-C is contained in chapters 3 and 4 of this thesis respectively.

2.3 Natural history of HGV/GBV-C infection

2.3.1 Transmission

There have only been a limited number of studies performed to date assessing the natural history of HGV/GBV-C. However, HGV/GBV-C is known to be transmitted from person-to-person by the inoculation of blood products after transfusion(s) or by exposure to the virus during intravenous drug usage (103,106-108). In addition, there is evidence showing that the virus can be found in saliva (103,106,109,110) and semen (111). Vertical transmission has been shown (112,113), and studies investigating sexual transmission of HGV/GBV-C suggest that the virus is also transmitted by sexual contact, with the receptive sexual partner at greater risk of acquiring the virus (113-115).

2.3.2 Viral carriage and clearance

Current evidence suggests that the majority of HGV/GBV-C infections result in chronic carriage of the virus, even in immunocompetent individuals (103,116-118). Most of the infected individuals show evidence of ongoing infection for a period of years, and if there is viral clearance, it is associated with the appearance of serum antibodies specific for the second envelope glycoprotein of HGV/GBV-C (anti-E2) (116,118-124).

The presence of anti-E2 is a marker of past infection, suggesting that this antibody is responsible (or partially responsible) for viral clearance (116,121,124). Large studies have repeatedly shown very few individuals to be positive for both markers of infection, and when both RNA and anti-E2 are demonstrated at the same time, it is normally interpreted as being indicative of a seroconversion process. In addition, studies have assessed the protective effect of detectable serum anti-E2 (125). These have shown that the presence of this antibody protects against re-infection with HGV/GBV-C. As discussed in chapter 3, the HGV/GBV-C genome has a conserved envelope coding (E1 and E2) region. Therefore unlike HCV, HGV/GBV-C has little ability to evade immune capture by altering the immunogenic epitopes.

2.3.3 Animal models

Animal studies of HGV/GBV-C using chimpanzee and tamarin infection models have not defined any disease association for this virus. However, the virology and immunology patterns demonstrated in these animal studies appear to mirror that seen in human infection with regard to infection routes and chronic viraemia (126). A newly discovered primate variant of HGV/GBV-C (named GBV-Ctro) has recently been discovered in chimpanzees and partially characterised (127). However, it has not been demonstrated to cause disease in these primates as yet.

2.4 HGV/GBV-C epidemiology

The defining of global HGV/GBV-C infection patterns remains a "work-in-progress". However, HGV/GBV-C prevalence studies have been conducted in countries from each continent and certain aspects regarding risk factors and infection patterns have been clarified. The majority of prevalence studies have been performed by hepatovirologists, based on the initial understanding that this virus was the latest of the primary hepatitis viruses. This is reflected in the bias towards studies assessing markers of infection in patients with liver disease.

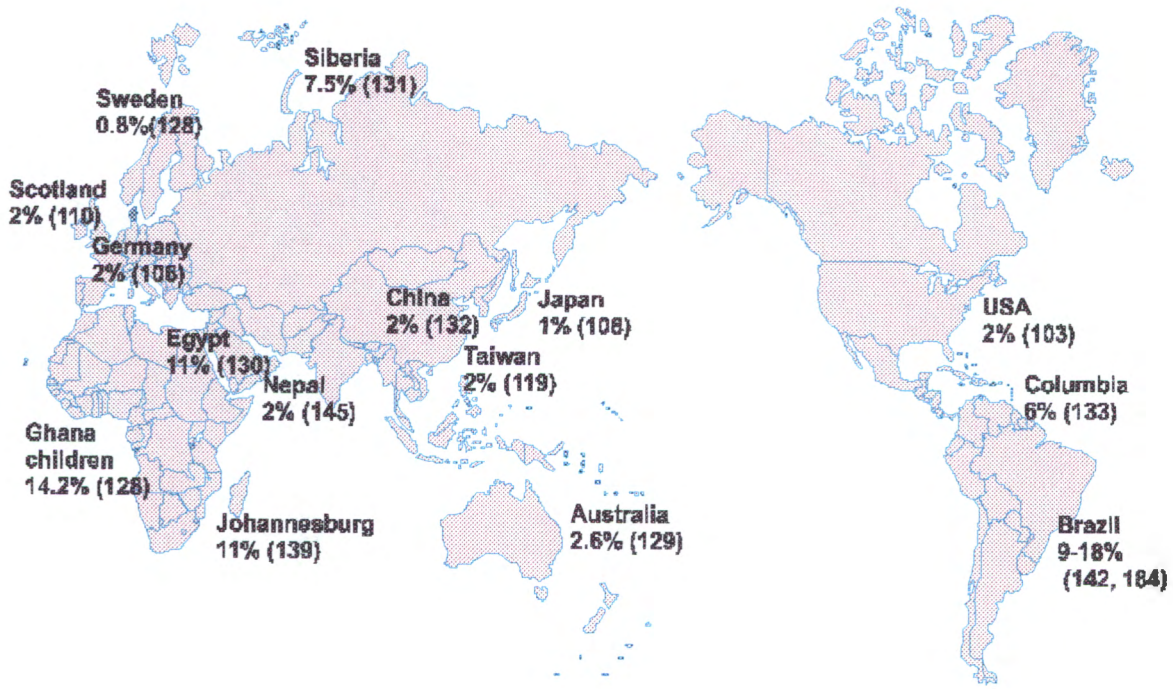
In keeping with the natural history of HGV/GBV-C reviewed above, studies have assessed either one or both of the following markers of infection:

- viraemic status by RT-PCR, and/or
- past infection, by detecting antibodies to the E2 protein.

The following map (Figure 2.1) displays a selection of the published HGV/GBV-C prevalence figures, giving the RT-PCR (current infection) results for adults according to geographic region. Although all of the studies represented below used RT-PCR for assessment of viraemia, it is important to note that they were performed using different laboratory methodologies / commercial kits and with different sampling procedures for their study subjects, and therefore it may be argued that they are not directly comparable. In addition, much of the RT-PCR work presented in this map was performed shortly after the discovery of HGV/GBV-C and thus with limited knowledge of the genetic sequence of HGV/GBV-C. As a consequence, primer design may not have been optimal in all studies. Studies were only chosen for representation in the above diagram if they

were performed with the aim of elucidating the community prevalence of HGV/GBV-C, and not in a specific cohort of patients or at-risk groups.

Figure 2.1: Prevalence of HGV/GBV-C viraemia. (Numbers in brackets represent the journal reference number).



Significant differences are evident from the figure between the developed and under-developed countries. It can be seen that the prevalence of HGV/GBV-C viraemia in developed communities of Europe, North America, and Australia varies between <1% and 3% (110,128,129). By contrast, the available prevalence data for developing nations in Africa, South America and parts of Asia show significantly higher rates of HGV/GBV-C carriage that range between 5% and 18% (130-133).

Fewer studies have assessed past exposure to HGV/GBV-C, as demonstrated by the presence of serum anti-E2 antibodies. However, where performed, the figure for past exposure is normally higher than that for viraemia (129,134,135).

2.4.1 HGV/GBV-C risk factors

The main routes of transmission and body fluids responsible for transmission of HGV/GBV-C have been mentioned above i.e. blood products and possibly semen and saliva. The data for HGV/GBV-C is in keeping with other viruses such as HBV and HCV that are transmitted parenterally. Those at highest risk of being exposed to HBV and HCV e.g. intravenous drug abusers, are those who show the highest exposure rates for HGV/GBV-C as well (136-138). Most groups of patients with repeated exposure to blood products, such as haemophiliacs show increased prevalences of HGV/GBV-C (139,140). Interestingly, higher prevalence have even been shown for patients who have received virus-inactivated blood products, therefore suggesting that HGV/GBV-C may be (partially) resistant to these treatments (136,140). However, many infected individuals give no history of a known risk factor (141,142).

Other HGV/GBV-C risk factors have also been identified in addition to those above:

- Children of infected mothers are at risk of acquiring HGV/GBV-C by vertical transmission of the virus. These conclusions have been based on both the prevalence of comparative infant groups as well as molecular analysis of viruses extracted from the mother and infant (112,143).

- Both peritoneal and haemodialysis patients appear to have higher prevalences, and although certain studies have ascribed this phenomenon to the blood transfusions given to these patients, others studies show the infections to be independent of treatment (139,144,145).
- Transmission of HGV/GBV-C from patients to staff may also occur. Needle stick injuries have resulted in transmission of the virus to health care workers (146), while certain institutions have shown higher prevalences of HGV/GBV-C in dialysis staff (147).
- HIV infected individuals in developed countries show increased exposure to HGV/GBV-C (137,148,149). However, this higher prevalence may be related to concomitant risk factors such as intravenous drug usage.
- As discussed, intravenous drug abuse is a risk factor for HGV/GBV-C infection. While the use of these drugs is very limited in South Africa (150) the principle of percutaneous trauma and carry-over of blood requires further investigation in the South African setting. There is widespread use of scarification (ritual or therapeutic scarring of the skin) in many Black communities. The cleanliness of the blades used in this procedure is often doubtful and carry-over of infected blood may occur. In addition, circumcision is a routine practice in much of southern Africa for men during initiation periods in adolescence. The blades used for these surgical procedures may not always be changed after each circumcision, thereby increasing the risk of viral transmission (151).

2.4.2 Association with liver disease

2.4.2.1 Hepatitis

Primary publications:

The two primary publications describing the discovery of GBV-C and HGV both made the statement that the newly described viruses were associated with hepatitis (102,103). The first dealing with GBV-C (102) entitled, *"Isolation of novel virus-like sequences associated with human hepatitis*" investigated the serum sample from the surgeon (GB) and other sera from patients with non-A-E hepatitis. A significant (unspecified) proportion of these proved HGV/GBV-C PCR positive.

The initial description of HGV (103) entitled, "*Molecular cloning and disease association of hepatitis G virus: a transfusion-transmissible agent*; described a significant positive association between HGV/GBV-C infection and post-transfusion non-A-E hepatitis, chronic non-A-E hepatitis, acute non-A-E hepatitis, as well as co-infection with HBV and HCV.

As a result of these two publications, many investigations followed which assessed liver patients, blood donors and the general community to confirm or refute this position.

2.4.2.2 Later data:

A proportion of the publications following immediately after the discoveries of HGV and GBV-C gave support for the initial hypothesis that HGV/GBV-C was responsible for inducing hepatitis. However, many had both selection and

methodological bias, as they frequently investigated hepatitis patients in the absence of adequate controls or without adequate investigation of risk factors (152,153). However, even studies with adequate controls included suggested that HGV/GBV-C may be implicated in the pathogenesis of non-A-E hepatitis (154).

Most of the later studies have had more acceptable methodologies, and a greater proportion have sampled their study subjects prospectively. The majority of these have not shown an association with hepatitis (141,145,155,156). Two large clinical studies published in the New England Journal of Medicine stand out (141,156). The study by the "USA Sentinel Counties Study of Viral Hepatitis" assessed all samples from patients attending state health department clinics with acute hepatitis (156). When the data was analysed and applied to all cases of acute hepatitis, only 0.3% of individuals were infected with HGV/GBV-C alone. This was not sufficient to implicate the virus in causing hepatitis, given the prevalence in the other groups. In the second study, H. Alter et al. assessed the role of HGV/GBV-C in post-transfusion hepatitis (141). It is clear from that data that HGV/GBV-C transmission to transfusion recipients is a frequent occurrence but that the virus is not a causative agent of post-transfusion hepatitis. In addition, when co-infected with HCV and HGV/GBV-C from the same blood unit, the presence of HGV/GBV-C did not worsen the clinical or biochemical hepatitis.

In addition, many studies from different regions of the world including patients with all known HGV/GBV-C genotypes, have not shown an association between HGV/GBV-C infection and raised liver enzymes (157-159). Indeed, studies have

documented lower ALT values in infected than uninfected blood donors and patients (110,160).

2.4.2.3 Co-infection with HAV, HBV and HCV

Many cohorts have been studied to assess the natural history of chronic HBV and HCV using clinical, biochemical, histological and virological variables. The use of stored serum from these studies has allowed extensive retrospective analysis of the possible additional effect of HGV/GBV-C infection in addition to HBV or HCV. These studies have been uniform in their findings that super-infection or (co-infection) with HGV/GBV-C has no effect on the liver using any one of the four variables above (158,161-163). The same findings apply in the post-liver transplant setting (164,165).

The acute hepatitis study by M. Alter et al. using the "Sentinel Counties Study of Viral Hepatitis" samples also assessed the possible role of co-infection with hepatitis A, B or C and HGV/GBV-C (156). As with all other clinical settings, no hepatitis-inducing causative role could be found for HGV/GBV-C.

2.4.2.4 Hepatocellular carcinoma

The available published data suggests that HGV/GBV-C does not cause hepatocellular carcinomas. Studies from geographic regions representing all HGV/GBV-C genotypes have shown no association (166-170). In addition, co-infection of HGV/GBV-C with either HBV or HCV did not appear to increase the carcinogenic potential of HBV or HCV (171). Only two studies have shown an association with hepatocellular carcinomas, but in both cases many of the

individuals were co-infected with either HBV or HCV (172,173). While the authors of these two publications note the association, no attempt is made to designate this infection as being causative.

2.4.3 Association with other diseases

No disease has been consistently associated with HGV/GBV-C infection to date. There has been speculation that HGV/GBV-C infection is associated with haematological disorders such as lymphoma and myeloysplasia (174-176). However, although certain groups have shown this association, a causative mechanism has not been postulated and further work is required to confirm or refute this hypothesis. It has been postulated that HGV/GBV-C may play a role in renal disease independently of the frequent transfusions that many renal patients receive (144,177-179). However, this also requires further in-depth assessment.

2.4.4 Treatment

No treatment for HGV/GBV-C is currently advocated, as there is no rationale for such intervention in the setting of no apparent pathogenic potential. Retrospective studies have investigated patients treated with interferon for chronic HBV or HCV infection who were co-infected with HGV/GBV-C (180). These show an apparent interferon-mediated HGV/GBV-C viral suppression, but a recurrence of viraemia in most cases after cessation of treatment. No other successful treatment schedules have been reported.

2.5 Study rationale and preliminary work

No work has been performed on HGV/GBV-C infection in the Western- and Eastern Cape Provinces of South Africa to date, and prior to initiating the study, no work had been performed on this virus in southern Africa.

This study, assessing HGV/GBV-C prevalence was divided into three distinct phases. Only phase 3 is presented in this chapter.

- Phase 1 laboratory assessment of HGV/GBV-C carriage was performed on the serum samples collected in Keiskammahoek only. These preliminary results were published in the *Journal of Medical Virology in 1997, volume 53; pages 225-228.* This phase 1 RT-PCR and detection process was performed using the first generation Boehringer Mannheim (now Roche) Hepatitis G Virus primer and capture probe set (Roche Molecular Biochemicals, Germany). The nucleotide sequence of this set was based upon the HGV/GBV-C homology data available at the time.

- In phase 2, contained in chapter 3 of this thesis, a portion of the 5'NCR of South African PCR positive samples was sequenced to ensure that the 5'NCR primers and probe were appropriate for South African variants of HGV/GBV-C. The sequence data showed that the antisense primer used in phase 1 needed modification, due to a lack of homology at five out of the 19 nucleotide positions. (This analysis was confirmed independently by Dr. V. Schlueter of the Roche Molecular Biochemicals group, who also generated sequence data on South African variants.) The antisense primer was substituted in phase 3

by another (42 bases upstream) that was shown to be homologous to all known variants.

- This chapter contains the complete data generated during phase 3 with primers and probes that were homologous with all known (including South African) variants of HGV/GBV-C. Of note, the prevalence figures contained in this chapter differ significantly from that published in the preliminary analysis of phase 1, due to the addition of more positive samples not previously detected.

2.6 Materials and Methods

2.6.1 Communities sampled

This study was performed on the same serum samples from the Western and Eastern Cape Provinces, as described in the previous chapter of this thesis. However, while the hepatitis A-E study evaluated four communities, the HGV/GBV-C assessment contained in this chapter investigated the samples from one urban and one rural community.

2.6.1.1 Western Cape

The urban suburbs of Nyanga and Guguletu were chosen in Cape Town. The volunteers were from both the urban formal housing and informal squatter communities.

2.6.1.2 Eastern Cape

The Eastern Cape area of Keiskammahoek was chosen as the rural comparison site. As in chapter 1, this included people living in formal housing within the

Keiskammahoek town, as well as those living as subsistence farmers in isolated clusters of traditional mud house villages.

2.6.2 Sampling methods

The sampling methods are identical to that described in the previous chapter of this thesis for Keiskammahoek and Cape Town. The only exception is that two of the 251 Keiskammahoek samples contained insufficient serum for HGV/GBV-C analysis. Thus the rural group was reduced to 249 samples.

2.6.2.1 Sample preservation

The serum was stored at -80°C as described in chapter 1. Of note, the serum used for this HGV/GBV-C analysis was a different aliquot to that used for hepatitis A-E testing, and was only thawed for the first time for the HGV/GBV-C analysis.

