GB VIRUS C / HEPATITIS G VIRUS (GBV-C/HGV) INFECTION IN KWAZULU NATAL, SOUTH AFRICA. ITS DIAGNOSIS, DISTRIBUTION AND MOLECULAR EPIDEMIOLOGY

by

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Submitted in partial fulfillment of the requirements for the degree of Doctor in Philosophy in the Department of Medicine, Nelson R Mandela School of Medicine, Faculty of Health Sciences, University of Natal, Durban, South Africa

DECLARATION

The experimental work in this thesis was carried out in the University of Natal, Nelson R Mandela School of Medicine, Faculty of Health Sciences, Departments of Medicine and Virology. This thesis is the candidate's own original work and has not been submitted in any form to another university. Selected results from the thesis have been presented at national and international scientific meetings and published in scientific journals either as reviews, abstracts or full manuscripts. Researchers who were closely associated in these studies are either co-authors in these publications or are duly acknowledged in the text.

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DEDICATION

This thesis is dedicated to the memory of

Marjorie Nadia Sathar

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PRESENTATIONS

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ETHICAL CONSIDERATIONS

The studies were approved by the Ethics Committees of the University of Natal and the National Blood Transfusion Services. Written informed consent was obtained from all subjects included in the study.

LIST OF ABBREVIATIONS

α, alpha

~, approximately

δ, delta

°C,degrees centigrade

λgt11, Lamda gt11 (43 kilobase linear double stranded vector)

-, minus, negative

$[NH_4]_2SO_4$, ammonium sulfate

3′ NCR, three prime non-coding region

^{33}P, isotope labelled phosphate

5′ NCR, five prime non- coding region

A, absorbance

AIDS, acquired immunodeficiency syndrome

AIH, auto immune hepatitis

ALD, alcoholic liver disease

ALP, alkaline phosphatase

ALT, alanine aminotransaminase

AST, aspartate aminotransaminase

AUG, adenine uracil guanine

bDNA, branched DNA

bp, base pair

BVDV, Bovine Viral Diarrhoea Virus

c/s/s, counts per second per second

CAT, chloramphenicol acetyltransferase

CD, cluster designation

CDE, Genetic Data Environment

cDNA, complementary DNA

CDTect, carbohydrate deficient transferrin

CLD, chronic liver disease

GBV-A,GB Virus A

GBV-B, GB Virus B

GBV-C, GB Virus C

GGT, gamma glutamyl transferase

GLOB, globulin

H_2O_2, water

HAEM, haemodialysis

HAV, Hepatitis A Virus

HBV,Hepatitis B Virus

HCC, hepatocellular carcinoma

HCHV, Hog Cholera Virus

HCl, hydrochloric acid

HCV, Hepatitis C Virus

HEV, Hepatitis E Virus

HFV, Hepatitis F Virus

HGV, Hepatitis G virus

HIV, human immunodeficiency virus

hr, hour

IFN, interferon

IL, interleukin

IRES, internal ribosome entry site

ISDR, interferon sensitive determining region

IVDA, intravenous drug abusers

JEV, Japanese Encephalitis Virus

KCl, potassium chloride

KZN, KwaZulu Natal

LCx, ligase chain reaction

M, male

MEGA, Molecular Evolutionary Genetic Analysis

MEIA, microparticle enzyme immunoassay

mg/ml, miligrams per millilitre

$MgCl_2$, magnesium chloride

min, minute

ml, milliliter

mm, millimole

mM, millimolar

M-MLV RT, Moloney Murine Leukemia Virus Reverse Transcriptase

mRNA, messenger RNA

mths, months

n, number

NaCl, sodium chloride

NANB, non-A,non-B hepatitis

NANE, non-A, non-B, non-C, non-E hepatitis

ng, nanogram

nm, nanometer

NS3, non structural proteins

nt, nucleotide

OLT, orthotopic liver transplant

ORF, open reading frame

PBMC, peripheral blood mononuclear cell

PCR, polymerase chain reaction

PHYLIP, Phylogeny Inference Package

pmol, picomole

RDA, representational difference analysis

RNA, ribonucleic acid

rpm, revolutions per minute

RTD-PCR, real time detection PCR

RT-PCR, reverse transcription PCR

rTth, *Thermus thermophilus* polymerase

Anti-HBs, antibody to HBsAg

sAg, surface antigen

SD, standard deviation

sec, second

SISPA, sequence independent single primer amplification

T cells, Thymus cells

TAE, Tris-acetate-EDTA buffer

TBE, Tris-borate-EDTA buffer

T-BIL, total bilirubin

TRANSF, transfusion

Tris, Hydroxymethylaminomethane

Tris-HCl, Tris

U, units

U/L, units per liter

ug, microgram

ug/ml, micrograms per milliliter

ul, microliter

uM, micro molar

umol/l, micromoles per liter

UTR, untranslated region

uv, ultra violet

V, volts

v/v, volume per vloume

w/v, weight per vloume

WNV, West Nile Virus

XLA, X-linked agammalobulinmia

YFV, Yellow Fever Virus

ABSTRACT

Recently a new *Flavivirus*, GB Virus C also referred to as Hepatitis G virus (GBV-C/HGV) was identified in humans with indeterminate hepatitis. Whilst in non-African countries this discovery led to an enormous enthusiasm to elucidate an association with liver disease, very little was known about the prevalence and pathogenicity of GBV-C/HGV infection in KwaZulu Natal, South Africa, where Hepatitis B Virus (HBV) infection is endemic and infection with the Human immunodeficiency virus (HIV) is a catastropic health problem.

Sera from patients with liver disease (chronic liver disease [n = 98]; alcoholic liver disease [n = 50]); high risk groups (haemodialysis patients [n = 70]; HIV positive mothers and their babies [n = 75]) and control groups (alcoholics without liver disease [n = 35] and blood donors from the four racial groups [n = 232]) were screened for GBV-C/HGV RNA and Anti-E2 antibodies by reverse transcription polymerase chain reaction (RT-PCR) and an enzyme linked immunosorbent assay (ELISA), respectively. Overall 43.9% (43/98) of patients with chronic liver disease; 60 % (30/50) of patients with alcoholic liver disease; 47.1% (33/70) of haemodialysis patients; 60% (21/35) of alcoholics without liver disease and 31.9% (74/232) of blood donors (Africans] 44/76; 5.9%); Asians (5/52; 9.6%); Whites (15/49; 30.6%) and "Coloureds"[mixed origin] (9/54; 16.6%)]) were exposed to GBV-C/HGV infection as determined by the detection of Anti-E2 &/or RNA in serum. There was a significant difference in the prevalence of GBV-C/HGV infection (RNA &/or anti-E2) between African blood donors and the other racial groups ($p < 0.001$), between blood donors and haemodialysis patients ($p = 0.02$) and or patients with chronic liver disease ($p =$

0.04). There was no significant difference in the prevalence of GBV-C/HGV between African blood donors (45/76, 59.2%) and alcoholics with and without liver disease (30/50, 60% and 21/35, 60%, respectively). Anti-E2 antibodies and GBV-C/HGV RNA were almost mutually exclusive. GBV-C/HGV infected dialysis patients tended to have had more transfusions ($p = 0.03$) and had a longer duration of dialysis than non infected patients, indicating that the majority of patients on maintenance haemodialysis acquire their GBV-C/HGV infection through the transfusions they receive. There was no evidence for *in utero* and/or *intrapartum* transmission of GBV-C/HGV. However, there is some mother-to-infant transmission of GBV-C/HGV, though it is very probable that in KZN GBV-C/HGV is transmitted by as yet undefined non-parenteral routes.

Sequence and phylogenetic analysis of the 5′ non-coding region (5′ NCR) and E2 gene segments of the GBV-C/HGV genome identified an additional "genotype" (Group 5) of GBV-C/HGV that is distinct from all other known GBV-C/HGV sequences (Groups 1-4). Although there is a high prevalence of Group 5 GBV-C/HGV isolates in KZN, there was no significant difference in liver biochemistry between GBV-C/HGV infected and non-infected patients with liver disease or between blood donors in each of the four racial groups. There was no significant differences in CD4 (461.12 ± 163.28 vs 478.42 ± 181.22) and CD8 (680.83 ± 320.36 vs 862.52 ± 354.48) absolute cell counts between HIV positive patients co-infected with GBV-C/HGV and those not infected with GBV-C/HGV, respectively. However, significantly higher relative CD3 [$80.0 \pm 4.17\%$ vs $70.99 \pm 19.79\%$] ($p = 0.015$), gamma delta T cells ($\gamma\delta$T) [$3.22 \pm 1.30\%$ vs $2.15 \pm 29.12\%$] ($p =$

0.052) and lower CD 30 [35.45 ± 17.86% vs 50.59 ± 9.20%] (p = 0.041) status were observed in GBV-C/HGV positive compared to GBV-C/HGV negative HIV infected patients, respectively.

Although there is a high prevalence of novel Group isolates of GBV-C/HGV in KZN, the lack of elevated liver enzymes and clinical hepatitis in blood donors and haemodialysis patients suggests that GBV-C/HGV is not associated with liver disease. HBV and not GBV-C/HGV modifies the course of alcoholic liver disease. The relatively higher number of CD3 cells and increased γδT expression, together with a decrease in CD 30 cells tends to suggest an association with protection and or delayed progression of HIV disease in GBV-C/HGV infected patients. Whilst GBV-C/HGV is not associated with liver disease, it may be an important commensal in HIV infected patients.

CHAPTER 1

CHAPTER 1

LITERATURE REVIEW

The first reference to hepatitis was ascribed to Hippocrates. The earliest reference to hepatitis in Western Europe was in a letter written in 751 AD by Pope Zacharias to St Boniface, Archbishop of Mainz. Although hepatitis was known as an infectious disease, the current knowledge of human hepatitis began with observational studies of epidemics during World Wars I and II and the demonstration later by transmission studies with bacteria free filtrates that the disease was cuased by a virus (Zuckerman, 1977). The lack of animal models of infection and *in vitro* systems for propagating hepatic viruses hindered much of the early work on the causative agents of viral hepatitis in humans. The first successful transmission of viral hepatitis from humans to non-human primates was achieved by Deinhardt *et al* (1967), when serum from a 34 year-old surgeon (whose initials were GB) with acute hepatitis was inoculated into tamarins (*Saguinus spp*). Animals inoculated with GB serum developed hepatitis, as did animals inoculated with sera of tamarins with GB serum-induced hepatitis. In the 1970s two types of hepatitis transmissions were differentiated on the basis of epidemiological observations. Type A (Hepatitis A virus) was transmitted faecal-orally and Type B (Hepatitis B virus) was transmitted parenterally. The discoveries of hepatitis A virus (HAV) and hepatitis B virus (HBV) as candidates for non - A, non - B (NANB) human hepatitis in the 1970s led to a waning in the interest of the GB agent, but indicated the existence of further forms of hepatitis (non-A, non-B [NANB]).

In the late 1980s and 1990s, two NANB agents, Hepatitis C Virus (HCV) (Choo *et al*, 1989; Kuo *et al*, 1989) and Hepatitis E Virus [HEV](Reyes *et al*, 1990; Dawson *et al*, 1992) were established as principle etiologic agents in NANB hepatitis. Hepatitis F Virus (HFV), a new enterically transmitted virus which is hepatotropic was implicated as an additional agent in NANB hepatitis, but these findings were not substantiated (Arankalle *et al*, 1994, Deka *et al*, 1994). Despite the development of very sensitive and specific assays to detect viral proteins and antibodies to these proteins or nucleic acids, it became apparent that approximately 15-20% of parenterally transmitted cases of NANB hepatitis and 10-15% of community acquired hepatitis cases could not be attributed to either of these viruses (Alter and Bradley, 1995). After exclusion of known viruses and rigorous application of criteria to exclude drug exposure, alcohol abuse, autoimmune diseases and other conditions that cause liver damage, the evidence strongly suggested that yet another hepatitis virus or viruses may be responsible for these cases of non-A, non-B, non-C, non-E (NANE) hepatis. To examine this possibility, recombinant DNA technology was used to identify a novel virus from the serum or plasma of patients with non-A-E hepatitis.

1.1 Discovery of GB Virus -C (GBV-C)

Tamarins clearly developed hepatitis after inoculation with the GB serum, after extensive serial passage of the agent in these animals, a question was raised about the human origin of the hepatitis-causing agent in them (Parks and Melnick, 1969). Additional passage and cross-

challenge experiments indicated that the GB agent was distinct from any known human hepatitis viruses (Karayiannis *et al*, 1989; Deinhardt *et al*, 1975).

A modified version of the polymerase (PCR) technique known as representational difference analysis (RDA) (Lisitsyn *et al*, 1993) was used to selectively amplify unique nucleotide sequences present in one complex source (in this case, a pool of known infectious tamarin GB plasma at the eleventh passage) and absent in another (plasma taken from the same animals before inoculation) (Simons *et al*, 1995a). Genomic representations of the related source material were generated by PCR amplification of the restriction-digested DNA to which specific oligonucleotide primers were ligated. Through a series of subsequent subtractive hybdrization and selective amplification steps, the unique sequences present in one representation were exponentially amplified, while the sequences held in common with a second representation were linearly amplified. The enriched DNA was then cloned and analysed. Simons *et al* (1995b) identified and cloned as complementary DNA (cDNA), two RNAs with features of flavivirus RNAs in tamarin GB plasma and named these RNAs, hepatitis GB viruses A (GBV-A) and B (GBV-B). Both apparent viral genomes have an overall organization similar to the genomes of HCV and other flaviviruses, and have limited nucleotide sequence homology with one another and with HCV (Muerhoff *et al*, 1995). GBV-A and GBV-B have not been found in humans, although GBV-B was serially transmitted in tamarins and caused hepatitis, GBV-A could not be readily transmitted to tamarins (Schaluder *et al*, 1995). Whether either is the causative agent of hepatitis in the original human patient, GB, remains unproven.

Following cloning of GBV-A and GBV-B from tamarin serum, a related virus was discovered in serum of a human in West Africa (Simons *et al*, 1995b). Serum samples from numerous patients living in this region were first screened for antibodies against recombinant proteins encoded by GBV-A and GBV-B (Pilot-Matias *et al*, 1996a); immunoreactive samples were further screened for RNA by reverse transcription (RT)-PCR, using degenerate primers derived from the homologous sequences shared by GBV-A, GBV-B and HCV from the NS3/helicase region of these viruses. Amplification products were obtained from immunoreactive sera whose sequences were different from the other viruses (Simons *et al*, 1995c). Sequence and phylogenetic analysis of the PCR products from GBV-A, GBV-B and HCV together with additional members of the Flaviviridae, were consistent with this sequence being derived from a virus which is more closely related to GBV-A than to GBV-B, or any of the HCV genotypes. The new virus was named GBV-C (Simons *et al*, 1995c).

1.2 Discovery of Hepatitis G Virus (HGV)

The independent discovery of another human virus, named hepatitis G virus (HGV) which is almost identical to GBV-C, was more straightforward than that of GBV-C. Independently, in an effort to identify additional agents for post-transfusion NANB hepatitis, Linnen *et al* (1996a) performed molecular cloning with plasma from a patient designated PNF2161. This patient was originally believed not to be infected with HCV (based on a negative test for serum antibody to HCV with a first-generation immunoassay). Subsequent testing with a second-generation immunoassay for anti-HCV and PCR assay

(for the 5′ nontranslated region of HCV) for HCV RNA in serum demonstrated that this patient was chronically infected with HCV. The extracted viral RNA was reverse transcribed with random primers to prepare cDNA and subsequently amplified following a sequence-independent single-primer amplification (SISPA) strategy, that was designed to amplify any double-stranded DNA. The amplified product was used to construct a λgt11 library, which was then immunoscreened with serum from the patient, and a single immunoreactive clone was identified. An anchor PCR was employed to generate multiple overlapping clones whose sequences were combined to produce the 9392 nucleotide (nt) long HGV genome (Linnen *et al*, 1996a). In this way, a virus like RNA sequence was identified and designated as HGV.

When HGV sequences were compared to GBV-A and GBV-B, these viruses were found to be significantly similar. Comparison of HGV with the original 311 nucleotide (nt) reported for GBV-C (Simons *et al*, 1995b) found that these sequences were nearly identical (85% and 100% at the nucleotide and amino acid levels, respectively) suggesting that these viruses were closely related (Linnen *et al*, 1996a). Comparisons of the full-length sequences of GBV-C and HGV revealed that both these viruses were indeed independent isolates of the same virus (Zuckerman, 1996). The nt sequence comparisons between HGV and GBV-C and other members of the Flaviviridae showed intermediate homologies between GBV-C and GBV-A (48%), and between GBV-C and HGV (43%) compared to the lower homology found between any other two GBV-C/HGV clones (26%-32%) (Table 1.1).