2.6.3 Reagents and Control Plasmas

Unless otherwise stated, all reagents and HGV/GBV-C control sera used in this prevalence study were supplied (free of charge) by Boehringer Mannheim (now Roche), Mannheim, Germany. The HGV/GBV-C control sera included RNA negative, low titre positive and high titre positive samples.

2.6.4 RNA Extraction

RNA was extracted from 200µl serum using Total RNA Isolation Reagent (Advanced Biotechnologies Ltd, London, UK) according to the manufacturer's instructions. The RNA was precipitated in isopropanol overnight at -20°C, pelleted, washed with 75% ethanol and resuspended in 20µl diethyl pyrocarbonate (DepC) treated ultra pure water.

2.6.5 cDNA synthesis

A cDNA synthesis step was performed in a 20µl solution containing 10µl of the extracted RNA with final concentrations of 50nM random hexamers, 200µM PCR nucleotide mix, 1 U RNase inhibitor, 1X reverse transcription (RT) buffer [50mM Tris-HCl, 8mM $MgCl_2$, 30mM KCl, 1mM dithioerythritol (pH 8.5)] and 10 U Maloney murine leukemia virus (MMLV) reverse transcriptase. This was incubated for 10 minutes at room temperature, 30 minutes at 42°C and 5 minutes at 95°C.

2.6.6 HGV/GBV-C PCR

PCR was performed using primers specific for the 5'NCR of HGV/GBV-C. In brief, 5µl of the cDNA solution was added to a 45µl mastermix containing (final concentrations) 1X PCR digoxigenin (dig) labeling mix (200µM dATP, dCTP, dGTP each; 190µM dTTP, 10µM DIG-11-dUTP), 200nM of both forward and reverse primers, 2.6 U Expand High Fidelity polymerase and 1X PCR buffer [10mM Tris-HCl, 50mM KCl, 1.5mM $MgCl_2$ (pH 8.3)]. Samples were subjected to

40 amplification cycles with three temperature settings of 94°C, 55°C and 72°C each for 45 seconds, followed by a 7 minute elongation period at 72°C. The primers and capture probe used in this study are as follows: The suitability of the primers for amplification South African HGV/GBV-C variants is described above.

	Sequences
5'NCR forward	5'-CGGCCAAAAGGTGGTGGATG-3'
5' NCR reverse	5'-AACACCTGTGGACCGTGCG-3'
5'NCR capture probe	**5'-Biotin-GGTAGCCACTATAGGTGGG-3'**

These primers and probes were provided in the Roche Molecular Biochemicals second generation Hepatitis G Virus Primer and Capture Probe set.

2.6.7 Detection of amplification products

The PCR amplification products were detected using the PCR ELISA DIG Detection system using a standard microtitre plate format with microtitre wells coated with streptavidin. The amplification products (20µl) were diluted 1:3 with denaturation solution (50mM NaOH) and incubated for 10 minutes at room temperature. Hybridisation buffer (440µl) containing 75ng/ml of the biotinylated probe was added and 200µl of this solution pipetted into microtitre plate wells. The plates were incubated for three hours at 42°C and then washed five times. The provided anti-digoxigenin-peroxidase conjugate in Tris-HCl buffer (pH 7.5) was added to the plates, incubated at 37°C for 30 minutes and then washed five times to remove unbound conjugate. The enzyme substrate [1.9mM 2,2' azino-di(3-ethylbenzthiazoline sulfonate), diammonium salt; ABTS] in 100mM

phosphate citrate buffer (pH 4.4)-3.2 mM H_2O_2 (as sodium perborate) was added and the colour developed for 30 minutes at 37°C. The absorbance was measured at 405nm and samples considered positive if the sample optical density was >3 times the negative control optical density.

Serum samples were assessed in batches of 12 inclusive of negative and positive controls. The results of the RT-PCR were only accepted if the results of the control sera were within acceptable limits, according to the manufacturer's instructions.

2.6.8 Statistical Methods

Statistical analysis was performed using the Epi-Info version 6 software program (Center for Disease Control, Atlanta, USA) and the Statistica software program (Statsoft, Tulsa, USA).

- The differences in prevalence were analysed using the Chi squared test.
- Demographic data were tabulated and factors associated with increased risk of infection were assessed using discriminant analysis by stepwise method.
- Stratified analysis was used to assess the interdependence of risk factors.
- A *p*-value of <0.05 was considered to indicate statistical significance.

2.7 RESULTS

2.7.1 Demographic data

A cohort of 484 people were studied, of whom 249 were from the rural region of Keiskammahoek and 235 from the urban area of Cape Town.

- The rural sample was divided into the 160 (64.3%) individuals recruited during the random community sampling and the 89 (35.7%) recruited from the OPD. All of the random community sample volunteers lived in traditional mud houses with no access to in-door water supply nor waterborne sewerage. The majority (60%) of the rural OPD sample group lived in these same conditions, while 30% lived in formal housing.
- The urban group consisted of 135 individuals (57%) living in the formal housing sector and 100 (43%) individuals living in squatter settlements.

The mean age of the entire group was 43.8 years (range 18-92). However, the mean ages of the rural and urban groups (47 and 40 years respectively) was significantly different ($p=0.02$). The male:female ratios of the rural and urban groups were 1:1.6 and 1:2.6 respectively. No significant differences ($p>0.05$) were shown between the urban and rural groups with regard to a history of ritual scarring (73.0% vs. 69.8%), major trauma (16.7% vs. 14.9%) and a history of hepatitis or any form of liver disease (4.0% vs. 3.8%). However, the urban group showed greater exposure to blood products (12.9% vs. 20.4%; $p=0.02$).

2.7.2 Prevalence

One hundred and thirty of the 484 samples were HGV/GBV-C PCR positive, giving an overall prevalence of 26.9%. The rural and urban prevalences of 20% and 34% were significantly different (p=0.0005).

2.7.2.1 Community versus OPD sampling

The prevalence did not differ significantly in the rural group between the OPD and community sampling groups (20.9% and 19.4% respectively), thereby validating the OPD sampling procedure for the analysis of HGV/GBV-C viraemia. No significance was demonstrable between the urban squatter and formal housing groups (p=0.17).

2.7.3 Risk Factors

2.7.3.1 Univariate analysis

Univariate analysis of the questionnaire variables showed a significant relationship between HGV/GBV-C infection and younger age (p=0.016) and the use of illicit drugs (p=0.045). All positive drug histories related to marijuana usage and none of the recruits gave a history of intravenous drug abuse. Further analysis showed that this significant association with drug use was only valid for the urban group. Those living in squatter communities had a lower prevalence of HGV/GBV-C (p=0.002). However, this statistical significance was not evident when controlling for urban / rural place of residence.

The use of pit latrines for sewerage disposal was associated with a significant reduction in HGV/GBV-C prevalence as compared to waterborne sewerage and bucket latrines ($p=0.003$). There were very small numbers of rural inhabitants with access to waterborne sewerage and equally few urban inhabitants using pit latrines. Thus, although the numbers prevented formal assessment, the association with sewerage disposal method may be a confounding factor relating to rural / urban life.

Univariate analysis showed no relationship between infection and gender, water source, employment, a history of hepatitis or any form of liver disease, past blood transfusions, scarification, excessive alcohol consumption or major trauma.

2.7.3.2 Discriminant analysis

Discriminant analysis was performed by forward and reverse elimination, and the following results were obtained using forward elimination (standardised coefficients in brackets): drug usage (0.583), younger age (0.498), past blood transfusions (0.295) and female gender (0.136). The positive predictive value of these four factors was 59.6% and the negative predictive value was 61.6%. Reverse elimination analysis did not show any combination of these factors to have significant predictability.

2.8 Discussion

This data shows the prevalence of HGV/GBV-C in adult Black South Africans to be extremely high and to have significant variation between urban and rural regions. It was expected that the South African prevalence would be higher than that seen in the USA and Europe, based on the published data from other developing countries (103,128,130,139,142). However, the mean prevalence of this cross-section of South African Black adults (26.9%) is higher than any other published figure to date, excluding those studies assessing extremely high risk groups such as intravenous drug abusers. This study aimed to assess the carriage rate of HGV/GBV-C, and does not address the issue of past infections, as demonstrated by the presence of serum anti-E2. It would be expected that HGV/GBV-C exposure rates would be significantly higher than the figure shown here for carriage.

This prevalence figure is also significantly higher than the South African figure generated by Casteling et al. in Johannesburg blood donors (11.1%) (139). While this may appear to be a major discrepancy, the higher prevalence of HGV/GBV-C in Black communities is in keeping with other data on parentally transmitted viruses in the transfusion service. Those attending blood transfusion services around the world represent a highly selected community, as a consequence of the stringent exclusion criteria applied to every donor (181). The same phenomenon applies in South Africa, where blood donors are predominantly from the middle class Caucasian population, while the data contained in this thesis describes the epidemiology of viral markers in extremely poor Black South Africans.

For example, the following comparison data is available from the Western Province Blood Transfusion Service (WPBTS) in Cape Town:

- hepatitis B carriage is approximately 3-5% in the adult Black community (chapter 1), while first-time blood donors of the WPBTS have a prevalence of 0.87% (personal communication with Dr. A. Bird, WPBTS).
- As discussed in chapter 1 of this thesis, anti-HCV antibodies are detected in 1.8% of the adult Black community of the Western Cape, while the prevalence is only 0.41% in blood donors of the same province (78).
- The HIV prevalence in 1997 amongst antenatal clinic attendees in the Western Cape was 6.3% (Department of Health 1997 survey), while the percentage positive donations (out of 140 000) at the WPBTS in 1997 was 0.045% (personal communication with Dr. A. Bird, WPBTS).

The prevalence figures of this study were validated by the presence of adequate controls. Firstly, all batches were run in groups of 12 sera, including a positive and negative control. If either of these was not within the expected norms, the batch was repeated. Secondly, all normal precautions were taken to prevent DNA contamination of samples (182) including the use of four different rooms for the different stages: a) mastermix preparation; b) RNA extraction and cDNA synthesis; c) PCR; and d) detection. The primers and probe used in this study to detect HGV/GBV-C were designed according to consensus sequence data generated South African variants.

The urban and rural groups were well matched with regard to the histories obtained for scarification, liver disease and major trauma. However, the urban group comprised more women and a greater proportion of younger individuals with a greater percentage who had received blood transfusions. While it is difficult to explain the gender differences, the younger age of the urban group is in keeping with the movement of young adults to major urban areas in seek of employment. The fact that few give a history of liver disease is in keeping with the experience of developing countries, where almost all are exposed to HAV very early in life, and many exposed to HBV before the age of five (94,183). Both infections are most commonly subclinical when acquired early in life.

The random community sampling was performed to validate the OPD sampling procedure. This showed no difference in prevalence between the two sampling procedures, thereby validating the OPD recruiting exercise for HGV/GBV-C. No significant difference was demonstrated between the urban formal housing group and the urban squatter communities. Squatter communities live in conditions that are less hygienic than their counterparts in formal housing, and do not have a potable water supply within the house. This comparison of the urban communities suggests that the life-style associated with inferior living conditions alone is not responsible for the increased risk of acquiring HGV/GBV-C in urban communities.

Analysis of the questionnaire variables showed the use of marijuana and younger age to be associated with infection. None of the drug usage was intravenous. This is in keeping with reports from South Africa showing there to be very little

current abuse of such drugs (150). The relationship with drug use was only valid for the urban group, suggesting that the association was probably life-style related, rather than the use of the drugs themselves. The association with younger adults acquiring the virus has been documented in other studies (123,184). It is important to note that the associations between infection and age and living in an urban city are variables that are independent of each other. Discriminant analysis showed a history of transfusions and female gender to be predictors of HGV/GBV-C positivity. However, even when combining the drug usage, age, transfusions and gender in this analysis, the positive predictive value of the combination was poor.

The use of a pit latrine for sewerage disposal as well as living in a traditional mud house were associated with a lower prevalence of HGV/GBV-C using the univariate analysis. However, the discriminant analysis did not show these to be significant factors. In addition, these associations were shown to be statistically insignificant when controlling for urban / rural living.

In summary, HGV/GBV-C carriage rate is high amongst adult Black South Africans of the Western and Eastern Cape Provinces. The prevalence rates differed significantly between urban and rural volunteers. Certain factors appeared to be associated with infection, although no combination of the collected variables was shown to be adequately sensitive in predicting infection.

CHAPTER 3

Molecular characterisation of the 5' non-coding region of South African variants of HGV/GBV-C

3.1 Summary

HGV/GBV-C has been characterised as a novel flavivirus, and to date three known genotypes have been characterised from West Africa, Europe/USA and Asia respectively. These genotype analyses have been predominantly based on the sequence variation of the 5'NCR of the virus. Although point mutations and point insertions/deletions have been documented in all flavivirus non-coding regions, no major deletions have been shown to date. The 5'NCR of HGV/GBV-C appears to regulate protein translation via an internal ribosomal entry site (IRES).

A 344bp PCR product of the 5'NCR of HGV/GBV-C, representing more than 60% of the 5'NCR was cloned, sequenced and analysed from 32 HGV/GBV-C PCR positive volunteers. Wild-type virus amplicons were detected in all samples. However, 5/32 (15.6%) also amplified another smaller PCR fragment that ranged between 205 and 231bp. A deletion of between 113-131bp was detected in isolates generating the smaller bands. Sequence analysis showed all cloned PCR fragments to be HGV/GBV-C-specific. RNA secondary structure analysis of the 5'NCR showed the deletions to be over the regions previously named domains II and III. This data suggest that nucleotides 303 to 444 may be non-essential for 5'NCR functioning and maintenance of the IRES.

Phylogenetic analysis demonstrated a novel fourth South African genotype, distinct from genotypes 1-3 with DNA distances of >0.1000. ALT and AST values for the wild-type and mutant samples were normal.

3.2 Introduction

HGV/GBV-C variants have been shown to have genetic relatedness to previously described viruses such as HCV, yellow fever virus and dengue virus, all of which are known members of the family *Flaviviridae*. Thus, much of our current understanding of HGV/GBV-C biology is based on prior knowledge of the molecular features of these (104).

The structural and genetic constituents of HGV/GBV-C and other flaviviruses is reviewed below. However, the literature review relating to flavivirus (and HGV/GBV-C) replication will be contained in the following chapter of this thesis.

3.3 Flavivirus characteristics

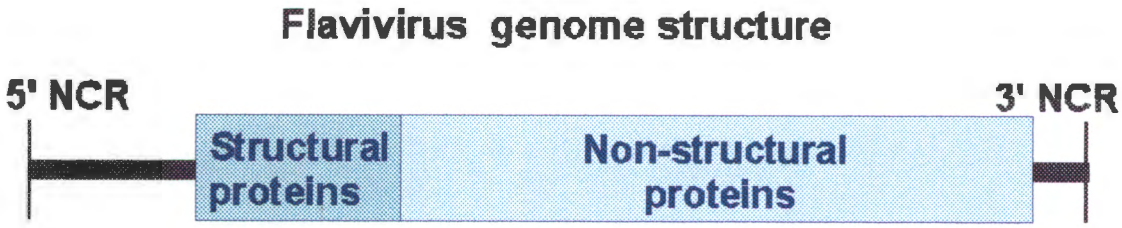
3.3.1 Physicochemical

Not all members of the family *Flaviviridae* have been visualised to date. However, the electron microscopy data from those that have been described show enveloped virus particles that are 40-60 nm in diameter, spherical and smooth in appearance. The buoyant density of this family of viruses ranges from 1.19 to 1.23 g/ml and virions are composed of 6% RNA, 66% protein, 9% carbohydrate and 17% lipid (185).

3.3.2 Genome and protein description

Flaviviruses contain one single-stranded, positive sense RNA molecule. These genomes vary in length and are approximately 9,5-12,5kb (186). Figure 3.1 below is descriptive of the overall genetic structure.

Figure 3.1:



3.3.2.1 Non-coding regions

The 3' and 5' ends of flaviviruses' single open reading frames (ORFs) are flanked by non-coding regions that are thought to have the capacity to regulate viral replication. Both of these flanking regions have a high degree of homology within the specific genus, and in addition have limited sequence similarities with other members of the family *Flaviviridae* outside of the genus (186). Certain members of the family *Flaviviridae* have a poly-A tail at the end of the 3'NCR, while in others this is absent (186-188). A type 1 cap of the 5'NCR is thought to regulate ribosome binding in a subset of flaviviruses (see below). Flavivirus 5'NCRs vary in length from below 100 bases to more than 400 (186,189,190). The type of ribosomal binding is thought to be in part determined by the length of the 5'NCR.

3.3.2.2 Core proteins

The gene for flavivirus core proteins is contained in the coding region adjacent to the 5'NCR and upstream of the envelope coding region. As a result of its proximity to the 5'NCR and IRES, it is the first viral protein to be translated (186). Core proteins are responsible for the formation of the nucleocapsid structure (191). While there are conserved RNA regions coding for both hydrophobic and hydrophilic amino acid domains within the core protein, there is little RNA homology between the different flaviviruses (191).