Table 1.1 Amino acid sequence homology (%) between HGV and GBV-C and other members of the Flaviviridae (Linnen *et al* 1996a; Leary *et al*, 1996a,b)

	GBV-A	GBV-B	GBV-C	HGV
GBV-B	27			
GBV-C	48	28		
HGV	43	28	100 (NS3)	
HCV	26	32	29	27

GBV-GB Virus A, B,and C; HGV- Hepatitis G Virus; HCV- Hepatitis C Virus

1.3 Molecular Analysis of GBV-C/HGV

1.3.1 Genomic organisation

The genomic organisation of GBV-C/HGV and HCV is similar and both viruses belong to the *Flaviviridae* family. The genome of the virus consists of single stranded RNA and has a positive polarity. Similar to other members of the Flaviviridae, GBV-C/HGV genome is approximately 9500 nt (Table 1.2 and Fig. 1.1) in length and contains a single open reading frame (ORF) that encodes the viral polyprotein.

Table 1.2 Comparison of the genomic organisation of GBV-C and HGV with other members of the *Flavivirdae* [adapted from Linnen *et al* (1996a) and Simmons *et al* (1996)]

	GBV-A	GBV-B	GBV-C	HCV
Genome	9653	9143	9103-9395	9401
5′ NCR (nt)	594	445	281-551	341
3′ NCR (nt)	198	83	313-315	72
Polyprotein	2954	2864	2842-2933	3011
Core	absent	present	absent	present
Host	tamarins	tamarins	humans	humans

GBV-GB Virus A, B, and C; HGV- Hepatitis G Virus; HCV- Hepatitis C Virus
5′ NCR-5 prime non-coding region; nt-nucleotide

Based on amino-acid sequence homology, characteristic amino-acid motifs and hydrophobicity/hydrophilicity plots, homologues of nearly all HCV proteins have been found in GBV-C/HGV (Linnen *et al*, 1996a; Leary *et al*, 1996b). The viral polyproptein is preceded by a 5′ non-coding region (5′ NCR), followed by a long open reading frame (ORF) terminating with 3′ NCR (Fig.1.1) (Linnen *et al*, 1996a). The polyprotein is cleaved into smaller viral proteins necessary to complete the viral life cycle with different functions by host-encoded signal peptidases and viral proteases. Polyprotein cleavage was studied by expressing GBV-C/HGV cDNA constructs in mammalian cells using vaccina virus or baculovirus (Kim and Fray, 1998). These fragments include the envelope proteins (E1 and E2) at the amino or N-terminal end followed by nonstructural (NS) proteins (NS2, NS3, NS4, and NS5) at the carboxy or C- terminal end (Fig. 1.1).

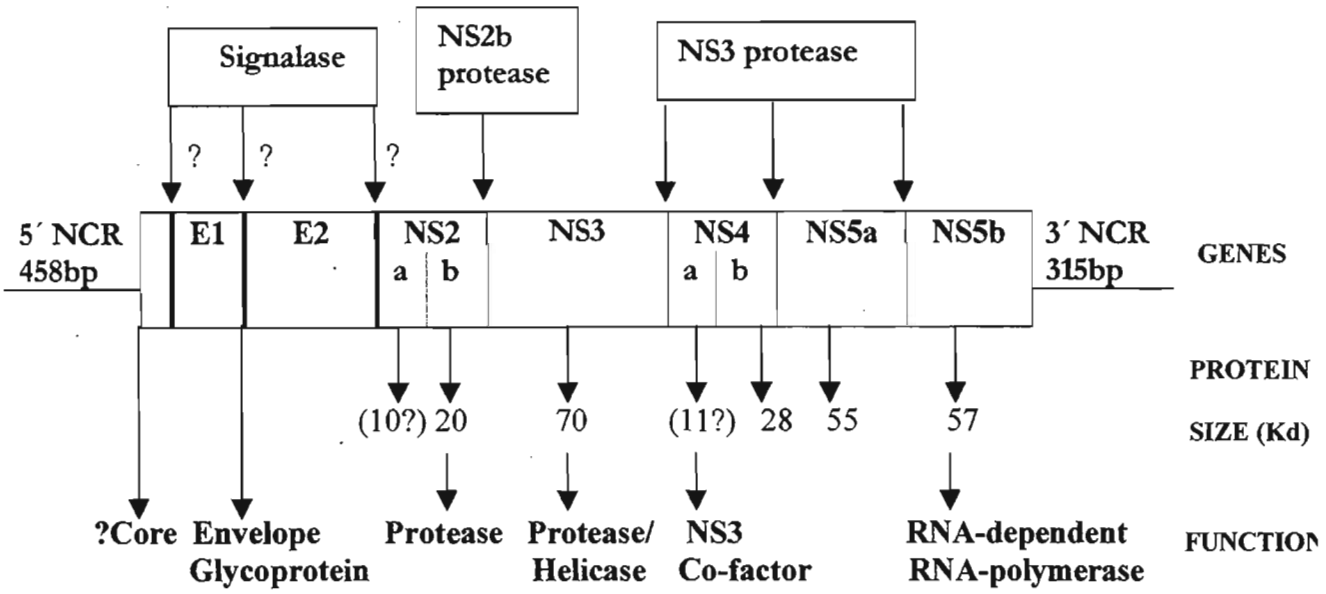


Fig. 1.1 HGV (PNF2161) genome. Proteolytic processing and functions of the structural and non-structural proteins (nt-nucleotide; NCR-non coding region; E–envelope genes; NS - non-structural genes) [adapted from Kim and Fry, 1998)

1.3.2 Sequence characteristics

The GBV-C/HGV genome is unusual. The region between the 5′ NCR and the envelope proteins is absent or truncated [Fig.1.1] (Leary *et al*, 1996b, Linnen *et al*, 1996a; Simons *et al*, 1995a). This region normally encodes the nucleocapsid or core protein that encases the viral genome. Nucleotide sequence alignments from the middle of the 5′ NCR to within the El encoding region show that the deduced amino-acid sequences upstream of El varied in length. Close scrutiny shows that all isolates examined so far share a consensus initiation codon 14 amino-acids upstream of El (Muerhoff *et al*, 1996; Linnen *et al*, 1996a; 1997; Kim *et al*, 1997; Okamoto *et al*, 1997; Pickering *et al*, 1997), which represents the shortest peptide encoded by some isolates. In order to determine the site of polyprotein initiation, Simons *et al*. (1996) performed *in vitro* translation experiments using constructs in which the 5′ NCR sequences, which included coding sequences also, were fused in frame with chloramphenicol acetyltransferase (CAT). CAT activity was abolished when the AUG nearest to El was rendered inactive. Similarly, truncation of sequences upstream of El that removed this initiation codon had a similar effect. These experiments established that the AUG nearest to El is the authentic initiation codon for polyprotein synthesis and confirmed the absence of a nucleocapsid protein. The only other flavivirus without an obvious core gene is GBV-A (Simons *et al*, 1996). Other Flaviviruses have an initiator methionine at the first amino acid of a basic core protein.

Thermodynamic modelling to determine the secondary RNA structure of the 5′ NCR shows that this region has a complex structure consisting of a number of stem-loops

suggestive of a regulatory function (Simons *et al*, 1996; Pickering *et al*, 1997). Translation of bicistronic RNAs containing 5′ NCR sequences within the intercistronic space was consistent with the presence of a weakly active internal ribosome entry site (IRES) not only in HGV, GBV-C, but also in GBV-A (Simons *et al*, 1996).

The NS2 protein is a zinc protease that cleaves the polyprotein at the NS2/NS3 junction. The activity of the protein was abolished when the two conserved amino-acids histidine and cysteine associated with this activity were mutated (Kim *et al*, 1997). The N-terminal end of NS3 encodes a protein which functions as a chymotrypsin-like serine protease and contains the characteristic conserved catalytic triad of the three amino-acids H,D and S, (Leary *et al*, 1996b; Linnen *et al*, 1996a; Kim *et al*, 1997). The carboxyl end of the NS3 region encodes a protein with motifs associated with helicase activity, whilst the NS5 region of the gene contains the GDD motif characteristic of RNA-dependent RNA polymerases. Finally, the envelope glycoproteins of the virus bear little similarity to those of HCV and are less heavily glycosylated. Such observations suggest either that GBV-C/HGV is a core-less virus, or that a core-like structure is appropriated from cellular proteins or a co-infecting viruses. The 3′ UTR of HGV is 350 nt long, thus, longer than that of HCV. It does not have a poly-A tract or a poly-U-tail (Tanaka *et al*, 1995). Neither the 5′ nor the 3′ UTR bear any identity to those of HCV.