3.3.2.3 Envelope proteins

Flaviviruses are contained in a lipid envelope that renders them susceptible to destruction by organic solvents and detergents (185). The envelope proteins are believed to be important for receptor binding and internalisation of the virus (192,193), but in addition, are the major immunological targets for neutralising antibodies (194). As a consequence certain flaviviruses, such as HCV, have developed mechanisms of escaping immune capture by way of constantly varying the amino acid sequence of this region through regular mutations in the nucleotide sequence (195,196). The envelope coding sequences are found with the other structural components of flaviviruses in the 5' one third of the genome. Comparison of corresponding envelope coding regions of different viruses within the family *Flaviviridae* show them to have areas of sequence homology (186,191).

3.3.2.4 Non-structural proteins

The NS2, NS3, NS4, NS5A and NS5B genetic regions of flaviviruses are packaged in the 3' two thirds of the genome, and are known to code for the non-structural components of the viruses. The functions of all of these components are not well documented. However, the NS2 protein is composed of two portions, A and B, both of which appear to have proteolytic activity, with the NS2B portion having maximal activity when complexed with the NS3 protein (197,198). The NS3 region codes for a non-structural protein (predicted 68-70 kd) with at least helicase, protease and RNA triphosphatase functional capacity (104,199-201). The NS5B protein is the largest and most conserved (~103 kd) of the non-structural components and is believed to be the RNA-dependant RNA polymerase protein (202,203). This understanding is based on the finding of a highly conserved sequence motif (GDD) that is characteristic of known viral RNA-dependant RNA polymerases. Little is known about the functions of the NS4 proteins of flaviviruses.

3.4 Literature review of HGV/GBV-C molecular characteristics

3.4.1 General

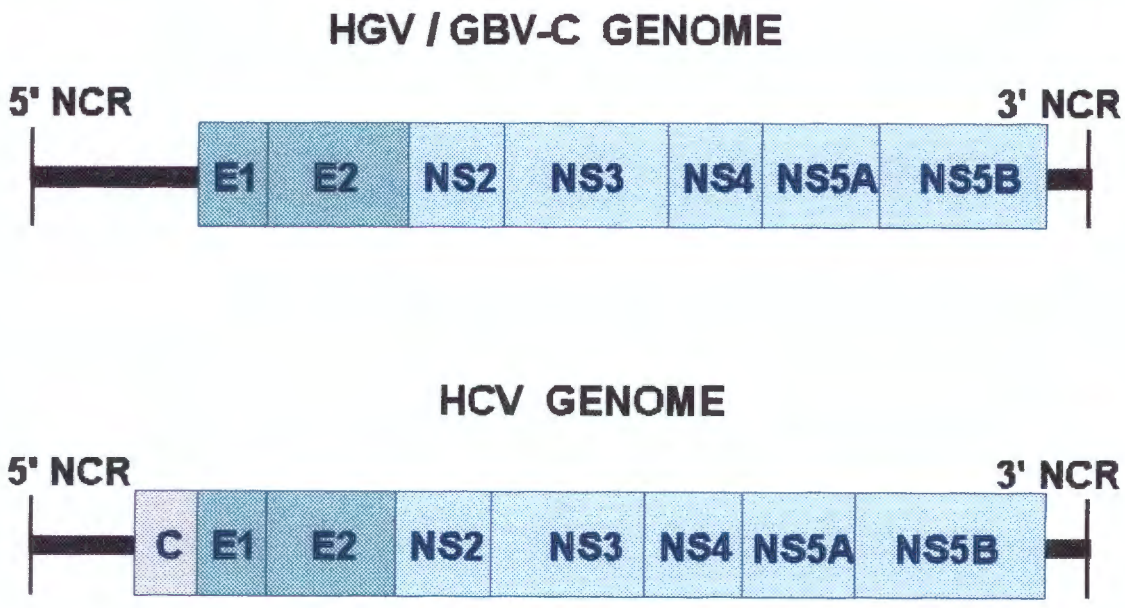
The discovery of GBV-A, GBV-B, GBV-C and HGV has resulted in a greater understanding of the phylogenetic relationships within the family *Flaviviridae* and its genera. Full and partial nucleotide sequences of various geographical isolates of these viruses have been published and are available for comparison. Initial sequence data suggested that the HGV/GBV-C isolates may be novel members of the genus *Hepacivirus* within the family *Flaviviruses* (103,104,204,205).

However, it is now evident that there is only approximately 30% nucleotide homology with HCV, thereby excluding there being such a close association (104,206).

Although it is thought that the full genetic sequence of HGV/GBV-C is known, the vast majority of analytical work has been based on the 5'NCR sequences. Less is known about the sequence and functional aspects of the other components of HGV/GBV-C. As the work to be presented focuses on the 5'NCR of South African variants of HGV/GBV-C, the literature review below will focus on this component.

Figure 3.2 below demonstrates the genetic structure of two flaviviruses aligned for the purpose of comparison. The HGV/GBV-C genome is placed above and is compared to that of HCV clade 1 (adapted from Leary et al. Journal of Medical Virology (104)).

Figure 3.2:



3.4.2 The 5' non-coding region

The most conserved regions of the HGV/GBV-C genome are the 5'- and 3'NCRs. The 5'NCR has a nucleotide homology of greater than 95% between isolates of different geographic origins (103-105,207). As a consequence, many diagnostic PCR assays are designed with primers specific to this region, so as to optimise the sensitivity of genome amplification (208).

While there is good overall homology in the 5'NCR, this region has three variable regions (V1-3) where significant differences between different isolates are demonstrable (206). The nucleotide substitutions are in the most, consistent amongst isolates from similar geographic regions and genotypes (105). As described below, the substitutions in this region are mostly covariant, thereby allowing for maintenance of RNA secondary structure (209). Single base insertions and deletions have been demonstrated in the 5'NCR of all flaviviruses. However, no major deletions have been shown to date in the 5'NCR of any HGV/GBV-C, HCV, GBV-B or GBV-A isolate.

3.4.2.1 5'NCR boundaries

The extreme 5' RNA sequence of the HGV/GBV-C 5'NCR was described subsequent to the initial discoveries of HGV and GBV-C, using a modified RACE technique (rapid amplification of cDNA ends) (206). In describing this region, the investigators have found evidence suggestive of an RNA hairpin loop at the extreme 5' end of the RNA that is similar to that seen in the models of HCV

5'NCR structure (206,210). It is possible that this hairpin loop is important in self-priming of the RNA during RNA replication (206,210).

There remains a level of uncertainty with regard to the position of the 3' end of this NCR, as the 5'NCR of HGV/GBV-C has multiple potential start codons that are in-frame with the downstream open reading frame, available for translation initiation (209). The initial uncertainty was in part due to an unsuccessful search for the viral core protein coding sequence. As discussed below, there is no evidence supporting the presence of a typical core protein sequencing region for both HGV/GBV-C and GBV-A, although GBV-B has sequence typical of a core region. Hsieh et al. (1997) suggested a 465 nucleotide long non-coding region for HGV/GBV-C (206). However, subsequent evidence has suggested that the start codon for the HGV/GBV-C open reading frame is 93 nucleotides further downstream (209,211).

3.4.2.2 Regulation by the 5'NCR

The 5'NCRs of flaviviruses are thought to regulate translation of their respective single downstream open reading frames (205,210). The open reading frame codes for both functional and structural proteins together. These proteins are cleaved later and modified post-translationally.

The mode by which the 5'NCRs of different flaviviruses regulate translation appears to vary.

- The flaviviruses with shorter 5'NCRs (e.g. dengue and yellow fever) are thought to use an eukaryotic ribosome scanning mechanism. In this model of

ribosome activity, the ribosome binds at the 5' cap structure of the RNA and scans the region until the functional (not necessarily the first) AUG codon. At this point, the translation of the downstream open reading frame begins (212).

- The flaviviruses with longer 5'NCRs are thought to initiate translation in a similar fashion to that employed by picornaviruses, where the ribosome binds to an internal ribosomal entry site (or landing pad) created by the secondary structure of the RNA (213). HGV/GBV-C has a very long 5'NCR and initial experimental evidence suggests that it uses this second strategy to regulate translation (205).

3.4.2.3 5'NCR secondary structure

The secondary (and tertiary) structure of the 3' terminal portion of the 5'NCR RNA, as well as the initial portion of the coding region, appears important for ribosome binding in flaviviruses (205,209). Thus mutations / alterations to this RNA sequence may change the structure to varying degrees. The necessity to maintain the structure of the ribosome landing pad is almost certainly the reason for the conserved nature of the entire 5'NCR. Although the ribosome binding site is relatively close to the start codon (210), the entire non-coding region is thought to act as a scaffold for the maintenance of the terminal portion structure. It is thus not surprising that Fukushi et al. (1994) have documented that a complete 5'NCR of HCV is required for maintenance of the internal ribosomal entry site (214). The highly conserved nature of both the HGV/GBV-C and HCV 5'NCRs suggests the same may hold for HGV/GBV-C protein translation.

Different groups have attempted to predict the RNA secondary structure of the HGV/GBV-C 5'NCR of isolates falling into the genotypes 1 and 2 (205,209). These proposed structures have similarities with that proposed for the 5'NCR of GBV-A (205) and show relatively little variation between isolates. Maintenance of the RNA secondary structure has been shown to be important. Therefore it is no surprise that when comparing these proposed structures with the nucleotide sequence, the majority of nucleotide substitutions are covariant substitutions (209). Thus, although there is diversity over the three variable regions of HGV/GBV-C, the RNA structure remains relatively constant.

It is difficult to say with certainty that the RNA structure proposed in these mathematical analyses is a fair indication of the in-vivo structure, as inevitably the RNA form is not static, and is dependant on energy levels and the biochemical cellular milieu (215). Accurate determination of the RNA structure is also dependant on having sufficient of the upstream RNA sequence in the analysis, so as to adequately represent the formation of the entire region (215). The different energy levels and cellular environments will inevitably have an effect on the structure of this region and in doing so, either allow replication of the virus or not. Conventional software programs predict the secondary, but not tertiary structure of RNA molecules. Thus, the results are probably an over simplification of the complexities of RNA structure.

3.4.3 Core

The genetic relatedness of HGV/GBV-C and HCV is evident from the diagram above (Figure 3.2), with the exception of the coding segment for the core protein

that is absent in HGV/GBV-C (103,205,207,216). Analysis of the entire genome of HGV/GBV-C (and GBV-A) has not demonstrated any RNA region with sequence suggestive of a nucleocapsid protein. This is unexpected, as these are the only two flaviviruses that have been described without a core coding sequence (101,205). The possible reason for its absence and the functional implications thereof remain unexplained. It has been speculated that HGV/GBV-C may have a core protein that is very different from those previously described before for other flaviviruses, or that it may be reliant on either a cellular protein or the core protein of another virus to act as a surrogate core protein (209).

3.4.4 Envelope

HGV/GBV-C has two coding regions for envelope proteins. The E1 gene of HGV/GBV-C is shorter than that of the E2 coding region and has greater homology with the corresponding E1 region of HCV than the E2 region (104). The E1 and E2 coding regions are located just downstream of the 5'NCR and unlike envelope proteins of other flaviviruses such as HCV, show a high degree of RNA homology between different HGV/GBV-C isolates (217-219). HCV has a hypervariable region in this area, and this is thought to facilitate escape from immune capture, as described above.

The lack of nucleotide variability in the HGV/GBV-C E1 and E2 genes is reflected in great homology in envelope amino acid sequences. As these envelope proteins are targeted by the immune system for viral clearance (217), this offers the virus no mechanism of temporary escape from immune capture. As a consequence, while antibodies to HCV envelope proteins are mostly a marker of

chronic infection, antibodies to the E2 protein of HGV/GBV-C are invariably associated with viral clearance (130). Thus, as described in chapter 2, the presence of viral RNA and antibodies are almost mutually exclusive.

3.4.5 Non-structural regions

The genes coding for the non-structural components of HGV/GBV-C are found in the two-thirds of the genome downstream of the E2 region (104). Although there is a degree of homology with HGV/GBV-C and other flaviviruses, the complete functions of all of the components remains unclear.

The similarity of the NS2 RNA segment of HGV/GBV-C to other flaviviruses suggests that this region codes for a viral protease. However, full clarity with regard to it's function in HGV/GBV-C biology remains unclear. The NS3 region has the greatest homology with the equivalent HCV genome segment and probably codes for both a protease and a helicase protein (104).

The functional aspects of the NS4 and NS5A segments remain undescribed. Analysis of NS5A sequences generated from central African HGV/GBV-C isolates show a 36 nucleotide insertion (220). The NS5B region is thought to code for the RNA-dependant RNA polymerase protein (104). This enzyme is central to the production of RNA replicative intermediaries during viral replication. The negative strand RNA is then used by the same enzyme for production of further virion RNA (see chapter 4 for further description).

3.4.6 The 3' non-coding region

Comparatively little data is available on the genetic variability of the 3'NCR of HGV/GBV-C, although the available data does suggest that it is highly conserved (104,221). Although initially thought to be only approximately 60 bases in length (104), it was later found to be in excess of 310 bases (211,221). HGV/GBV-C has neither a poly-A nor a poly-U tail configuration (104,211,221). The same is seen in GBV-A, once again showing its closely shared origins with HGV/GBV-C.

3.4.7 Genetic relatedness of different HGV/GBV-C isolates

3.4.7.1 Phylogenetic analysis

Phylogenetic analyses of GBV-A, GBV-B, GBV-C, HGV and HCV have shown the following:

- HGV and GBV-C are variants of the same virus (thus HGV/GBV-C) that form a distinct group separate from HCV; and
- HGV/GBV-C is most closely related to (but distinct from) GBV-A.

These analyses demonstrate the presence of three genotypes which show consistent geographical clustering with West Africa, USA/Europe and Asia. These have been named genotypes 1, 2 and 3 respectively (105,207,211,221,222). The genetic variability of genotypes 1 and 2 has led to certain investigators subgrouping each of these into genotypes 1A/B and 2A/B respectively.

The most consistent phylogenetic analysis results for HGV/GBV-C are reported to be those generated from the sequence of either the complete genome or the 5'NCR (222,223). Phylogenetic analysis of shorter HGV/GBV-C fragments from the E2, NS3 or NS5B region produce divisions that do not cluster according to geographic origins (216,223).

3.4.7.2 DNA distance

DNA distance analysis allows another measurement of variation in the genome and more specifically the evolutionary distance between the genotypes described. Evolutionary differences have been described for the HGV/GBV-C genotypes 1-3, using 5'NCR sequence (207). Table 3.1 below describes their findings (modified from A.S. Muerhoff; Journal of Hepatology, 1996, Vol 25, page 382). It is evident that the DNA distance within each genotype is significantly less that that seen between genotypes.

Table 3.1: DNA distances values for HGV/GBV-C genotypes 1-3.

HGV/GBV-C genotype	1A	1B	2A	2B	3
1A	**0.056**	0.092	0.133	0.123	0.115
1B		**0.053**	0.137	0.127	0.128
2A			**0.039**	0.065	0.101
2B				**0.034**	0.092
3					**0.012**

3.5 Study motivation

No isolates from Southern Africa have been included to date in the studies characterising the 5'NCR region of HGV/GBV-C.

3.6 Aim

Thus, this study aimed to investigate the molecular characteristics of the 5'NCR of South African variants of HGV/GBV-C, and compare them to those previously described.

3.7 Materials and methods

3.7.1 Specimens:

3.7.1.1 Sample number and profile

Thirty two HGV/GBV-C RNA positive serum specimens were obtained from volunteers recruited in the Western and Eastern Cape Provinces of South Africa. The serum specimens were taken from those who were HGV/GBV-C RT-PCR positive, as described in chapter 2 of this thesis. These included individuals residing in both rural villages and the metropolitan urban area of Cape Town.

3.7.1.2 Control samples

Positive and negative control sera were supplied by Roche Molecular Biochemicals, Germany.

3.7.2 RNA extraction

RNA was extracted from 200 μl serum using Total RNA Isolation Reagent (Advanced Biotechnologies Ltd., London, UK), as described in chapter 2 of this thesis.

3.7.3 cDNA synthesis

A cDNA synthesis step was performed using MMLV reverse transcriptase and random hexamer primers, as described in chapter 2 of this thesis.

3.7.4 HGV/GBV-C PCR and Detection

3.7.4.1 General parameters

A nested PCR was performed on all 32 samples using the primers described below (224). Five microlitres of the above cDNA solution (or outer product) was added to a 45μl mastermix containing (final concentrations) 200μM dATP, dCTP, dGTP and dTTP each, 2.6 U Expand High Fidelity polymerase and 1X PCR buffer [10mM Tris-HCl, 50mM KCl, 1.5mM $MgCl_2$ (pH 8.3)] (all Roche Molecular Biochemicals, Germany) and the relevant forward and reverse primers. Samples were subjected to 35 amplification cycles as described in chapter 2 of this thesis.