1.3.3. Phylogenetic relationships

1.3.3.1. Relatedness to the *Flaviviridae*

Phylogenetic analysis take into account the number of genetic changes that have occurred to change one amino acid sequence into another. The larger the number of changes, the further the genetic distance between the two sequences. If differences between multiple sequences are compared, sequences that share groups of similar changes as compared to the other sequences will be closer to each other than those that do not contain shared changes. The relative evolutionary distances between GB viruses and other members of the *Flaviviridae* are readily apparent on analysis of the unrooted phylogenetic tree in which the length of the traced path from one sequence to the next represents the amount of genetic distance between the sequences (Fig.1.2). Phylogenetic analysis of *Flaviviridae* polyproteins shows that HGV and GBV-C are more similar to the tarmarin GBV-A than either HCV or GBV-B which are about equally distant (Fig 1.2). Even more distant are members of the flaviviruses (yellow fever and dengue 1) and the pestiviruses (Linnen *et al*, 1996a; Leary *et al*, 1996b) (Fig 1.2). The divergence between GBV-A, GBV-B and GBV-C and other *Flaviviridae* members, including HCV group, demonstrates that GBV-C/HGV cannot be considered genotypes of HCV. This analysis suggests that the GB agents may be classified into separate genera within the *Flaviviridae* or into subgenera in a genus including HCV. However the lack of a core protein in GBV-A and GBV-C could necessitate the placement of these viruses in their own family.

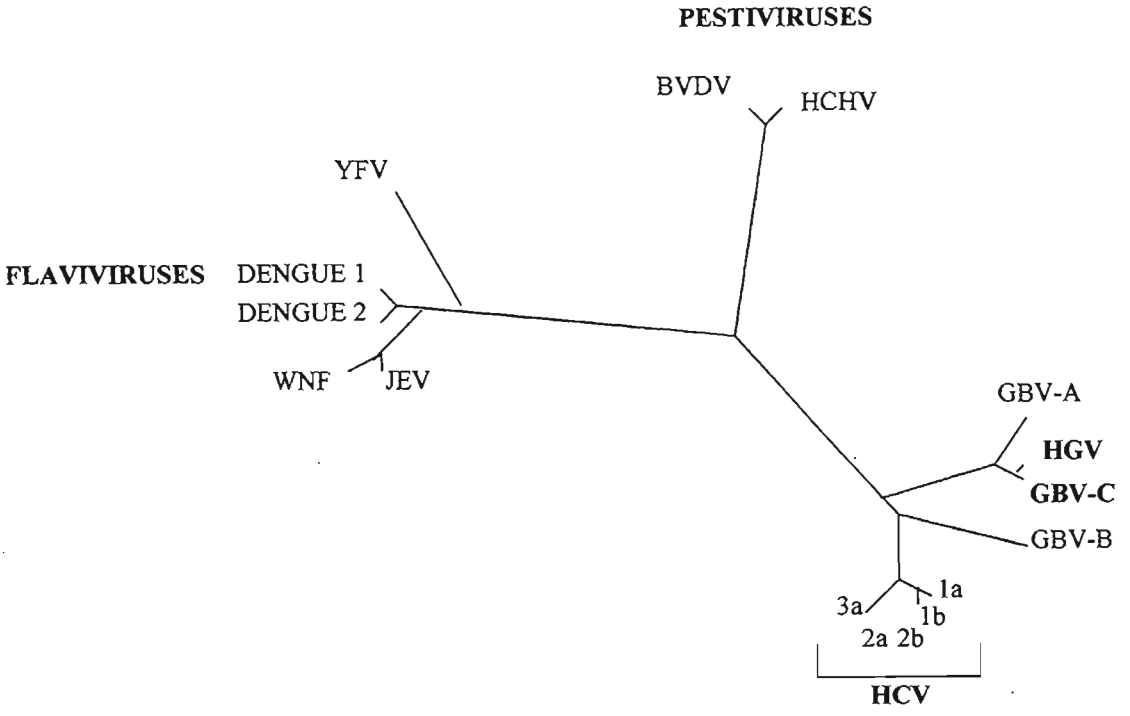


Fig. 1.2 Unrooted phylogenetic tree of the NS3 RNA helicase domains of the GB viruses and other members of the Flaviviridae. **Flaviviruses:** Japanese Encephalitis virus (JEV), West Nile virus (WNV), Yellow Fever virus (YFV) and Dengue virus type 1 and 2.**Pestiviruses:** Hog cholera virus (HCHV), Bovine Viral Diarrhoea virus (BVDV). **HCV :** genotypes 1a,1b, 2a, 2b and 3a (adopted from Simons *et al*, 2000).

1.3.3.2. Phylogenetic groups

Initial studies on the phylogenetic structure of GBV-C/HGV were based on the sequence analysis of short fragments of the NS3 (~118nt) and or the NS5b (~354nt) regions of the genome, where the initial PCR primers for the detection of this virus were located (Simons *et al*, 1995b Leary *et al*, 1996b; Linnen *et al*, 1996a). Congruent phylogenetic trees were not noted between isolates from different parts of the world in these studies (Berg *et al*, 1996a; Kao *et al*, 1996; Schreier *et al*, 1996; Tsuda *et al*, 1996; Kinoshita *et al*, 1997; Pickering *et al*, 1997; Viazov *et al*, 1997a).

Previous studies have identified three (Okamoto *et al*, 1997; Susuki *et al*, 1999) four (Charrel *et al*, 1999) or five (Takahashi *et al*, 1997) phylogenetic groupings of GBV-C/HGV, although some of these groupings are weak and inconsistent between different studies. Whereas HCV genotypes can be distinguished by phylogenetic analysis of a variety of sub-genomic regions as small as 222 nt, variants of GBV-C/HGV can not be reliably identified in this way. Systematic analysis of six complete GBV-C/HGV genome sequences revealed that congruent phylogenetic relationships were obtained for only a minority of 300, 600 and 1200 nt fragments, and that the optimal region was all or part of the 5′ NCR (Muerhoff *et al*, 1997 Smith *et al*, 1997a). Based on the assumption that the sequence analysis of the 5′ NCR was predictive of the analysis of the complete genome sequence, sequence analysis of 5′ NCR provided limited evidence that GBV-C/HGV can be separated into three major groups that

correlated with the geographic origin of the isolates (Muerhoff *et al*, 1996, 1997; Smith *et al*, 1997a). The evolutionary relationship between some of these isolates is presented graphically in Fig. 1.3. Group 1 consists of isolates from West and Central Africa and includes the original GBV-C isolate. Group 3 isolates have been found predominantly in South-East Asia and Japan. Group 2 isolates have found in Europe and North America (HGV like), but also includes isolates from Japan, Pakistan and East Africa. In addition Group 2 can be subdivided into Groups 2a and 2b; the sub-division of Group 1 is less certain (Muerhoff *et al*, 1997).