3.7.4.2 Primers

sense	5'-AGGTGGTGGATGGGTGAT-3'
antisense	5'-TGCCACCCGCCCTCACCCGAA-3'
nested sense	5'-TGGTAGGTCGTAAATCCCGGT-3'
nested antisense	5'-GGAGCTGGGTGGCCCCATGCAT-3'

3.7.4.3 PCR amplicon size and region

The final PCR product (inclusive of nested primers) spanned a predicted 344 base pair (bp) region of the 5'NCR from base 139 to 483 (numbering according to that of accession number U76893) (206).

3.7.4.4 Visualisation

The PCR products were separated by electrophoresis in 2% agarose gel and visualised under ultra-violet light using ethidium bromide staining.

3.7.5 Southern Blot

The PCR fragments were confirmed as being HGV/GBV-C specific by Southern blot hybridization analysis as follows.

3.7.5.1 Transfer of PCR products

The PCR products in the agarose gel were denatured for 30 minutes in a 0.5M NaOH 1.5 M NaCl solution and subsequently neutralised for 40 minutes in 20 mM NaOH 1M ammonium acetate. The PCR products were transferred from the agarose gel to a Hybond-N nylon membrane (Amersham, UK) by capillary method, and cross-linked to the nylon membrane by applying 2400 watts of ultraviolet light.

3.7.5.2 Prehybridization

The nylon membrane was placed in the rotating tube of a hybridisation oven for 4 hours at 42°C in a 40 millilitre solution containing 6X standard sodium citrate (SSC), 0.1% blocking reagent (Roche Molecular Biochemicals, Germany) and 1% sodium dodecyl sulphate (SDS) (see appendix for reagents).

3.7.5.3 Probe and probing conditions

A denatured digoxigenin-labeled HGV/GBV-C PCR product was used as a probe for confirmation that the amplicons visualised on the gel were HGV/GBV-C sequence specific. This PCR product was generated as described in the previous chapter of this thesis from an HGV/GBV-C positive sample (ZAN22), and spanned 186bp of the 5'NCR of HGV/GBV-C.

The cDNA probe was denatured by boiling for 5 minutes followed by immediate freezing on dry ice. The probe was then added to the prehybridisation solution and incubated overnight at 42°C.

3.7.5.4 Stringency washes

The nylon membrane was removed from the hybridisation solution and washed twice at 42°C for 15 minutes in a solution containing 2 X SSC 0.1% SDS. This was followed by a second round of stringency washes with identical temperature and time parameters using a 0.1X SSC 0.1% SDS solution. Finally, the membrane was washed for 5 minutes at room temperature in washing buffer (buffer 1containing 0.3% Tween-20) (see appendix for buffer 1).

3.7.5.5 Detection system

All detection steps below were performed at room temperature. The membrane was incubated in 1% blocking (see appendix) buffer for 30 minutes, followed by a further 30 minutes incubation after the addition of anti-digoxigenin Fab fragments (1:10 000 dilution) (Roche Molecular Biochemicals, Germany). The membrane

was washed twice in washing buffer for 15 minutes and equilibrated for 2 minutes in buffer 3 (see appendix). A 1:100 dilution of CDP-Star (Roche Molecular Biochemicals, Germany) in buffer 3 was added and incubated for 5 minutes. The excess liquid was removed and the membrane sealed in a plastic covering. The membrane was exposed to medical X-ray film (Agfa, Belgium) for 1 minute, followed by processing of the film by conventional methodologies.

3.7.6 Cloning

PCR products were cloned using the pCR-Script Amp SK^+ cloning kit (Stratagene, La Jolla, CA) as suggested by the manufacturer. Transformed *Epicurian coli*® XL1-Blue cells were grown overnight on agarose 2 X yeast-tryptone broth containing 100μM ampicillin, 100μM 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) and 0.5μM isopropyl-1-thiol-β-D-galactopyranoside (IPTG) (see appendix). Bacterial colonies were selected according to blue / white differentiation and grown for 16 hours in 100ml 2 X yeast-tryptone broth containing 100μM ampicillin.

Plasmid DNA was extracted (Nucleobond, Macherey-Nagel GmbH, Düren, Switzerland) and subjected to restriction enzyme digestion with Pvu II (Roche Molecular Biochemicals, Germany) to confirm the inclusion of the desired cDNA fragment size. Digested plasmids were separated in 2% agarose gels containing ethidium bromide and visualised by ultraviolet light illumination.

3.7.7 Sequencing

3.7.7.1 Samples

Eight of the samples containing the predicted HGV/GBV-C fragment size were sequenced. In addition, a clone from all five of the samples shown to have a smaller inserted fragment was sequenced.

3.7.7.2 Sequencing method

Sequencing was performed in both the forward and reverse directions using the Pharmacia™ ALFexpress® automated sequencer (AM version 3.01, Pharmacia Biotech) by Ms. D. James of the University of Cape Town Department of Microbiology. Any sequence discrepancies were resolved by repeat sequencing.

3.7.8 Supplementary testing of mutant samples

Serum samples found to contain a virus with the deletion (see results) were re-evaluated in the following manner for confirmation of this finding.

3.7.8.1 Repeat testing

The complete RT-PCR process was repeated using the same methodology.

3.7.8.2 Heat denaturation

The RNA suspensions were heat denatured at 95°C for five minutes followed by immediate cooling on ice, prior to reverse transcription so as to reduce the secondary structure of the RNA region during reverse transcription.

3.7.8.3 Supplementary nested PCR

Samples that generated only a mutant virus amplicon with the above protocol were subjected to a separate supplementary nested PCR. This supplementary PCR assay used the same "outer" reaction described above. However, the nested PCR was designed with an antisense primer internal to the deleted region, thereby preventing amplification of the mutant and excluding the possibility that the mutant virus was "out-competing" the wild type virus in the PCR reaction. The following antisense primer was used in the nested reaction: 5'-AGAGAGACATTGAAGGGCGACG-3'.

3.7.9 Sequence Analysis

3.7.9.1 Numbering system and comparison sequences

The nucleotide numbering used in the analysis below was derived from either:

A) the position within the PCR product or;

B) according to the numbering system of accession number U76893 (206).

Sequences that were used for comparison with the South African samples were representative of all three genotypes previously described: (accession numbers) U59518-21, U59529-33, U59543-53, U76893, D87251, D87708-14 and D90601. The original HGV isolate, PNF2161 (U44402) and the GBV-C isolate (U36330) were also included in the analysis.

3.7.9.2 Sequence alignment and phylogenetic analysis

Alignment of sequences was performed using ClustalW version 1.6 (European Molecular Biology Laboratory, Heidelberg, Germany) with final manual alignment by eye.

Phylogenetic analysis was performed with ClustalW and Treecon for Windows version 1.1 (Antwerpen, Belgium), using the neighbour joining method and Kimura algorithm, with gaps included. Phylogenetic trees were visualised using the Treeview computer program version 1.2 (IBLS, University of Glasgow).

3.7.9.3 Bootstrap analysis

ClustalW was used to assess the statistical reliability of the tree using the Felsenstein bootstrap method based on 100 repetitions.

3.7.9.4 Inclusion of mutant samples in analysis

The tree and bootstrap values were initially generated comparing only the wild-type South African variants with the other published sequences. Deletion isolates were then included individually (using the same methodology) with the deletion gap considered to be one mutational event.

3.7.9.5 DNA distance analysis

Intra- and inter-genotype DNA distance values were generated with ClustalW.

3.7.10 RNA secondary structure analysis

The predicted RNA secondary structure was generated using the *MFold* computer program (215). The *Mfold* program uses the energy rules, as suggested by Turner et al. (225). This program can be accessed at the following URL address: [http://www.ca.aecom.yu.edu/GCGdoc/Program_Manual/RNA_Secondary_Structure/mfold.html].

3.7.11 Biochemistry

Serum ALT and AST were measured (by the Chemical Pathology Department staff of the University of Cape Town) using the Roche Diagnostics 747 serum analyser.

3.8 Results

3.8.1 Initial analysis

3.8.1.1 Wild type

Twenty seven of the 32 samples (84.3%) amplified the predicted 344bp PCR fragment (Figure 3.3).

3.8.1.2 Mutant

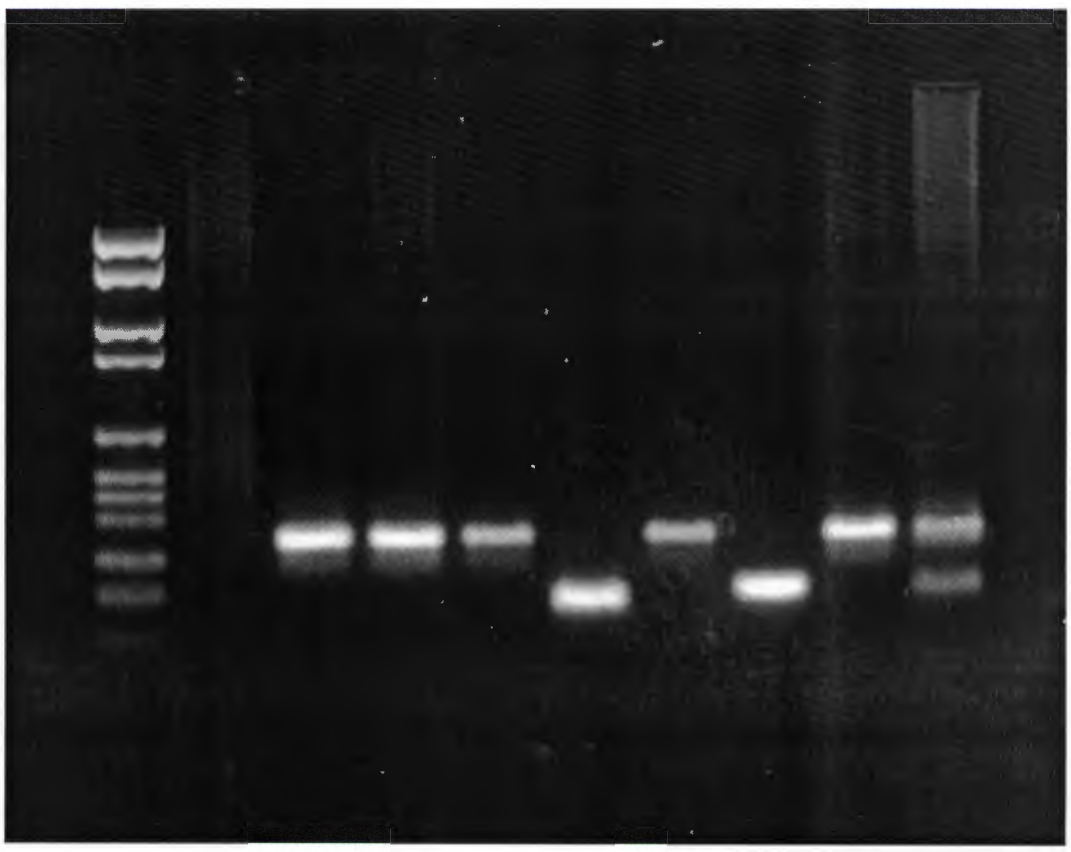
Of the remaining five samples, three (9.4%) generated smaller PCR fragments of 214, 215 and 231bp respectively (ZAF87, ZAN11 and ZASq13). The last two samples (6.3%) (ZAF3 and ZAF115) each generated two PCR bands: one of the

predicted fragment size of 344bp and another of 205 and a 211bp respectively (Figure 3.3).

The samples consistently amplified the same size amplicons, and manipulating the reverse transcription conditions as described in 3.7.8 above did not alter this.

Figure 3.3:

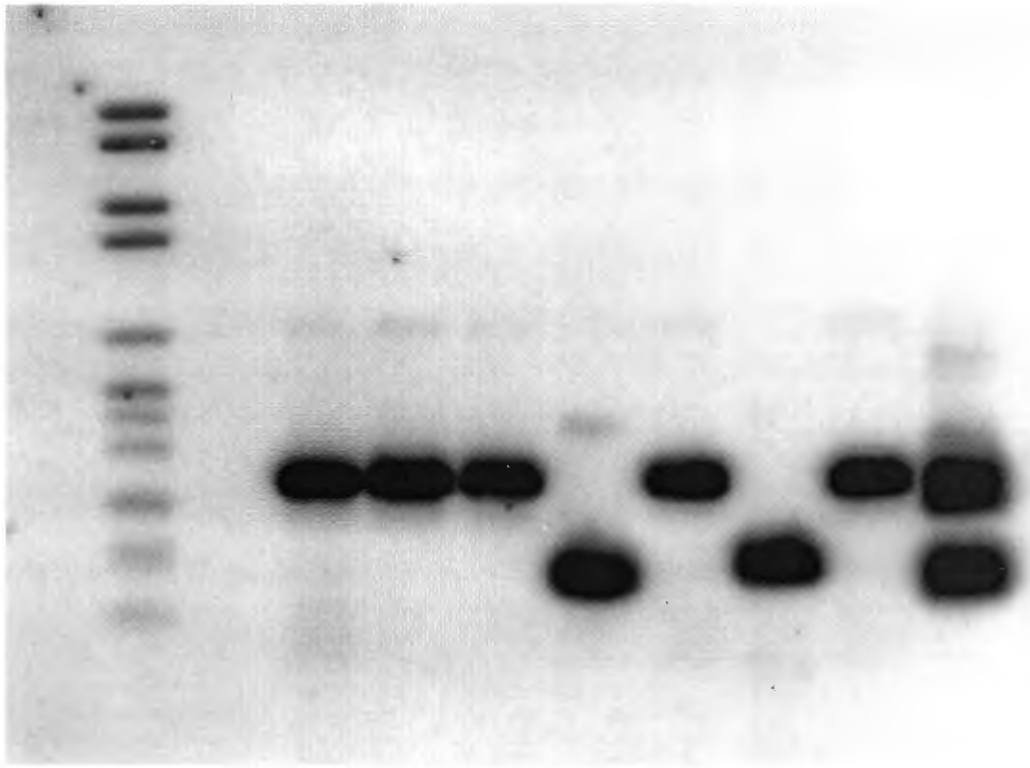
This photograph is of a representative agarose gel electrophoresis pattern for serum samples containing wild-type virus, mutant virus and both. Lanes I to 10 contain the following in order: molecular weight marker VI (Roche Molecular Biochemicals, Germany), negative control, positive control, and samples ZAK28, ZAN7, ZAN11, ZAN22, ZASq13, ZAK12 and ZAF3.



3.8.2 Southern blot

Southern blot analysis (after nested PCR) showed that both the predicted 344bp PCR fragments as well as the smaller fragments were HGV/GBV-C specific. Samples ZAF87, ZAN11 and ZASq13 showed no evidence of the wild-type cDNA fragments by Southern blot. Figure 3.4 below demonstrates the Southern blot findings using the same samples as shown in Figure 3.3 (scanned with a black and white filter).

Figure 3.4: Southern blot pattern demonstrating specificity of PCR amplicons.



3.8.3 Cloned fragments

Restriction fragment analysis of clones confirmed the sizes of the inserted PCR fragments.

3.8.3.1 Samples with single PCR bands

Many clones from each sample were analysed, and all clones from the samples with a single PCR band contained only one cDNA insert size (whether the predicted size or smaller).

3.8.3.2 Samples with two PCR bands

Different clones from samples ZAF3 and ZAF115 contained either the 344bp or the smaller fragments.

3.8.4 Supplementary analysis of mutant samples

Wild-type virus was not detected in samples ZAF87, ZAN11 and ZASq13 using the above amplification protocol. However, wild type virus was detected when using the supplementary PCR with the primer internal to the deleted region.

3.8.5 Sequence analysis

3.8.5.1 Wild-type

Sequence analysis of the cDNA clones showed all to be HGV/GBV-C-specific. The sequence alignment of eight wild type HGV/GBV-C isolates, including the wild type (wt) sequence of ZAF3 and ZAF115, is shown in Figure 3.5A below.