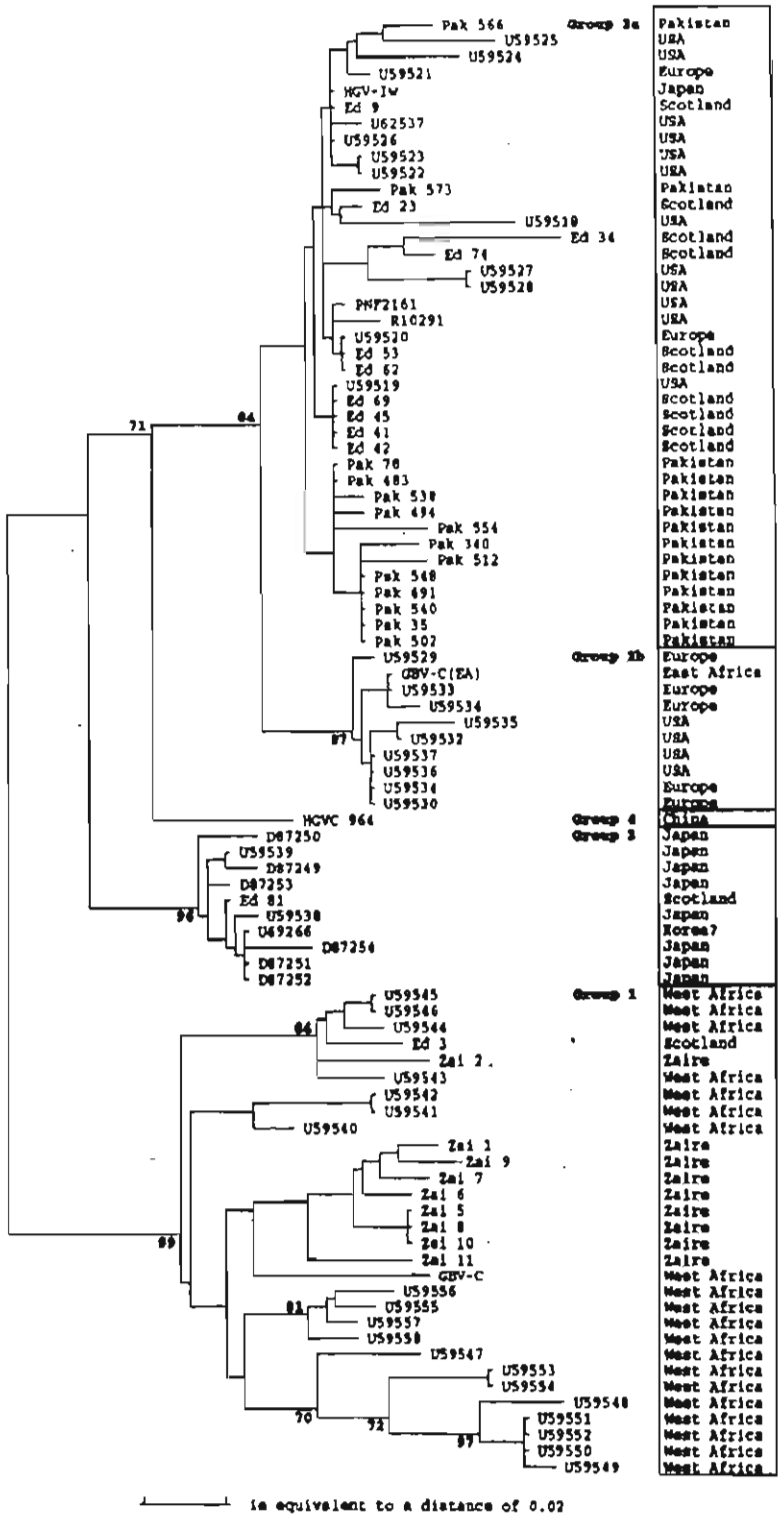


Fig.1.3 Consensus phylogenetic tree of the 5′NCR of geographically disparate isolates of GBV-C/HGV. The tree was generated by comparison of positions –366 to –235 of the 5′NCR. The percentage of bootstrap replicates in which major groupings were observed amongst 500 replicates is indicated. Sequences are identified by accession numbers or by the prefixes Pak for Pakistan, Ed for Edinburgh and Zai for Zaire. The geographical origin for each sequence is indicated at the right (Smith *et al*, 1997a).

1.4 Biophysical and biochemical characteristics of GBV-C/HGV

Biophysical and biochemical characterisation of GBV-C/HGV has been hampered by the lack of adequate cell culture or animal models to generate large amounts of highly purified virions. Currently, human plasma is the best source of GBV-C/HGV. Because viral titers are $< 10^9$/ml. Viral RNA is detected by RT-PCR. Flaviviruses are characterised physically by a lipid-containing envelope and core proteins that surround and protect the RNA viruses from serum RNases (Westaway *et al*, 1985). The lipid envelopes of Flaviviruses are sensitive to disruption by chloroform and by ionic and non-ionic detergents. Chloroform has been reported to reduce infectivity of HCV (Bradley *et al*, 1983). In addition chloroform has been used to reveal HCV nucleocapsids (Hijikata *et al*, 1993). Several biological detergents (e.g. sodium deoxycholate, Brij-58, NP-40, Triton X-100 and Tween-80) have been used to reveal nucleocapsids of Flaviviruses as indicated by a shift in detectable RNA to higher buoyant densities (Miyamoto *et al*, 1992; Takahashi *et al*, 1992).

The presence and/or absence of core protein in GBV-C/HGV has been debatable. The length of the GBV-C/HGV ORF upstream of the E1 protein is truncated or absent in some isolates. The distribution of genetic polymorphisms among various isolates suggests that the region is not a coding region (Okamoto *et al*, 1997). *In vitro* translation data suggest that only the AUG at position 552 is capable of initiating translation (Simons *et al*, 1996). It has been suggested that either the virus does not have a nucleocapsid, or that it uses a unique capsid protein,

perhaps obtained from another virus with which the infected person is co-infected, or even a cellular gene product (Simons *et al*, 1996).

Immunoprecipitation experiments suggest that GBV-C/HGV is associated with low density lipoproteins in plasma but not human antibodies because anti-lipoprotein A1 and anti-apolipoprotein B antibodies shift PCR signal to the precipitate while anti-human IgG antibody does not (Hijikata *et al*, 1996; Sato *et al*, 1996). Xiang *et al* (1998) using sucrose gradient centrifugation, isopycnic banding in caesium chloride, and saline density flotation centrifugation demonstrated extremely-low-density GBV-C/HGV virion particles (1.07 to 1.09 g/ml) and a nucleocapsid density of approximately 1.18 g/ml, similar to HCV. One major difference between the particle types was that GBV-C/HGV was consistently more stable in cesium chloride than HCV. An observed shift in RNA buoyant density in sucrose gradients following treatment with Tween-80 in the presence of RNase inhibitor (Sato *et al*, 1996) provided additional physical evidence of a GBV-C/HGV nucleocapsid. Lectin columns bind GBV-C/HGV consistently with the presence of envelope glycoproteins (Sato *et al*, 1996). In their more recent study Xiang *et al* (1999) evaluated the buoyant density of GBV-C/HGV, and found very low density particles consistent with virions, and intermediate density particles consistent with nucleocapsids in GBV-C/HGV infected patients. In addition, for the first time, Xiang *et al*, (1999) using electron microscopy demonstrated an apparent nucleocapsid within an enveloped particle. To assess the origin of the protein content of this particle, patient plasma was evaluated for reactivity with a synthetic oligopeptide representing a conserved

region near the amino terminus of the predicted ORF (Xiang *et al*, 1999). Specific antibody was detected in some individuals, similar to the data presented by Feucht *et al* (1997a) who identified antibody against a recombinant core protein in GBV-C/HGV-infected patients. These data indicate that GBV-C/HGV particles contain nucleocapsids. In some isolates, the region upstream of the GBV-C/HGV E1 protein coding region appears to be expressed, and this region may represent the structural protein of the nucleocapsid. Sequential filtration and RNA titration studies suggest that the GBV-C/HGV virion is between 50-100nm in diameter, being unchanged after passing through a 100nm filter and being reduced to 10-1000 fold after passing through a 50nm filter (Melvin *et al*, 1998).

For HCV, the free nucleocapsid is detected by the PCR signal to an increased buoyant density after detergent treatment (Melvin *et al*, 1998). The peak of GBV-C/HGV RNA in sucrose gradients was observed at a buoyant density of 1.05-1.13g/ml (Melvin *et al*, 1998). GBV-C/HGV RNA was reduced following treatment with chloroform or with five detergents indicating that GBV-C/HGV has a lipid-containing envelope (Melvin *et al*, 1998). Sucrose gradients and self-forming caesium chloride gradients of detergent-treated GBV-C/HGV showed a shift in the RNA peak to heavier buoyant density only when RNasin and high detergent concentrations were present (Melvin *et al*, 1998). The treated material was non filterable and RNA had density of > 1.5g/ml (Melvin *et al*, 1998). These results indicate that GBV-C/HGV is a small, lipid-enveloped RNA virus, and that the GBV-C/HGV RNA was

present in an extended or aggregated form and do not support the existence of a nucleocapsid structure in the GBV-C/HGV virion (Melvin *et al*, 1998).

Until additional biochemical studies are undertaken to identify the amino terminus of the proteins contained in the nucleocapsid structures shown on the electronmicrographs (Xiang *et al*, 1999), the origin of these structures will remain debatable.

1.5 Diagnosis of GBV-C/HGV

In contrast to what is found for HCV, the identification of serological markers indicative of ongoing GBV-C/HGV infection have not been successful to-date. Thus, the identification of viraemic samples has relied on the detection of viral RNA. Several nucleic acid-based assays have been developed for this purpose. Although labour-intensive, many of these assays are sensitive and specific. An antibody marker of viral clearance has been identified.

1.5.1. Detection of Ribonucleic acid (RNA)

The reverse-transcription polymerase chain reaction (RT-PCR) is the only diagnostic tool available to detect current GBV-C/HGV infection in human serum, plasma, or tissues. Several of the assays start with the synthesis of complementary DNA (cDNA) using random hexameres with reverse transcriptase; the purpose of which was to generate relatively large

number of DNA sequences to which GBV-C/HGV specific primers would anneal. The cDNA then could be efficiently amplified with thermostable DNA polymerase. Because some of the genomic regions were not well conserved among various GBV-C/HGV isolates, degenerate primers (primers with bases containing more than one nucleotide at a certain position) were used. To increase the specificity of the annealing between mismatched nucleotide sequences, a thermal cycling strategy named "touchdown" PCR was used. The annealing temperature is incrementally decreased with each PCR cycle, preferentially allowing the best matched primer template to form (Leary *et al*, 1996b).

There are also assays that use primers targeting the NS5B replicase region of the GBV-C/HGV genome (Genelabs Technologies INC, USA; Boehringer Mannheim, Germany). The PCR product is detected by dot blot hybridisation using a riboprobe or an enzyme-linked test (semiquantitative Enzymun-Test). Another strategy depends on the PCR amplification of two target regions (5′ NCR and NS5A) from the same clinical specimen (Schlueter *et al*, 1996). This technique is applicable for routine screening in clinical laboratories.

The first generation RT-PCR assays utilised primers from the NS3 and NS5 (Simons *et al*, 1995b; Linnen *et al*, 1996a) regions of the genome to detect viral RNA. However, because the targets of these assays are coding regions, silent mutations noted in the various GBV-C/HGV isolates resulted in a number of false-negative results. The second generation RT-PCR assays target highly conserved regions within the NS5A (Linnen *et al*, 1996a) or 5' UTR (Schlueter *et*

al, 1996); the latter region appears to be more sensitive and more widely used in this respect (Kao *et al*, 1997a). These assays are more sensitive and specific than the first generation assays. However, both the first and second generation assays rely on Southern hybridisation for the detection of GBV-C/HGV PCR products. The inclusion of Southern hybridisation results in assays that are tedious, labour-intensive and time consuming.

Kao *et al* (1997a) compared sensitivity and specificity of PCR assays using primers from different regions of viral genome, viz. 5′ NCR, E2 and NS3. The positive rates by 5′ NCR, NS3 and E2 primers were 100%, 98% and 84%, respectively, and the sensitivity of the PCR assays using 5′ NCR primers were 10-100 times more likely to detect GBV-C/HGV RNA than that of NS3 and E2 primers. These results were confirmed by other investigators (Bhardwaj *et al*, 1997; Cantaloube *et al*, 1997).

Because GBV -C/HGV was detected in blood donors, the question arose whether blood banks should include GBV-C/HGV in their screening. Screening of blood donations is only feasible with serological tests, not with PCR. Third generation RT-PCR assays have been developed which permit a higher level of throughput, in conjunction with ease of use. In these assays GBV-C/HGV specific PCR products are captured by oligomer hybridisation for the detection of sequences within the 5′ NCR. The assay described by Schlueter *et al* (1996) utilises a two step RT-PCR assay in which digoxigenin-labelled dUTP is incorporated during the PCR amplification. A biotinylated capture oligomer hybridises with all GBV-C/HGV specific

products that may be present. The GBV-C/HGV oligomer complex is captured on a streptavidin-coated surface. After extensive washing, captured PCR product is detected immunologically using an anti-digoxigenin-peroxidase conjugate (Schlueter *et al,* 1996) [Boehringer Mannheim,Germany].

Abbott Laboratories described a single tube assay that only requires the addition of serum-derived nucleic acid to pre-aliquoted reaction vials. Reverse transcription, PCR amplification and oligomer hybridisation occur in the same tube containing recombinant *Thermus theromphilus* (rTth) polymerase, adamantine-labeled sense and anti-sense oligonucleotide primers for the 5′ NCR of GBV-C/HGV, and carbazole-labelled capture oligomer. Detection of GBV-C/HGV product employs the automated LCx detection system (Abbott Laboratories, Abbott Park, IL, USA) utilising a microparticle enzyme immunoassay (MIA). These third generation assays provide data within 5 hours (hr) as opposed to the 2 days minimum required for the first- or second- generation RT-PCRs.

Apart from detection of GBV-C/HGV RNA based on RT-PCR, branched chain DNA (bDNA) signal amplification has been developed. Although convenient, it is not as sensitive as RT-PCR assays. While RT-PCR remains the "gold standard" for detecting GBV-C/HGV, its sensitivity is not fully resolved.

1.5.2. Detection of antiviral antibodies (Anti-E2)

Five different prokaryotically expressed recombinant GBV-C/HGV proteins were employed for serological studies utilising ELISA and Western blot (Dawson *et al*, 1996; Pilot-Matias *et al*, 1996b). Although some regions of GBV-C/HGV have been identified as immunogenic, efforts to develop a screening immunoassay for the detection of GBV-C/HGV antibodies have been unsuccessful because only 25% of GBV-C/HGV infected individuals develop antibodies to these prokaryotically-expressed proteins. Most of the seropositive individuals produce antibodies against only a single antigen, and no single antigen has been identified thus far that is consistently recognised by individuals exposed to GBV-C/HGV (Dawson *et al*, 1996).

The phylogenetic relationship between GBV-C/HGV and HCV suggested that useful markers of GBV-C/HGV exposure might be found in regions homologous to those identified for HCV. With this in mind, an antibody response to GBV-C/HGV directed against the envelope glycoprotein, E2, has been detected following its expression as a recombinant protein in Chinese hamster ovary cells (Pilot-Matias et al, 1996b; Tacke *et al*, 1997a). The secreted E2 protein has been purified and used in a solid phase enzyme-linked immunosorbent assay (ELISA) for the detection of anti-E2 (Dille *et al*, 1997). Interestingly, almost all sera positive for anti-E2 are negative for viral RNA, and vice versa, implying that anit-E2 is associated with virus clearance and is perhaps, protective or neutralising (Pilot-Matias *et al*, 1996b; Tacke *et al*, 1997). However, the specificity for anti-E2 has not been established.

1.5.2.1 GBV-C/HGVAnti-E2. A protective and or neutralising antibody and a marker for recovery ?

Analysis of serial samples for both RNA and anti-E2 suggests that GBV-C/HGV infection follows one of two paths; acute infection followed by recovery (appearance of GBV-C/HGV E2 antibody), or acute infection progressing to chronicity (persistence of GBV-C/HGV RNA). Follow-up of 16 post transfusion patients for up to 16 years revealed that individuals who develop an anti-E2 response become GBV-C/HGV-RNA-negative, while those who do not develop anti-E2 are persistently infected (Tacke *et al*, 1997b). The presence of anti-E2 and the subsequent loss of viraemia have been confirmed by other investigators (Dille *et al*, 1997; Gutierrez *et al*, 1997; Hassoba *et al*, 1997). Anti-E2 appears to be a long-lasting circulating antibody and once acquired generally tends to persist (Masuko *et al*, 1996, Lefrere *et al*, 1997).

In chronic HCV infection the co-existence of E2/NS1 antibody and viraemia suggests that anti-E2 is not a neutralising/protective antibody, but serves as a marker of active HCV replication (Yuki *et al*, 1996). GBV-C/HGV Anti-E2 on the other hand, has been described as a marker of viral clearance and is considered to be protective against GBV-C/HGV reinfection. In 54 recipients who underwent orthotopic liver transplantation (OLT) the presence of anti-E2 pre-transplant was associated with a relatively low rate (15%) of post transplantation GBV-C/HGV infection compared to 46% in anti-E2 negative (pre transplant)

patients (Hassoba *et al,* 1998). Post transplantation immune suppression apparently had only a minor effect on the prevalence of anti-E2 in patients who were anti-E2 positive prior to transplantation (Hassoba *et al,* 1998). A negative association between the presence of GBV-C/HGV RNA and the presence of anti-E2 was found in all patients tested pre- and post transplantation, suggesting viral clearance (Hassoba *et al,* 1998). Anti-E2 appears to be a neutralizing antibody whose presence at the time of liver transplantation protects against acquisition of GBV-C/HGV infection post-OLT (Bizollon *et al,* 1998; Hassoba *et al,* 1998; Tillman *et al,* 1998; Silini *et al,* 1998). No new GBV-C/HGV infections were noted among subjects with anti-E2, despite ongoing drug use (Thomas *et al,* 1998). These data suggest that anti-E2 is a protective or neutralising antibody. Because high titres of GBV-C/HGV anti-E2 antibodies appear to be protective in humans, the possibility of using human or animal immune serum globulin for prophylactic purposes is intriguing. Other possibilities such as use of recombinant GBV-C/HGV E2 glycoprotein or synthetic subunit vaccines should be explored if GBV-C/HGV is proven to be a public hazard.