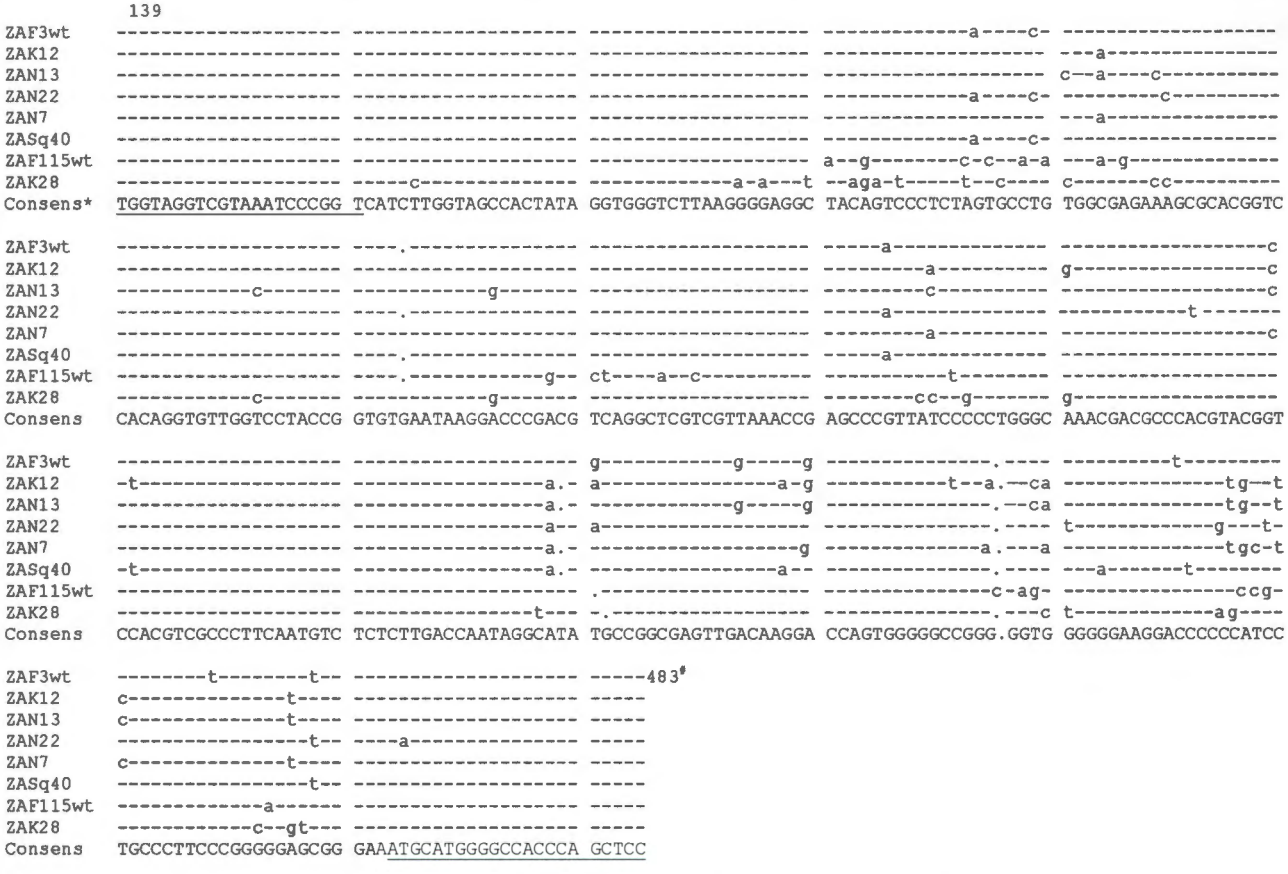
3.8.5.2 Mutant type

Deletant sequences from clones ZAN11, ZASq13 and ZASq87, as well as those clones containing the smaller fragment of ZAF3 and ZAF115 (designated ZAF3mut and ZAF115mut) are shown in Figure 3.5B. All deletions were over the same nucleotide region, with minor variation.

3.8.5.3 Variable regions

The degree of sequence heterogeneity in variable regions 2 and 3 (V2 and V3) is significantly higher than that seen in the other 5'NCR segments of the South African isolates. The variable regions account for only 20% of the length of the amplicons (excluding primers). However, 52% of the point mutations are in V1 and V2. Thus in variable regions 2 and 3, there is a point mutation on average every 1.8 bases, compared to a point mutation every 7.4 bases outside these regions.

Figure 3.5A: Sequence alignment of wild-type variants



One base removed from numbering to accommodate shift at position 414.

Figure 3.5B: Sequence alignment of deletant variants

One base removed from numbering to accommodate shift at position 308.

3.8.5.4 Deleted regions

Overview:

Analysis of the sequences show the deletions to end almost uniformly at base 305 or 306 (of the predicted PCR product). However, each of the deletions starts at a different position over a span of 28 bases. The deleted sequence is over a region of the 5'NCR that is initially well conserved, and then terminates after variable region III (206).

Specific deletions:

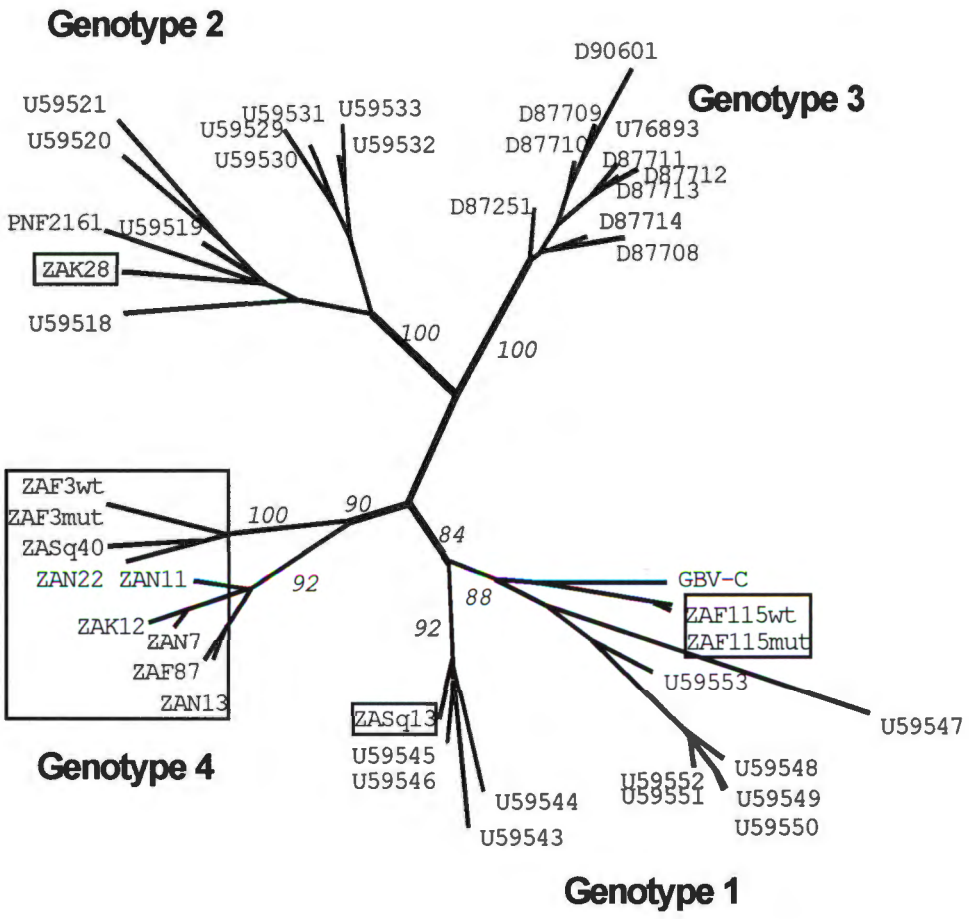
Thus, using the numbering system of accession number U76893 (206), the deletion in clone ZASq13 was from base pair position 333 to 443. The deletions in clone ZAN11, ZAF87, ZAF3 and ZAF115 all terminated at base position 444, but started at nucleotide 314, 313, 303 and 310 respectively (Figure 3.5B).

3.8.5.5 Phylogenetic analysis

Tree configuration:

Phylogenetic analysis, as shown by the unrooted tree below (Figure 3.6), showed 9/13 (69%) of the South African isolates to cluster into a separate, distinct group when compared to isolates from West Africa, Asia and Europe/USA. The South African cluster was not an artifact induced by the deletants, as the same phylogenetic relationships were present when the analysis was performed without the inclusion of the deletant isolates. The tree is consistent with previous phylogenetic analyses demonstrating geographic clustering of genotypes 1-3 with West Africa, Europe/USA and Asia respectively (105,207).

Figure 3.6: Phylogenetic tree comparing South African HGV/GBV-C variants with genotypes 1-3.



Bootstrap values:

The statistical reliability of the tree was confirmed by bootstrap analysis. Figure 3.6 demonstrates the bootstrap values (in italics) where that figure exceeded a score of 80. The divisions into genotypes 2 and 3 remain as previously published, as does genotype 1, where the sub-division within genotype 1A and 1B is demonstrable. The South African group also has a possible subdivision

within the new cluster, with samples ZAK12, ZAN7, ZAF87 and ZAN13 distinguishable from others in the grouping.

3.8.5.6 DNA distance assessment

The mean DNA distance values within and between the three known genotypes and the SA cluster are shown in Table 3.2, as a mean and standard deviations (in brackets). The DNA distances between the South African group and the other genotypes were all >0.1000; i.e. as large as that previously described between genotypes 1-3 (207), thus demonstrating the presence of a novel fourth genotype in South Africa.

Table 3.2: DNA distance values for HGV/GBV-C genotypes.

	n	1	2	3	ZA
1	12	0.0677 (0.0349)	0.1200 (0.0101)	0.1109 (0.0181)	0.1126 (0.0217)
2	10		0.05139 (0.0137)	0.0941 (0.0122)	0.1055 (0.0113)
3	10			0.0274 (0.0088)	0.1113 (0.0089)
ZA	9				0.0543 (0.0210)

Samples falling outside of South African cluster:

Four SA isolates did not fall within the new SA clustering (Figure 3.6). Sample ZAK28 was closely associated with the HGV-like isolates (genotype 2), while the samples ZASq13, ZAF115wt and ZAF115mut were related to the GBV-C-like variants, previously described in West Africa (genotype 1).

Position of mutants:

Three of the deletion isolates (ZAN11, ZAF3 and ZAF87), clustered with the new South African group, while ZASq13 and ZAF115mut fell into genotype 1. The different viruses (wild-type and deletion) contained in the samples ZAF3 and ZAF115 were shown to be very closely associated phylogenetically, suggesting that the deletion isolates may have been formed within the host (see Figure 3.6).

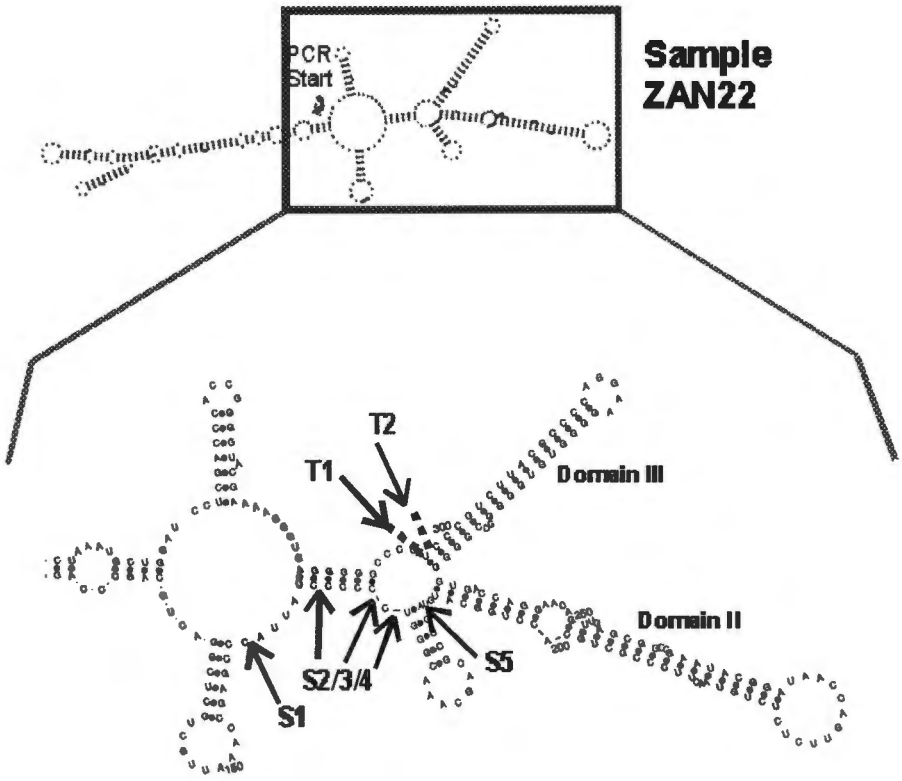
3.8.6 RNA secondary structure

RNA secondary structure analysis (Figure 3.7) showed the predicted 5'NCR region of the wild type isolates to have a structure comparable to that previously described (209).

3.8.6.1 Deleted region

The deletion involved two major stem loop structures (domains II and III) and an additional smaller upstream loop structure. The start positions of the deletions are marked S1-5, while the termination positions are marked T1 and T2 in Figure 3.7. Comparative analysis of the nucleotide sequence in Figure 3.5A and the predicted RNA structure in Figure 3.7 showed 85% of the nucleotide changes in the variable regions (II and III) to be covariant substitutions, thus allowing for the maintenance of the stem loop structure.

Figure 3.7: Predicted RNA secondary structure of the 5'NCR showing overall structure and deleted regions.



3.8.7 Biochemistry

ALT and AST values for all 32 wild-type and mutant HGV/GBV-C samples were within the normal limits. No differences were demonstrable between the samples within the genotype 4 and the mutant samples.

3.9 DISCUSSION

The 5'NCR of flaviviruses has been extensively investigated because of their putative role in regulation of translation. The analysis of this data shows certain features not described before for HGV/GBV-C and the family *Flaviviridae*.

3.9.1 Phylogenetic and evolutionary distance analysis

Previous phylogenetic analyses of HGV/GBV-C have shown the presence of only three genotypes (105,211,221,222). The data from this study is supportive of those analyses, but in addition describes a fourth genotype that predominates in Southern Africa. It is important to note that only West African isolates were included in the published studies to date and none were from South Africa.

The DNA distance analysis shows the evolutionary distance between the South African cluster and the other genotypes to be as great as those previously demonstrated between genotypes 1-3, i.e. >0.1000. The data suggests that South African genotype is a less heterogeneous group than genotype 1, but equally heterogeneous than genotype 2. As discussed in the literature review, both genotypes 1 and 2 have been subdivided by investigators into two subdivisions, A and B. Figure 3.6 above gives an early indication that the South African genotype may also be subdivisible. However, further isolates will be required to confirm or refute this. The geographic origin of an organism is often presumed to be that area where maximum genetic diversity occurs. The data above showing additional HGV/GBV-C heterogeneity in Africa supports the hypothesis that HGV/GBV-C may originate in this continent (220).

It is not surprising that four of the South African isolates are phylogenetically related to genotypes that predominate outside of the region, as South Africa is a heterogeneous society with strong trade and cultural roots in both Africa and Europe/USA, with substantial movement of individuals between these regions. HCV clade 5 predominates in Southern Africa while it is not common elsewhere (85). This data provides the first evidence to suggest the same for HGV/GBV-C.

The finding that the deletion isolates do not form a separate cluster is noteworthy and suggestive of the capacity of multiple genotypes to form this mutation. The selective advantage (if any) of this mutant genetic form has yet to be elucidated. Individuals containing both the wild type and mutants were not limited to genotype 4. For ZAF3 and ZAF115, the closeness of the two isolates in each of these individuals is suggestive of the deletion being generated after infection with a single strain, and not being due to separate infections. It remains unclear whether the mutant has the capacity to produce infectious particles, and thus whether individuals can be infected with the deletion isolate alone.

3.9.2 Sequence analysis

3.9.2.1 Wild-type sequence

The primers used to amplify HGV/GBV-C sequence were designed for the conserved region between variable regions 1 and 2 (V1 and V2) and the conserved region downstream of V3. Thus the clones contain sequence over two of the three 5'NCR variable regions (V2 and V3) previously described (206). The South African sequence data show similar patterns to those previously described, with a high degree of heterogeneity in V2 and V3. The variable regions account

for only 20% of the length of the amplicons (excluding primers). However, 52% of the point mutations are over the same region. These variations in V2 and V3 do not allow discrimination between the samples from the Western and Eastern Cape Provinces, and this almost certainly reflects the very significant movement of people between these two provinces.

3.9.2.2 Mutant isolates

Minor variation in the 5'NCRs is well established (105,206,207). However, this study documents the first major deletion found in the 5'NCR of HGV/GBV-C isolates. No such deletion has been demonstrated to date in any member of the family *Flaviviridae*.

The deleted sequence in the five isolates shows variation in the position where it begins, and to a lesser extent the point where consensus sequence is re-started. The five samples show the initiation position of the deletion start point to span a region of 28 bases. The end point of the five deletions is more constant, with all but one ending at the same point and the last only one base away. The data suggest that the bases between 303 and 444 of the 5'NCR are non-essential for a functionally competent internal ribosomal entry site.

It was not possible to detect the mutant virus in the absence of the wild-type. The search for small quantities of the wild type virus in ZAF87, ZAN11 and ZASq13 was performed using three methods, of which only one was successful. In the first instance, a nested PCR assay followed by Southern blot hybridization was unable to detect wild type RNA transcripts. Secondly, purifying many clones from

each of these samples and performing restriction fragment length analysis revealed none of the predicted full length cDNA fragments. The wild-type transcripts were only detected in samples ZAF87, ZAN11 and ZASq13 when using a primer internal to the deletion. This is suggestive that the mutant cDNA "out-competed" the wild-type cDNA during the PCR process. This may have been due to a vast difference in RNA titre and/or a more efficient amplification due to size differences.

The possibility that these deletions are due to reverse transcription or PCR errors exists. However, efforts were made to exclude any possible errors in these processes. All serum samples showed consistent results with multiple repeat testing using the standard protocol. Heat denaturation of the RNA immediately prior to RT did not alter this. In addition, changing the PCR denaturation and annealing conditions gave identical results. Had the deletion findings been due to a RT artifact, it would have been expected that over 15% of the samples would have shown these deletions and that samples would have given different results after changing the test parameters. The variation in the deletion length also suggests a more dynamic biological role involved that requires further investigation.

3.9.3 RNA secondary structure and IRES

The fact that these HGV/GBV-C isolates are able to accommodate such a large deletion in the 5'NCR distinguishes HGV/GBV-C from HCV, as HCV requires a complete 5'NCR to remain translation efficient (214). It has been suggested that the HGV/GBV-C 5'NCR has weak IRES activity (205). IRES regions have been

shown to be within a short distance of the ATG start codon (210). Thus this data suggests that the IRES is well downstream of HGV/GBV-C base 444, so as to preserve this structure.

There is significant variability in specific regions of the 5'NCR. However, comparison of the sequence data and the predicted secondary structure of the RNA shows approximately 85% of the variation to comprise covariant substitutions, thus facilitating preservation of the RNA secondary structure.

The presence of these deletions over domains II and III supports the proposal that the 5'NCR is longer than the 465 bases initially proposed (206,209,211). The South African deletions remove domains II and III and end only 25 bases upstream of the proposed ATG codon at base 465, thereby almost certainly altering the RNA secondary structure of the region. However, if the true start position is 93 bases further (209,211), the preserved region downstream of the deletion would be extended to 118 bases and thereby possibly keep domains IV and V sufficiently unaltered to preserve IRES function. It must be noted that the South African nucleotide sequence used in this analysis did not include the extreme 5' portion of the 5'NCR. As this region may be an important component influencing the RNA structure, there may be differences demonstrated when included in the analysis.

3.9.4 Implications for primer design

The deletion occurs over an area initially proposed as being suitable for primer design (226). Thus, it is possible that groups using primers over this region have missed deletion isolates.

3.9.5 Biochemistry

Almost all evidence to date suggests that HGV/GBV-C neither replicates in the liver nor causes hepatitis (see next chapter). All serum samples investigated in this study had ALT and AST levels within the normal ranges, suggesting that both the members of this new genotype and the mutant isolates are not associated with hepatitis.

CHAPTER 4

Investigation of the replication sites of HGV/GBV-C

4.1 Summary

The tissue tropism of HGV/GBV-C has been investigated in a limited manner by a number of investigators, and no conclusive site of replication has been found. Recently, a more extensive investigation of the sites of replication of HGV/GBV-C was undertaken in selected organs removed from immunocompromised patients who had died of AIDS (n=4) or cirrhosis (n=2). The studies suggested that the spleen and bone marrow were sites of HGV/GBV-C replication. However, there is no data to date in normal subjects nor in an extensive set of human organs.

Therefore, this study investigated the sites of HGV/GBV-C replication in serum and twenty-three different organs collected during post-mortem examination of apparently healthy individuals (n=45) who died accidental deaths, a subset of whom had evidence of HGV/GBV-C infection. The four HGV/GBV-C PCR positive cadavers that were investigated for replication sites were anti-HIV and anti-HCV negative. Tissues were carefully collected to prevent cross contamination. A strand-specific RT-PCR assay for the detection of either HGV/GBV-C positive strand RNA (virion) or negative strand RNA (replicative intermediary) was employed. Strand specificity of the RT-PCR assay was assessed with synthetic positive-and negative strand HGV/GBV-C RNA generated from a plasmid, using T7 and T3 RNA polymerases.

Spleen and bone marrow biopsies were found to be positive for both negative- and positive strand HGV/GBV-C RNA in all four cadavers. In addition, one cadaver was positive for both RNA strands in the kidney, and another positive for both strands in the liver. No negative strand RNA was detected in the following:

brain, muscle, heart, thyroid, salivary gland, tonsil, lung, lymph nodes, gall bladder, pancreas, oesophagus, stomach, small bowel, large bowel, adrenal gland, gonad, aorta, skin and cartilage.

This study concludes that HGV/GBV-C is a lymphotropic virus that replicates primarily in the spleen and bone marrow.

4.2 Introduction to flavivirus replication

HGV/GBV-C is presumed to be a novel member of the family *Flaviviridae*, based on its molecular structure, as discussed in chapter 3 of this thesis. Thus, much of our current understanding of HGV/GBV-C replication is based on the available data from other pesti-and flaviviruses and HCV. A review of the structure and genetic characteristics of HGV/GBV-C is contained in the introduction and literature review of chapter 3 of this thesis.

4.2.1 Current understanding of flavivirus replication

4.2.1.1 Attachment and entry

Flaviviruses are thought to attach to permissive cells by way of specific cellular receptors (227,228). The cellular receptors for flaviviruses, other than HCV, remain unknown. CD81 has been shown to be a candidate receptor for HCV (229), and although entry into the cell appears to take place by receptor-mediated endocytosis (227,228), these early stages are not well characterised. After entry, flavivirus particles have been demonstrated by electron microscopy in cellular vesicles from where the nucleocapsid is released into the cytoplasm (230,231).

From this point in the flavivirus replication cycle, two processes are thought to occur in parallel:

- Production of viral proteins; and
- RNA replication.

4.2.1.2 Protein production

The positive sense virion RNA is used directly as messenger RNA for synthesis of proteins. Translation of the viral proteins begins at an AUG start codon at the 3' terminal end of the 5'NCR (186). In certain flaviviruses, more than one in-frame AUG start codon exists, and protein translation occurs from one or more of the sites. The 5'NCR acts a regulatory area, providing access for ribosomal entry upstream of the single open reading frame (186,205,210). The translation process results in the production of a single polyprotein that represents the entire flavivirus genome, apart from the 5'- and 3' non-coding regions. This protein undergoes post-translational cleavage and modification by both cellular and virally encoded proteases, resulting in the formation of mature structural and functional components (186). These structural and functional proteins are required for the synthesis of new virus particles. As discussed in chapter 3 of this thesis, the HGV/GBV-C and GBV-A genomes do not appear to code for a core protein, an important distinguishing feature not see in other flaviviruses.

4.2.1.3 RNA replication

Native flavivirus RNA molecules have positive polarity. A template for the production of further copies of native viral RNA is required, and is believed to be mediated by the production of a complementary negative strand of the viral RNA (192). The protein responsible for the RNA-dependant RNA polymerase activity is coded for in the NS5B region of the genome (203). These negative strand RNA molecules are genome-length and facilitate the production of new full length mirror image positive strand. These newly formed positive polarity RNA molecules are then utilised for either protein translation or the production of

further negative polarity replicative intermediaries (192). Synthesis of positive- and negative strand RNA molecules appears well regulated so as to produce fairly constant ratios of positive- to negative viral RNA strands of between 10:1 and 100:1 respectively (232-235). Thus, the presence of these replicative intermediaries in tissues is believed to be indicative of replication and hence cellular tropism. The detection of either positive or negative strand RNAs is possible by either strand-specific RT-PCR or in-situ hybridisation. (These methods are discussed below).

4.2.1.4 Assembly and release

It is currently believed that flavivirus RNA and protein synthesis and maturation occurs outside of the cell nucleus and that virus protein production is closely associated with intracellular membranes (192). For those flaviviruses that are readily detectable by electron microscopy, mature viruses are initially visualised in the lumen of the endoplasmic reticulum (236). Exocytosis occurs after transport of viral components from the endoplasmic reticulum to the cell surface (192).

4.2.2 Methods for detecting flavivirus replication

Flavivirus replication can be identified by the presence of negative strand viral RNA. This is possible using one of the following methodologies:

- Strand-specific RT-PCR (SS-RT-PCR); or
- In-situ hybridisation (ISH).

4.2.2.1 Strand-specific RT-PCR

Selective detection of positive- or negative strand viral RNA is possible by SS-RT-PCR (237-239). The process of detecting single strand RNA is technically demanding and the published limitations of the technique are discussed below. Strand-specific reverse transcription involves the selective conversion of either the positive- or negative strand of the genome into cDNA, by means of a single primer during the RT process. The residual RNA is subsequently destroyed and the RT enzyme inactivated. The process is then followed by conventional PCR with resultant selective amplification of one genome strand only.

The use of SS-RT-PCR is frequently preceded by "standard" non-strand-specific RT-PCR of the same tissues. If "standard" RT-PCR is repeatedly performed on an organ where replication of that virus does not occur, the PCR will alternate between a positive and negative result, due to the "blood contamination" within the tissues. However, for those tissues that act as the primary site of viral replication, cellular RNA accumulates far in excess of that seen in serum (240), and the RT-PCR is consistently positive, due to the vast abundance of viral RNA within the cells of that organ.

Limited work has been performed using SS-RT-PCR for HGV/GBV-C, although the methodology has been documented for hepatitis C (237,238). Detecting negative strand intermediaries by SS-RT-PCR has been controversial as these assays have historically been associated with significant false positive results. The first descriptions of SS-RT-PCR used either MMLV or Avian Myeloblastosis Virus reverse transcriptase enzymes. These enzymes operate optimally at

between 37°C and 42°C. These relatively low operating temperatures resulted in the following problems:

- false priming due to primer binding of the incorrect strand;
- false priming by extraneous nucleotide sequences; and
- self-priming of the RNA (238).

Three methodologies have been documented to circumvent these problems and specifically detect one or other RNA strand.

A) Currently, the most widely accepted method for strand-specific reverse transcription involves the use of the newer heat-stable enzymes such as rTth (Perkin-Elmer Cetus) that are active at up to 70°C (238,241). This method improves the specificity of the primer binding and reduces the chances of extraneous nucleotide sequences binding. In addition the higher temperatures support linearisation of complex RNA secondary structures, thereby minimising self-priming of the RNA.

B) The second methodology describes the use of "tagged" primers for single strand RNA detection (237). This method incorporates primers with extraneous sequences which are added to the 5' end of the desired primer. After reverse transcription, the PCR is performed using a primer specific for the "tag" region, and not the original viral sequence.

C) The third method describes the chemical modification of all free 3' ends of the RNA with borohydride, thereby preventing them binding to the target RNA (242). The strand specific primer is then added after the chemical modification process is completed, thereby allowing that primer to bind without competition from other nucleotides.

In each of these three methods, it remains necessary to denature the RT enzyme after completion of the cDNA formation, so as to prevent residual RT activity during the PCR process. In addition, certain groups also destroy the residual RNA prior to PCR with the addition of RNase.

4.2.2.2 In-situ hybridisation

The process of detecting RNA transcripts within the cells of a cut tissue section by in-situ hybridisation is well described for both messenger RNA and certain viral RNA (243,244). In brief, the in-situ hybridisation process involves the generation and use of labeled (e.g. digoxigenin) complementary RNA and/or cDNA probes which are applied to the tissue sections. This is followed by detection of any bound probe by way of immunological probing for the incorporated molecules in the probe. The use of single stranded RNA molecules that are complementary to either the negative- or positive strand of a flavivirus allows detection of specific strands of RNA.

Successful use of in-situ hybridisation is dependent on a variety of factors, including the method of tissue collection, the method of fixing the tissues, and the time delay from death to sample collection. It is thought by certain investigators that tissue fixing with paraformaldehyde is preferential to formalin or other solutions for detecting in-situ RNA (245). The tissues have to be digested and processed differently according to the above factors and optimal probe concentrations have to be estimated, so as to obtain a sufficient signal in the absence of non-specific background signal. As there is no amplification of the

RNA / cDNA prior to detection, the process of in-situ hybridisation is not as sensitive as SS-RT-PCR (244).

The sensitivity of nucleic acid detection in cut biopsy sections is improved if there is an in-situ amplification step. In this assay named in-situ PCR, the tissue sections are subjected to in-situ RT-PCR using a labeled nucleotide, followed by immunological detection of the label as described above for in-situ hybridisation (244).

4.3 Literature review relating to HGV/GBV-C replication

4.3.1 Overview

There is a paucity of data on HGV/GBV-C replication site(s). The overwhelming body of data demonstrates the lack of primary hepatotropism of HGV/GBV-C. The magnitude of the interest in HGV/GBV-C's role in liver disease has been based on:

- the initial clinical data that suggested that this virus was a causative agent for hepatitis; and
- the knowledge that the causative agent of many sporadic cases of (presumed viral) hepatitis is unknown (102,103).

However, as discussed earlier, it has become clear that there is little (or no) evidence to support HGV/GBV-C causing clinical hepatitis (141,156,246).

4.3.2 Published papers

Twelve papers have been published to date on the tropism (or lack thereof) of HGV/GBV-C in human tissues (239-241,247-255). However, in all but two of these papers, only liver and/or mononuclear cells were assessed.

4.3.2.1 Hepatotropism

Nine of the above papers present data from studies which attempted to detect the presence of HGV/GBV-C replication in liver biopsies, using different methodologies (239-241,247-251,253).

- Six of these nine research projects were unable to detect HGV/GBV-C replicative intermediaries using comparable SS-RT-PCR assays (239,241,248,249,251,253).
- The paper by Kobayashi et al. (1999) used SS-RT-PCR to assess circulating mononuclear cells and ISH to assess liver biopsies (250). They were able to detect HGV/GBV-C negative strand RNA in the liver biopsies of two immunosuppressed patients by ISH. However, the ISH assay demonstrated the signal to be exclusively from the peri-portal mononuclear cells and not the hepatocytes.

All seven studies therefore concluded that the liver was not the primary site of HGV/GBV-C replication.

Two further studies concluded the same, but based their conclusion on indirect evidence (240,247).

- Pessoa et al. (1998) investigated relative ratios of HGV/GBV-C RNA in serum and liver of infected patients and found that there was no accumulation of viral RNA in the liver, as would be expected if there was active replication (240).
- Berg et al. (1999) assessed the HGV/GBV-C relative viral titres between serum and liver tissue, and in addition, examined the viral titre dynamics after liver transplantation (247). The liver tissue was negative for HGV/GBV-C RNA and the viral kinetics were relatively unchanged in the post-operative period.

Both are highly suggestive that the liver is not the primary site of HGV/GBV-C replication.

4.3.2.2 Tropism of mononuclear cells

Three studies have assessed the possible tropism of HGV/GBV-C for mononuclear cells. Following their positive findings in the mononuclear cells of the peri-portal region of liver biopsies, Kobayashi et al. (1999) furthered their study to assess circulating mononuclear cells by SS-RT-PCR (250). They found two of the six infected individuals studied had evidence of HGV/GBV-C replication in these cells.

These findings contradict the two previous studies (253,254) where a total of 29 samples were assessed with no evidence of negative strand HGV/GBV-C RNA in the circulating mononuclear cells. It is difficult to interpret these conflicting results, as most of the studies use different methodologies with varying sensitivities and specificities. However, while certain individuals may have a subset of circulating mononuclear cells permissive to HGV/GBV-C replication, it

appears that in general, circulating mononuclear cells do not represent a primary site of HGV/GBV-C replication.

4.3.2.3 Tropism in extended tissue spectrum

Two studies (from the same group) have investigated the tissue tropism of HGV/GBV-C in a broader range of tissues (252,255). Laskus et al. (1998) collected tissues from twelve organs from four HGV/GBV-C infected cadavers undergoing post mortem examination and in addition, blood samples from six infected living individuals. In the second study, Radkowski et al. (1999) collected ten samples from two patients at post mortem examination. The tissues and circulating mononuclear cells from both of these two studies were investigated for HGV/GBV-C replication.

It is important to note two factors relating to sample bias in both of these studies:

- all cadaver tissues from the study by Laskus et al. were from individuals who had died of AIDS, and were thus severely immunocompromised.
- the six blood samples in the Laskus et al study were from HIV1 infected drug addicts.
- The study by Radkowski et al. used samples from two individuals who had died of end-stage liver disease. Patients with liver failure are known to be immune compromised (256,257).

The investigators used the same SS-RT-PCR methodology as previously described above with the thermostable RT enzyme rTth, and observed the following.

Laskus et al. study:

- None of the six blood samples were shown to contain circulating mononuclear cells with HGV/GBV-C negative strand RNA.
- Two out of four of the splenic biopsies and three of the four bone marrow biopsies were positive for replicative intermediaries, suggesting these to be the major sites of HGV/GBV-C replication.
- One of four liver biopsies was also positive for negative strand RNA.
- Where negative strand RNA was detected,
    - it was invariably in the presence of positive strand RNA, and
    - one to two logarithmic counts less concentrated than the positive strand RNA.
- The lymph nodes, pancreas, thyroid, adrenal gland, kidney, lung, muscle, skin and spinal cord were not found to contain negative strand HGV/GBV-C RNA intermediaries.

Radkowski et al. study:

- Both cadavers demonstrated the presence of negative strand RNA in the bone marrow and spleen.
- One of the two cadavers showed evidence of negative strand RNA in the lymph node.
- Where negative strand RNA was detected, it was invariably in the presence of positive strand RNA.

These studies are a valuable contribution to the HGV/GBV-C data. However, it is difficult to extrapolate this data to the general population who have no detectable immunosuppression.