1.6 Routes of GBV-C/HGV transmission

Since the discovery of GBV-C/HGV, attempts have been made to clarify its principle mode of transmission. In non-African countries the predominant route of transmission of GBV-C/HGV is parenteral. (Sathar *et al,* 2000) A high prevalence of GBV-C/HGV has been found in subjects with frequent parenteral exposure and in groups at high risk of exposure to blood and

blood products, including intravenous drug abusers (IVDA), patients on maintenance haemodialysis, multi-transfused individuals and haemophiliacs (Sathar *et al*, 2000). The high prevalence in blood donors worldwide, suggests that the principle route of transmission is via contaminated blood and blood products. However, maintenance of the virus at high levels in blood donors and the general population requires an effective non-parenteral route of transmission. Blood donors, however, are not representative of the general population since they are highly selective.

Various studies have pointed to the important role of sexual exposure as a likely route of transmission of GBV-C/HGV, in both non-HIV infected subjects without the risk for parenteral transmission (IVDU and multi-transfused individuals including haemophiliacs) (Kao *et al*, 1997b; Scallan *et al*, 1998; Sawayama *et al*, 1999) and HIV infected individuals with the risk for parenteral and sexual transmission (homosexuals, heterosexual and prostitutes) (Nubling *et al*, 1997; Ibanez *et al*, 1998; Nerurkar *et al*, 1998; Bourlet *et al*, 1999). Infection with GBV-C/HGV appears to be more frequent in patients with a sexual risk than those with parenteral exposure (Ibanez *et al*, 1998; Bourlet *et al*, 1999). In a study of 600 antenatal patients there was an overall prevalence (GBV-C/HGV RNA and/or Anti-E2 positive) of 11.8%. Since this group represents a young, sexually active population, the authors concluded that sexual or close contact might play a role in the transmission of GBV-C/HGV (Skidmore & Collingham 1999). Rubio *et al* (1997) reported a GBV-C/HGV prevalence of 21.7% among heterosexual partners of 150 index cases. Strak *et al* (1996) found

GBV-C/HGV prevalence of 10.9% among non-drug injecting homosexual and bisexual men. Scallan *et al* (1998) found a high prevalence of markers for GBV-C/HGV in non-intravenous drug using prostitutes (40%) and male homosexuals (47%). A positive correlation was demonstrated between GBV-C/HGV infection in prostitutes and the number of years of prostitution (Kao *et al*, 1997b; Sawayama *et al*, 1999) and the high frequency of paid sex (Wu *et al*, 1997). The near absence of GBV-C/HGV infection among heterosexual men (4%) and the comparatively higher prevalence among heterosexual women (15%) suggests that, as in HIV infection, the receptive partner is at high risk for acquiring GBV-C/HGV (Nerurkar *et al*, 1998). Interspousal transmissions of GBV-C/HGV have been reported (Kao *et al*, 1997c; Sarrazin *et al*, 1997). Whilst the role of semen in the transmission of GBV-C/HGV is controversial (Semprini *et al*, 1997; Hollingsworth *et al*, 1998; Eugenia *et al*, 2001), recent reports have suggested that human saliva may contribute to the spread of GBV-C/HGV RNA (Chen *et al*, 1997; Seemayer *et al*, 1998; Eugenia *et al*, 2001). Tucker *et al* (2000) did not detect GBV-C/HGV replicative intermediaries in the cadaver biopsies. Tucker *et al* (2000) looked in salivary glands and the gonads of GBV-C/HGV positive patients. Their results suggest that the virus may be present in the saliva and semen of infected individuals, but not transmitted by these routes. Despite the evidence for increased frequencies of GBV-C/HGV infection in association with sexual exposure, the mechanism of transmission remains unclear.

There is a higher risk of mother to infant transmission in high risk groups (Feucht *et al*, 1996; Viazov *et al*, 1997b; Fiscler *et al*, 1997; Zanetti *et al*, 1997; Wejstal *et al*, 1999). However, it

is not clear whether co-infection with HCV, HIV-1 or both or IVDU are the underlying cause for transmission of GBV-C/HGV from mother to infant. Nor is it clear as to whether transmission of GBV-C/HGV is influenced by breast-feeding or by the mode of delivery (Zanetti *et al*, 1997; Wejstal *et al*, 1999). Although the rate of perinatal transmission of GBV-C/HGV exceeds that of HCV, in most studies GBV-C/HGV did not induce liver disease in the infants studied (Zanetti *et al*, 1997; Viazov *et al*, 1997b; Wejstal *et al*, 1999).

Among 220 cases of needle-stick injuries, GBV-C/HGV RNA was detected in 21 (9.5%) donors (Shibuya *et al*, 1998). At the time of injury none of the 21 recipients was positive for GBV-C/HGV RNA or anti-E2, only 1/14 (7.1%) recipients became positive for GBV-C/HGV RNA which persisted for approximately 3 years without any evidence of liver disease (Shibuya *et al*, 1998). It has been suggested that iatrogenic transmission of GBV-C/HGV could possibly occur as a result of insufficient sterilization of needles and syringes (Ohshima *et al*, 2000). Confirmation that GBV-C/HGV is indeed an occupational hazard in hospital employees (Gartner *et al*, 1999; Schaade *et al*, 2000) will require more comprehensive longitudinal studies.

1.7 Prevalence of GBV-C/HGV infection

The prevalence of GBV-C/HGV infection in selected groups of subjects from some published studies are listed in tables 1.3 - 1.6. The frequency of positivity for RNA or anti-E2. varies

among groups, depending on the subjects' origins and the methods used to detect GBV-C/HGV markers. Generally, infection with GBV-C/HGV is significantly associated with a history of IVDA, exposure to blood transfusions, dialysis and with HCV infection. There is a higher prevalence of GBV-C/HGV RNA in blood donors and the general population of African countries (10-19%) compared to non-African countries (1-6%) (Table 1.3). The high prevalence in commercial blood donors (5-26%) (Table 1.4) is probably due to the increased risk of parenetral acquisition in this group. The prevalence of GBV-C/HGV anti-E2 antibodies in healthy individuals ranges from 3% -15.1% (Table 1.6).

The simultaneous detection of anti-E2 greatly extends the ability of RT-PCR to define the epidemiology of GBV-C/HGV (Table 1.6). For example, in non-African countries, 1-2.5% of blood donors is GBV-C/HGV RNA positive. Using anti-E2 assays, the same population of blood donors showed 3-9% seroprevalence. The overall prevalence of GBV-C/HGV in non-African blood donors was 4 -16%, compared to 20-30% in Africa (Table 1.6). In the high risk group of patients the overall prevalence of GBV-C/HGV infection ranged from 20-89% (Table 1.6). The combined overall prevalence of GBV-C/HGV infection is higher in African countries compared to non-African countries (Table 1.6). The simultaneous detection of GBV-C/HGV RNA and anti-E2 may represent the seroconversion state.

Thus, the total exposure to GBV-C/HGV should take into account both the number of PCR positive samples (i.e. viraemic/RNA positive) and anti-E2 positive samples (i.e. previously

infected but cleared) in a given population. GBV-C/HGV infection appears to be a common infection globally. The reason for the high prevalence of GBV-C/HGV in blood donors worldwide and the basis for the racial differences in GBV-C/HGV infection in blood donor populations are not known. Whether socio-economic factors are associated with prevalence of GBV-C/HGV is not known for certain, although a relationship was noted between GBV-C/HGV infection and the lack of water-borne sewage (Tucker *et al*, 1997). The differences in the prevalence of detecting GBV-C/HGV infection (Tables 1.3-1.6) may be due to the differences in the sensitivity of the various PCR protocols and primers (derived from various regions of the genome) used by various investigators; pre selection of patients in terms of status for other viral markers as well as different patient histories. Further investigations are required to determine whether genetically distinct isolates from different geographical regions of the world escape detection by current PCR methods and anti-E2 assays.