4.3.2.4 HGV/GBV-C permissive cell lines

Two groups have described human cell lines permissive to HGV/GBV-C replication (258,259). Ikeda et al. describe the use of two cell lines: MT-2C, a human T-cell leukemia type I-infected cloned T cell line; and PH5CH, a non-neoplastic human hepatocyte cell line (259). After inoculation of both cell culture systems with HCV- and HGV/GBV-C positive serum, both HCV and HGV/GBV-C genomes were detected by RT-PCR for more than 30 days. A limitation of the study was that HGV/GBV-C was not inoculated into the cells in the absence of HCV and therefore propagation of the virus in these systems may be dependent on the presence of HCV.

In the most comprehensive and complete data, Fogeda et al. have shown that human peripheral blood mononuclear cells are susceptible to HGV/GBV-C infection in-vitro (258). This group used SS-RT-PCR, sucrose ultracentrifugation and RNase sensitivity assays, in-situ hybridisation and Western blot assays to assess different markers of viral replication, and showed ongoing replication over a period of 30 days.

Although these cell culture systems have been described, there remains no data on the intracellular replication strategies of HGV/GBV-C, and thus although not

formally shown to date, it is presumed that HGV/GBV-C replicates in the same manner as other positive-stranded RNA flaviviruses.

4.4 Assumptions and hypothesis

Central to the design and methods employed in this study is the acceptance of two factors.

- The theory that during replication HGV/GBV-C, like other positive stranded flaviviruses, replicates its own RNA by way of a negative strand replicative intermediary. Thus those tissues permissive to HGV/GBV-C replication would have detectable negative strand HGV/GBV-V RNA, and not those tissues where no replication occurred.
- Tissue extracts may be HGV/GBV-C RT-PCR positive due to the detection of serum RNA within the tissues. However, in the absence of negative strand HGV/GBV-C RNA, this does not indicate viral replication.

4.5 Study aim

The aim of this study was to establish the sites of HGV/GBV-C replication in multiple cadaver tissue samples collected from individuals who appeared healthy before premature violent / traumatic death, using a strand-specific reverse transcription PCR assay.

4.6 Materials and Methods

4.6.1 Consent and legal considerations

Permission for the study was granted by the University of Cape Town Ethics and Research Committee. All tissues were collected in terms of the Human Tissues Act (Act No 65 of 1983), as amended by the Human Tissue Amendment Act of 1989.

4.6.2 Cadaver selection

Cadaver tissue biopsies were collected during routine post-mortem examination at a local city (Maitland) Police Mortuary. This sample collection phase was performed in collaboration with Professor G. Knobel and Dr. C. Eedes of the University of Cape Town Department of Forensic Medicine and Toxicology.

4.6.2.1 Cadaver inclusion criteria

All biopsies were obtained from adult individuals who had died by violent means. All adult cadavers examined at the South African Police Service Mortuary in Maitland were considered potential candidates for sampling.

4.6.2.2 Cadaver exclusion criteria

Cadavers were not recruited to the study if they:

- had died in hospital (even if admitted for treatment of trauma); or
- were under the age of 18 years; or
- if there were obvious signs of ante-mortem disease on examination; or
- if greater than 15 hours had passed between death and post-mortem examination.

4.6.2.3 Cadaver demographic data

The following data was recorded from the available information at the mortuary: age, gender, cause of death, recorded date and time of death, and time interval between death and post-mortem.

4.6.3 Tissues

Blood and the following tissue samples and were obtained from the first 12 cadavers recruited to the study:

> brain, tonsil, submandibular salivary gland, skeletal muscle, heart, lung, liver, gall bladder, bone marrow (from ribs), spleen, kidney, pancreas, adrenal, aortic arch, thyroid, lymph node, oesophagus, stomach, small intestine, large intestine, gonad, skin and cartilage (from epiglottis). All bowel, gall bladder and aortic biopsies were transmural.

Cadavers recruited subsequent to the initial twelve above had more limited tissue samples collected, based on the preliminary analysis of the first group. The samples from these subsequent cadavers included blood and the following organs: spleen, liver, kidney, skeletal muscle, lymph nodes and adrenal.

4.6.4 Prevention of RNA carry-over

The following measures were taken to prevent carry-over of viral RNA between biopsies at time of sampling. Each tissue was removed using a scalpel blade dedicated to that organ (i.e. 23 blades per cadaver). Any tissue in contact with

the forceps used to hold the tissue was discarded. Each tissue was cut into 1-2mm^3 pieces and placed in separate sealed containers and placed immediately on dry ice for transport to a laboratory -80°C freezer. The blood was separated by centrifugation and the serum and tissues were kept at -80°C until processed.

4.6.5 Hepatitis and HIV screening

Sera of the HGV/GBV-C positive cadavers were screened for antibodies to human immunodeficiency viruses (HIV) 1/2, antibodies to hepatitis C virus and for hepatitis B surface antigen, using the following Abbott Laboratories automated Axsym System assays:

- HIV "HIV 1/2 gO"
- HCV "HCV Version 3.0"
- HBV surface antigen "HBsAg (V2)"

4.6.6 Production of synthetic RNA controls

Synthetic negative- and positive sense control HGV/GBV-C RNA were produced as follows. A 343bp PCR product (ZAN22) of the 5'NCR of HGV/GBV-C was cloned and sequenced, as previously described in chapter 3 of this thesis.

4.6.6.1 Negative strand RNA

For the production of negative strand HGV/GBV-C RNA; the pZAN22 plasmid was linearized for one hour at 37°C using 10 units of the restriction enzyme, SacI, so as to produce RNA transcripts of appropriate lengths without excessive lengths of RNA complementary to the plasmid. The RNA transcripts were then

generated using T7 RNA polymerase and the T7/SP6 Transcription Kit, as recommended by the manufacturer (all reagents Roche Molecular Biochemicals, Germany).

4.6.6.2 Positive strand RNA

Positive strand HGV/GBV-C RNA was produced using the same plasmid (pZAN22) after digestion with 10 units of the restriction enzyme HindIII, as above. Transcription of the HGV/GBV-C RNA was then performed using T3 RNA polymerase and the buffers and reagents contained in the T7/SP6 Transcription Kit (as above).

Template DNA from both reactions was then removed by incubating the reaction at 37°C for 30 minutes with 10 units RNase-free DNase (Roche Molecular Biochemicals, Germany). Plasmid destruction was confirmed by performing PCR (as described below) on the synthetic RNA without RT. The synthetic RNA was quantified using the Beckman DU-40 spectrophotometer (Beckman Coulter Inc., California, USA), aliquotted into 5µl amounts and stored at -80°C until required.

4.6.7 Tissue and control RNA processing

4.6.7.1 RNA Extraction

Serum:

Total RNA was extracted from cadaver serum, as previously described in chapter 2 of this thesis.

Tissues:

RNA from tissue biopsies was extracted in a similar manner as described for serum. Each biopsy of 1-2mm^3 was macerated in a petri dish using a scalpel

blade dedicated to that tissue and then resuspended in 200μl of DepC treated water. This cell suspension was added to 1ml Total RNA Isolation Reagent (Advanced Biotechnologies Ltd, London, UK), vortexed for 30 seconds and then processed in the same way as serum samples. The extracted RNA was then resuspended in 50μl DepC treated water.

Four splenic and four bone marrow HGV/GBV-C PCR negative biopsies were also included and subjected to the same RT-PCR to ensure that there was no non-specific amplification of genetic material.

4.6.8 Reverse Transcription

Two reverse transcription reactions were utilised for the serum and tissue biopsies.

4.6.8.1 Non strand-specific reverse transcription

The extracted RNA from all biopsies was subjected to reverse transcription using random hexamer primers and MMLV RT, as previously described in chapter 2 of this thesis. This was followed by HGV/GBV-C PCR as below, so as to determine whether any (negative- or positive strand) HGV/GBV-C RNA was present.

These results were interpreted as follows:

1. Any extracted tissue sample that was HGV/GBV-C negative was re-tested to confirm that the RNA extraction and RT were successful. This was done by performing an RT-PCR assay for the presence of beta-actin mRNA, as

previously described (260). Samples that were found to be HGV/GBV-C negative and beta-actin messenger RNA positive were not tested further.

2. Samples found to be HGV/GBV-C positive by this non strand-specific reaction were then assessed for the presence of specific RNA strands, by the method below. Both positive- and negative strand synthetic RNA controls were included in every SS-RT-PCR batch to ensure the strand specificity of the assay.

4.6.8.2 Strand-specific reverse transcription:

All HGV/GBV-C PCR positive RNA extracts were assessed to discriminate between negative- and positive strand HGV/GBV-C RNA, by strand-specific RT-PCR using the newly developed Thermoscript™ RT enzyme system (GibcoBRL, USA).

Denaturation:

Five microlitres of extracted RNA was added to 5μl DepC treated water containing 50pmol of either the sense or antisense primer (below) and heated at 65°C for five minutes.

Reverse transcription:

A 10μl solution containing 1 X cDNA synthesis buffer, 10mM dithiothreitol, 40U RNaseOUT™ RNase inhibitor, 2mM dNTP mix, and 7.5U Thermoscript™ RT enzyme was added to the heated RNA and incubated at 65°C for one hour.

Reverse transcriptase inactivation:

The reverse transcriptase enzyme was then heat inactivated at 85°C for 5 minutes.

RNA destruction:

One unit of RNase H was added to destroy any residual RNA and incubated at 37°C for 20 minutes.

4.6.9 Polymerase chain reaction

A nested HGV/GBV-C PCR was performed, as previously described in chapter 3 of this thesis, using the following primers:

Sense	5'-TGGTAGGTCGTAAATCCCGGT-3'
Antisense	5'-GGAGCTGGGTGGCCCCATGCAT-3'
Nested sense	5'-GGTAGCCACTATAGGTGGG-3'
Nested antisense	5'-CTCGGTTTAACGGCGACGCT-3'

Using these primers, a positive result was predicted to generate a final PCR amplicon fragment of 136bps.

Amplicon visualisation

PCR products were separated by electrophoresis in 2% agarose gel and visualised under ultra-violet light using ethidium bromide staining.

4.7 Results

4.7.1 Cadavers

Forty-five cadavers were recruited to the study of which four were HGV/GBV-C RNA positive on serum analysis. The ante-mortem demographic data of the PCR positive individuals is displayed in Table 4.1. Of note, all four cadaver sera were anti-HIV negative and anti-HCV negative, while one was hepatitis B surface antigen positive. None showed any obvious signs of clinical disease at post mortem.

Table 4.1: Demographic data of HGV/GBV-C positive cadavers.

Cadaver numbers	One	Two	Three	Four
Age	30	36	25	45
Gender	Male	Female	Male	Male
Cause of death	Gunshot	MVA*	Gunshot	Stab
Time delay before sampling	12 hours	7 hours	8 hours	14 hours
Anti-HIV 1&2	Negative	Negative	Negative	Negative
Hepatitis B surface antigen	Negative	Positive	Negative	Negative
Anti-hepatitis C	Negative	Negative	Negative	Negative

* = motor vehicle accident

4.7.2 Tissues collected

Cadavers 1 and 2 had biopsies taken from serum and 23 organs. Serum and six tissues were obtained from the latter two cadavers. These tissues are shown in Table 4.2.

4.7.3 The assay

4.7.3.1 RT-PCR sensitivity

The RT-PCR assay sensitivity for synthetic RNA was shown to be 150 RNA copies per mililitre.

4.7.3.2 Reverse transcription specificity

The SS-RT-PCR assay was capable of specific amplification of either positive- or negative strand synthetic HGV/GBV-C RNA copies over a concentration of five logarithms per mililitre.

4.7.4 Non strand-specific RT-PCR

Multiple tissues from each cadaver were HGV/GBV-C PCR positive when performing the RT reaction with random hexamers and MMLV RT as described below and shown in Table 4.2.

4.7.4.1 Cadaver 1

The following tissues were PCR positive for cadaver 1: serum, skeletal muscle, tonsil, lymph nodes, liver, spleen, kidney, adrenal and bone marrow.

4.7.4.2 Cadaver 2

Cadaver 2 was found to be PCR positive in serum, skeletal muscle, heart muscle, thyroid, salivary gland, lung, lymph nodes, liver, spleen, kidney, adrenal, aorta, and bone marrow.

4.7.4.3 Cadavers 3 and 4

Cadavers 3 and 4 were both PCR positive in serum and all six biopsies taken at post-mortem.

4.7.4.4 HGV/GBV-C RT-PCR negative tissues

Table 4.2 also displays the tissues found to be RT-PCR negative, thereby excluding the presence of both negative and positive strand HGV/GBV-C RNA. All of these HGV/GBV-C PCR negative tissues were positive for beta-actin messenger RNA, thereby confirming that the RNA extraction and RT reaction were successful.

4.7.5 Strand-specific RT-PCR

4.7.5.1 Serum

None of the serum samples had detectable negative strand RNA by strand-specific RT-PCR.

4.7.5.2 Tissues

All four splenic and both bone marrow biopsies were strongly positive for both positive- and negative sense HGV/GBV-C RNA (Table 4.2). In addition, replicative intermediaries were found within the kidney of cadaver 3 and the liver of cadaver 4.

However, although positive strand HGV/GBV-C RNA was detected in the other tissues, no signal for negative strand HGV/GBV-C RNA was obtained. This suggests that the PCR signals were not due replication of the virus within tissues, but rather due to serum HGV/GBV-C RNA. Although relative titres of positive- and negative strands were not formally assessed, the intensity of the gel bands was greater for positive strand RNA than negative strand.

Although both bone marrow and spleen were strongly positive for replicative intermediaries, other lymphoid tissues such as tonsil and lymph nodes were negative. Cadavers 1 and 2 show no signs of positivity in any part of the bowel (including gall bladder and pancreas) and were thus not tested further for specific strands. The same applies to the testis and ovary of cadaver 1 and 2 respectively that were both RT-PCR negative. The sections of bowel and gall bladder tissue were transmural, and would thus have contained a variety of cell types including epithelium, mucous glands, muscle and lymph vessels (and residual bowel contents). The more vascular tissues, such as adrenal, muscle, liver and kidney were more likely to be PCR positive in the first RT-PCR using MMLV RT, but only showed positive strand RNA when using the supplementary SS-RT-PCR.

No amplicons were generated from the four splenic and bone marrow biopsies taken from HGV/GBV-C negative cadavers.

Table 4.2 gives a summary of the above results. Tissues found to repeatedly contain negative strand HGV/GBV-C RNA are shaded.

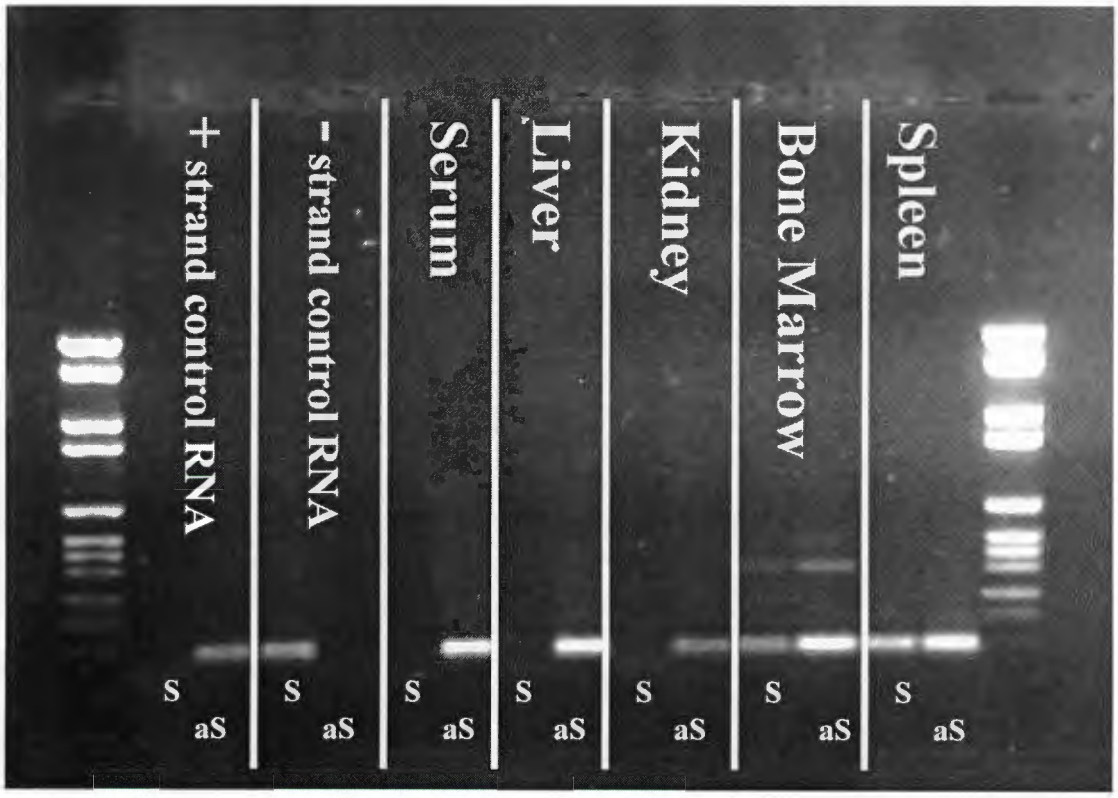
Table 4.2: Summary of tissues collected and results obtained.

Cadaver 1				Cadaver 2			
	Random Hexamers	Positive strand	Negative strand		Random Hexamers	Positive strand	Negative strand
Serum	Pos	Y	N	Serum	Pos	Y	N
Brain	Neg			Brain	Neg		
Skeletal muscle	Pos	Y	N	Skeletal muscle	Pos	Y	N
Heart muscle	Neg			Heart muscle	Pos	Y	N
Thyroid	Neg			Thyroid	Pos	Y	N
Salivary gland	Neg			Salivary gland	Pos	Y	N
Tonsil	Pos	Y	N	Tonsil	Neg		
Lung	Neg			Lung	Pos	Y	N
Lymph nodes	Pos	Y	N	Lymph nodes	Pos	Y	N
Liver	Pos	Y	N	Liver	Pos	Y	N
Gall bladder	Neg			Gall bladder	Neg		
Spleen	Pos	Y	Y	Spleen	Pos	Y	Y
Kidney	Pos	Y	N	Kidney	Pos	Y	N
Pancreas	Neg			Pancreas	Neg		
Oesophagus	Neg			Oesophagus	Neg		
Stomach	Neg			Stomach	Neg		
Small bowel	Neg			Small bowel	Neg		
Large bowel	Neg			Large bowel	Neg		
Adrenal gland	Pos	Y	N	Adrenal gland	Pos	Y	N
Ovary / testis	Neg			Ovary / testis	Neg		
Aortic arch	Neg			Aortic arch	Pos	Y	N
Skin	Neg			Skin	Neg		
Cartelage	Neg			Cartelage	Neg		
Bone marrow	Pos	Y	Y	Bone marrow	Pos	Y	Y
Cadaver 3				**Cadaver 4**			
Serum	Pos	Y	N	Serum	Pos	Y	N
Skeletal muscle	Pos	Y	N	Skeletal muscle	Pos	Y	N
Lymph nodes	Pos	Y	N	Lymph nodes	Pos	Y	N
Liver	Pos	Y	Y	Liver	Pos	Y	N
Spleen	Pos	Y	Y	Spleen	Pos	Y	Y
Kidney	Pos	Y	N	Kidney	Pos	Y	Y
Adrenal gland	Pos	Y	N	Adrenal gland	Pos	Y	N

Agarose gel photograph

Figure 4.1 is an example from the results of cadaver number 1. Each control or specimen is contained in two consecutive lanes. In lanes 2, 4, 6, 8, 10, 12 and 14 the reverse transcription reaction was performed with the sense primer (S). In lanes 3, 5, 7, 9, 11, 13 and 15 the reverse transcription reaction was performed with the antisense primer (aS). Lanes 2 and 3 show the results of positive strand control RNA, and lanes 4 and 5 show the results of the negative strand control RNA. Thereafter lanes 6-15 contain the results of the specified tissues. Lanes 1 and 16 contain molecular weight marker VI (Roche Molecular Diagnostics, Germany).

Figure 4.1: Agarose gel of SS-RT-PCR results from cadaver 1.



4.8 Discussion

This study is the first to document the sites of HGV/GBV-C replication in infected subjects without obvious disturbance of the immune system and in addition, increases the spectrum of tissues examined to date.

4.8.1 Tissue samples

4.8.1.1 Serum

Serum samples from each of the four HGV/GBV-C infected cadavers was RT-PCR positive using MMLV RT. However, strand-specific RT-PCR on the same serum samples showed only positive strand RNA and not negative strand RNA. Thus all further results for negative-strand RNA in tissue samples reflect intracellular RNA and not serum contamination.

4.8.1.2 Lymphoid tissues

All four splenic and both bone marrow biopsies were strongly positive for both negative- and positive strand RNA, suggesting that these represent the primary sites of HGV/GBV-C replication. The cell-specificity suggested by these cadaver findings is remarkably narrow, as seen by the negative findings in lymph node and tonsil biopsies. The data indicate that there is a primary cell tropism of HGV/GBV-C limited to the cells of the bone marrow and spleen and not a broad lymphotropism. The candidate cells for this tropism include the haematological stem cells, B-cells, plasma cells and mononuclear phagocytes, but almost certainly excludes the T-cells that predominate in the lymph nodes and tonsils.

4.8.1.3 Liver and kidney

Negative strand RNA transcripts were also consistently found in the liver of one cadaver and in the kidney of another. This may suggest that a subgroup of those infected may have low levels of replication within these two organs.

4.8.2 Comparison with literature

4.8.2.1 Extended tissue spectrum

There is remarkable consistency between our data and that previously generated from patients who died of AIDS or end-stage liver disease (252,255) suggesting that, unlike many viral infections which disseminate in immunocompromised individuals, HGV/GBV-C is unable to disseminate, on account of its high cell-specificity. However, although the study designs presented in this thesis and those of Laskus et al. and Radkowski et al. are similar, one potentially significant difference is that the other two studies allowed collection of cadaver tissue samples for up to 48 hours after death. The stability of intracellular HGV/GBV-C RNA over this prolonged time interval is unknown, and this factor may have led to under-reporting of the sites permissive to HGV/GBV-C replication in immunocompromised patients.

4.8.2.2 Liver and kidney

The overwhelming majority of biopsies assessed in the literature show no evidence of liver replication and the two studies that investigated the kidney (252,255) did not show evidence of replication. Insights obtained from the Kobayashi et al. data (250) and these findings in spleen, bone marrow, liver and kidney may lend assistance in interpreting these findings. Their study

demonstrated the presence of HGV/GBV-C replicative intermediaries in the mononuclear cells of the portal tract but not in hepatocytes. It is possible that the positive signal in liver and kidney is due to peri-portal macrophages in the liver (possibly Kuppfer cells) and their renal mononuclear counterparts, the mesangial cells. As strand-specific RT-PCR does not allow discrimination between the different cells of the biopsy, these positive findings represent the summation of the cellular components of each biopsy and thus may be due to any one of the cellular components.

4.8.3 HGV/GBV-C infection and haematological disturbances

The consistent presence of HGV/GBV-C replicative intermediaries in two lymphoid tissues allows the focus of future studies to move away from the liver so as to investigate the possible role of this virus in haematological abnormalities. The data contained in this chapter does not in any way suggest that HGV/GBV-C is pathogenic. However, if HGV/GBV-C does have a pathogenic role, the effect may be seen in the organs where replication is detectable.

The role of HGV/GBV-C in haematological disorders has received little attention to date and has focussed mainly on the acquisition of HGV/GBV-C during transfusions and transplantation (176,261-263). Although a few epidemiological studies have assessed the association of HGV/GBV-C infection and haematological disturbances, the data are inconclusive (264,265), and larger studies are required to supplement this.

4.8.4 Transmission and risk factors

It is clear that HGV/GBV-C is transmitted by blood (106,141,263). However, many infections occur in the absence of a known risk factor. In attempting to elucidate other transmission modes, it is important to note certain tissues that were negative for replicative intermediaries.

4.8.4.1 Saliva

HGV/GBV-C has previously been detected in saliva and transmission by this mode has been postulated (109,266). This data suggests that any detectable virus in saliva is not due to replication in the salivary glands. It is possible that, as in the case of HIV (267), HGV/GBV-C may be present in the saliva of infected individuals, but not normally transmitted by this route.

4.8.4.2 Gastrointestinal tract

Samples from the entire gastrointestinal tract from oesophagus to large bowel (apart from the liver of cadaver 3) were shown to be non-permissive to replication. These biopsies were transmural, and therefore contained all cellular components of the bowel (and any attached bowel contents on the inner surface). These findings suggest that it is unlikely that the bowel (or its contents) is involved in transmission or acquisition of HGV/GBV-C.

4.8.4.3 Sexual

It has been postulated that HGV/GBV-C may be sexually transmitted (114,115,268,269), and evidence suggests that the receptive sexual partner may be at greater risk of acquiring the infection (114). Sexual transmission may well

occur. However, this data is highly suggestive that the ovary and testis are not involved in virus replication and thus indirect transmission by sexual contact.

4.8.5 Assay reliability

The SS-RT-PCR process is technically demanding and has previously been associated with false positivity due to self priming of the RNA, false priming of the incorrect RNA strand and residual RT activity of enzymes during PCR (237,270). Much of the limitation was reverse transcriptase enzyme related. It is evident that the problems experienced were mainly due to the low operating temperatures of the enzymes available, and thus the advent of newer thermostable equivalents has resulted in increased specificity (237,271).

The assay used in this study was optimised using synthetic HGV/GBV-C RNA as template and showed repeatedly specific results over significantly different RNA concentrations when using the Thermoscript™ RT (GibcoBRL, USA) at temperatures at or above 65°C. Thus like with the use of the enzyme rTth, strand-specific results are possible with Thermoscript™ RT without the tagging of primers (237) or chemical modification of the 3' ends of RNA (242).

Thus, in summary, this study has assessed multiple biopsy specimens from apparently healthy individuals who died violent or traumatic deaths. The consistent finding of HGV/GBV-C replicative intermediaries in the spleen and bone marrow of these infected individuals suggests that these two organs represent the primary sites of HGV/GBV-C replication.

Chapter 5

Summary and Recommendations

The prevalence of serological markers of hepatitis A and B infection has been shown to be high amongst the adult Black communities of the Western and Eastern Cape provinces of South Africa. Although the data on hepatitis A is comparable with statistics generated in other regions of South Africa, the exposure rate to hepatitis B is lower than previously documented. The morbidity associated with hepatitis A infection currently remains extremely low in these communities, and thus new government sponsored intervention strategies to prevent infection do not appear necessary. However, it has to be borne in mind that, in the future with the upgrading of social services and living conditions of many previously disadvantaged communities, fewer individuals will be exposed to hepatitis A in early life, and thus will have a greater chance of a symptomatic infection later.

The prevalence of hepatitis B, although lower than other regions, remains unacceptably high and current strategies to vaccinate all children against hepatitis B as part of the Expanded Program on Immunisation should continue. In doing so, the long-term consequences of hepatitis B infection such as cirrhosis and hepatocellular carcinoma will be prevented. This strategy will also ensure that the hepatitis D virus is not introduced into the South African population.

Hepatitis C virus continues to be prevalent in these communities, and to a greater extent than that seen in the blood transfusion services. Preventing infection through adequate screening of blood products is essential, and is already in place. Further interventions to limit the spread of hepatitis C remain difficult, while the mechanisms of spread in the broader community remain unclear. The

health authorities should continue providing the infra-structure to monitor and treat those with cirrhosis and hepatocellular carcinoma induced by hepatitis C. In the interim, research projects investigating potential hepatitis C vaccines should be supported.

Hepatitis E virus exposure differs significantly between communities, with the poorest rural communities at greatest risk of acquiring the disease, because of inadequate access to chlorinated water and water-borne sewerage disposal. The hepatitis E virus data contained in this thesis have been presented to the Ministry of Water Affairs and Forestry of the South African Government. Subsequent to this presentation, the Ministry launched a government-sponsored water provision project in the rural village of Keiskammahoek, as a part of a broader program to improve the lives of these communities. The provision of chlorinated water to Keiskammahoek offers an opportunity to re-investigate the community at a later date to assess the potential favourable impact of this water provision.

HGV/GBV-C carriage was found to be high. The associations between infection and living in an urban setting, younger age and the use of illicit (non intra-venous) drugs are noteworthy. Recommendations to prevent infection must remain guarded in the setting of no definable, validated disease association described to date.

This molecular characterisation of South African HGV/GBV-C variants has shown two significant differences from those isolates previously described. Firstly, the majority of South African isolates are genetically different from those described in

West Africa and other geographic regions, requiring subdivision into a separate fourth genotype. If a disease association is documented and validated for HGV/GBV-C, it will be necessary to confirm that the findings are true for genotype 4 as well. Secondly, the 5'NCR of a minority of these isolates have a major deletion over domains II and III of the proposed RNA secondary structure, suggesting that this segment of the 5'NCR is non-essential for maintenance of the internal ribosomal entry site. However, these variants were not found in the absence of wild-type HGV/GBV-C, thus raising the possibility that these mutant viral RNA copies are not in fact replication competent. The finding of these deletion isolates offers an opportunity for further studies of ribosomal binding, by way of translation assays comparing deletant non-coding regions with the wild-type prevalent in South Africa.

The accumulated clinical and laboratory evidence has demonstrated that the initial presumed association with liver disease is incorrect. The liver does not act as the primary site of replication for HGV/GBV-C and there is no detectable liver disease in the presence of viraemia. This study has more organs tested than any other study to date. In addition, these are the only results generated from biopsies taken from individuals without of end-organ failure or AIDS. The data add to that of Laskus et al. (1998) and Radkowski et al. (1999) showing the sites of replication to be the bone marrow and spleen.

The viruses (both HGV and GBV-C) have a nomenclature and an association that was unfortunately linked to liver disease before this was proven. Thus, on the basis of our and other accumulated data, it is suggested that the use of the

names “the Hepatitis G Virus” and “GBV-C” both be limited in scientific publications, until formal naming and placing of the virus within the unified taxonomy system of the International Committee on Taxonomy of Viruses.

Appendix

Appendix 1 Keiskammahoek Districts

1. Keiskammahoek Town
2. Bomapass
3. Elukhanyweni
4. Cata I
5. Cata II
6. Upper Mnyameni
7. Lower Mnyameni
8. Upper Gxulu
9. Lower Gxulu
10. Bumbane
11. Ngxalawe I
12. Ngxalawe II
13. Nompha
14. Nqolo-Nqolo
15. Mqukwana
16. Ndlovini-Nothenga
17. Mthwaku
18. Ndlovini
19. Tyinira
20. Ngobozana

21. Ndema's Farm
22. Dontsa
23. Pierie Saw Mills
24. Fair View
25. Evelyn Valley
26. Sandile Purification
27. Fort Cox
28. Lower Zingcuka
29. Madubela
30. Sonjika's Farm
31. Rabe
32. Ngcamngeni
33. Burnshill
34. Lenye
35. Zanyokwe
36. Ngxondoreni
37. Rabula
38. Upper Ngqumeya
39. Lower Ngqumeya
40. Upper Zingcuka
41. Peter's Farm
42. James Farm
43. Tshoxa
44. Ngqudela
45. Gwili-Gwili

APPENDIX 2

MEDIA, ANTIBIOTICS, BUFFERS AND SOLUTIONS

All solutions that were autoclaved were done so at 15 pounds per square inch, for 15 minutes.

1: MEDIA

BROTHS

2X Yeast-Tryptone (2 X YT)

Yeast extract	10g
Tryptone	16g
NaCl	5g
Distilled water	1000ml

Autoclave

AGAR

2X YT Agar

2X YT	100ml
Agarose	1.5g

Autoclave

2: ANTIBIOTICS

AMPICILLIN

Solvent = water

working conc. = 100μg/ml

Store at -20°C

3: BUFFERS AND SOLUTIONS

CHLOROFORM-ISOAMYLALCOHOL

Mix at a ratio of 24:1

DEPC-TREATED WATER (0.1%)

DEPC	0.1ml
Distilled water	100ml

Stir for 30 min. at RT, then autoclave

ETHIDIUM BROMIDE (EtBr)

EtBr	0.1g
Distilled water	10ml

Shake well to dissolve

GEL TRACKING DYE IV (Gel-loading buffer)

Bromophenol blue	25mg

Sucrose	4g
EDTA (0.5M, pH8.0)	0.4ml
Distilled water	to 10ml

DENATURATION BUFFER

NaCl (5M)	150ml
NaOH (10N)	25ml
Distilled water	325ml

NEUTRALISATION BUFFER (1M ammonium acetate, 20 mM NaOH)

NaOH	8.0g
Tris-HCl (1M, pH 7.2)	770.8g
EDTA (0.5M, pH 8.0)	to 10 L

WASHING BUFFER 1

Maleic acid	11.6g
NaCl	8.76g
Distilled water	to 500ml

Adjust pH to 7.5 with 10N NaOH and autoclave

WASHING BUFFER 3

Tris-HCl (1M, pH 9.5)	10ml
NaCl (5M)	2ml
$MgCl_2$ (1M)	5ml

Distilled water	to 100ml

BLOCKING BUFFER (1%)

Blocking reagent	1g
Washing Buffer 1	100ml
Autoclave and store at 4°C	

PREHYBRIDISATION/HYBRIDISATION SOLUTION

SDS (10%)	2ml
SSC (20X)	6ml
Blocking stock solution (10%)	2ml
Distilled water	to 20ml

SODIUM DODECYL SULPHATE (SDS) (10%, w/v)

SDS	10g
Distilled water	100ml
Heat to +/- 80°C to dissolve	
Add HCL to pH7.2	

20X STANDARD SODIUM CITRATE (SSC)

NaCl	175.3g
Sodium citrate	88.2g
Adjust to pH 7.0 with 10N NaOH, and autoclave	

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