Table 1.3 Reported prevalence of GBV-C/HGV RNA in blood donors

Continent	Country	n	RNA+(%)	References
N America	USA	769	13 (1.7)	(Linnen *et al*,1996a,b)
S America	Brazil	11	2 (1.8)	(Lampe *et al*, 1997)
Africa	Egypt	82	16 (12.2)	(El-Zayadi *et al*, 1999)
	South Africa	248	32 (12.9)	(Mphahlele *et al*, 1998)
	South Africa	249	26 (10.4)	(Tucker *et al*, 1997)
	South Africa	167	21 (12.6)	(Lightfoot *et al*, 1997)
	South Africa	532	59 (11.1)	(Castelling *et al*, 1998)
	South Africa	232	44 (18.9)	(Sathar *et al*, 1999a)
Caribbean	Martinique	221	9 (4.1)	(Cesaire *et al*, 1999)
Asia	Japan	448	4 (0.9)	(Masuko *et al*, 1996)
	China	205	2 (1)	(Wang *et al*, 1997a,b)
	Thailand	69	3 (4.3)	(Raengsakulrarch *et al*, 1997)
	Vietnam	890	11 (1.2)	(Kakumu *et al*, 1998)
	Nepal	181	4 (2)	(Shrestha *et al*, 1997)
	Mongolia	121	8 (6.6)	(Kondo *et al*, 1997)
Australia		120	5 (4)	(Moaven *et al*, 1996)
Europe	Austria	92	3 (3)	(Schlueter *et al*, 1996)
	Germany	1048	14 (1.34)	(Roth *et al*, 1997)
	Germany	106	59 (4.7)	(Heringlake *et al*, 1996a)
	UK	125	4 (3.2)	(Jarvis *et al*, 1996)
	Italy	100	1 (1)	(Fiordalisi *et al*, 1996)
	Spain	200	6 (3)	(Saiz *et al*, 1997)

n-number; %-per cent; +-positive

Table 1.4 Reported prevalences of GBV-C/HGV RNA in high risk groups

Clinical Group	Country	RNA+ n (%)	References
Haemodialysis	Egypt	79 (30)	(El-Zayadi *et al.*, 1999)
	Europe		(Linnen *et al*, 1996b)
	South Africa	70 (24.3)	(Sathar *et al.*, 1999a)
	Brazil	65 (15.4)	(Lampe *et al.*, 1997)
	China	79 (54)	(Wang *et al*, 1997a)
	France	61 (57.5)	(De Lamballerie *et al*, 1996)
	Japan	519 (3.1)	(Masuko *et al.*, 1996)
	Indonesia	58 (55)	(Tsuda *et al.*, 1996)
Haemophiliacs	Scotland	95 (14)	(Jarvis *et al.*, 1996)
	Europe	49 (9)	(Linnen *et al*, 1996a)
	France	92 (17.4)	(Gerolami *et al.*, 1997)
	Japan	63 (24)	(Kinoshita *et al.*, 1997)
	Nicaragua	45 (38)	(Gonzales-Prez *et al.*, 1997)
	South Africa	102 (23.5)	(Castelling *et al.*, 1998)
IVDUs	Greece	106 (32.1)	(Anastassopoulou *et al.*, 1998)
	US	27 (4)	(Dille *et al.*, 1997)
	US	102 (14.7)	(Gutierrez *et al.*, 1997)
	Sweden	19 (16)	(Shev *et al.*, 1998)
	Europe	60 (33.3)	(Linnen *et al,* 1996a)
	Germany	99 (38)	(Tacke *et al.*, 1997a)
	Japan	49 (12)	(Aikawa *et al*, 1996)
Commercial Blood Donors	China	205 (8)	(Roth *et al.*, 1997)
	US	50 (26)	(Dille *et al.*, 1997)
	US	42 (5)	(Pilot-Matias *et al*, 1996b)
	US	711 (13.1)	(Gutierrez *et al.*, 1997)
HealthCare Workers	Egypt	30 (6.6)	(El-Zayadi *et al.*, 1999)
Drug Addicts	Nepal	72 (44)	(Shrestha *et al.*, 1997)
Prostitutes	UK	50 (18)	(Scallan *et al.*, 1998)
	China	140 (21)	(Wu *et al.*, 1997)
Homosexuals & Bisexual men	UK	52 (17)	(Scallan *et al.*, 1998)
	Germany	101 (11)	(Schlueter *et al.*, 1996)

n-number; %-per cent; +-positive; IVDU-intravenous drug user

Table 1.5 Reported prevalences of GBV-C/HGV RNA in patients with liver diseases

Clinical Group	Country	RNA+ n (%)	References
Acute/Chronic HBV	Europe	72 (9.7)	(Linnen *et al* 1996a)
	Egypt	63 (11,1)	(El-Zayadi *et al.*, 1999)
	Japan	83 (4)	(Sugai *et al.*, 1997)
	US	100 (32)	(Alter *et al*, 1997a)
	South Africa	106 (26.4)	(Mphahlele *et al.*, 1998)
Acute/Chronic HCV	Egypt	100 (14)	(El-Zayadi *et al.*, 1999)
	Germany	100 (9)	(Schleicher *et al.*, 1996)
	Italy	83 (26.5)	(Francesconi, 1997)
	Japan	88 (8)	(Sugai *et al.*, 1997)
	Russia	22 (41)	(Yashina *et al.*, 1997)
	Spain	143 (5.6)	(Saiz *et al.*, 1997)
	Taiwan	52 (10)	(Hwang *et al.*, 1997)
	US	116 (20)	(Alter *et al*, 1997a)
	South Africa	82 (30.5)	(Mphahlele *et al.*, 1998)
Acute/Chronic HAV	Japan	21 (0)	(Nakatsuji *et al.*, 1996)
	US	100 (25)	(Alter *et al*, 1997a)
Non A-E hepatitis	China	108 (16.7)	(Wang & Jin, 1997)
	Japan	43 (0)	(Nakatsuji *et al.*, 1996)
	Russia	28 (3.6)	(Yashina *et al.*, 1997)
	US	149 (8.7)	(Dawson *et al.*, 1996)
Chronic Liver Disease	Indonesia	149 (5)	(Tsuda *et al.*, 1996)
	Nepal	145 (3)	(Shrestha *et al.*, 1997)
	South Africa	92 (12)	(Sathar *et al.*, 1999a)
	Japan	226 (7.5)	(Nakatsuji *et al.*, 1996)
	US	326 (12.2)	(Linnen *et al*, 1996a)
	Italy	36 (39)	(Fiordalisi *et al.*, 1996)
Hepatocellular Carcinoma	Japan	111 (10)	(Kanda *et al.*, 1997)
	Japan	109 (10)	(Nishiyama *et al.*, 1999)
	Thailand	101 (6)	(Tangkijvanich *et al.*, 1999)
	China	114 (14,9)	(Cao *et al.*, 1998)
	Europe	57 (7)	(Brechot *et al.*, 1998)
	South Africa	135 (14)	(Lightfoot *et al.*, 1997)
Fulminant hepatitis	Japan	6 (50)	(Yoshiba *et al.*, 1995)
	Japan	10 (0)	(Kanda *et al.*, 1997)
	Germany	22 (50)	(Heringlake *et al.*, 1996)
	UK	23 (21.7)	(Haydon *et al.*, 1997)
	UK	20 (0)	(Sallie *et al.*, 1996)
	US	36 (38.8)	(Munoz S.J. *et al.*, 1999)
	Taiwan	32 (9)	(Liu *et al.*, 1999)

n- number; % - percent; + positive

Table 1.6 Reported prevalences of GBV-C/HGV RNA and Anti-E2 antibodies in blood donors, high risk groups and patients with liver disease

Clinical Group	Country	n	RNA+ (%)	Anti-E2+ (%)	Exposure* (%)	References
Blood Donors	Japan	200	2 (1)	10 (5)	12 (6)	(Tanaka *et al.*, 1998)
	Germany	200	5 (2.5)	7 (9)	33 (16.5)	(Tacke *et al.*, 1997a)
	US	199	3 (1.5)	9 (4.5)	11 (5.5)	(Gutierrez *et al.*, 1997)
	US	100	1 (1)	3 (3)	4 (4)	(Dille *et al.*, 1997)
	Spain	200	5 (2.5)	28 (14)	32 (16)	(Tacke *et al.*, 1997b)
	South Africa	248	32 (12.9)	30 (12.1)	52 (21.1)	(Mphahlele *et al.*, 1999)
	South Africa	232	44 (18.9)	35 (15.1)	74 (31.9)	(Sathar *et al.*, 1999a)
Commercial Donors	US	711	93 (13.1)	195 (27.4)	288 (40.5)	(Gutierrez *et al.*, 1997)
Plasmapheresis Donors	US	50	13 (26)	17 (34)	30 (60)	(Dille *et al.*, 1997)
	West Africa	30	10 (33.3)	4 (13.3)	14 (46.7)	(Dille *et al.*, 1997)
IVDU	Germany	99	38 (38)	41 (41)	75 (75)	(Tacke *et al.*, 1997a)
	US	27	1 (3.7)	23 (85.2)	24 (88.9)	(Dille *et al.*, 1997)
	US	102	15 (14.7)	76 (74.5)	91 (89.2)	(Gutierrez *et al.*, 1997)
Haemophliacs	Spain	62	22 (34)	20 (32)	33 (53)	(Tacke *et al.*, 1997b)
	France	92	16 (17.4)	33 (35)	47 (51)	(Gerolami *et al.*, 1997)
Haemodialysis	South Africa	70	17 (24.3)	18 (25.7)	33 (47.1)	(Sathar *et al.*, 1999a)
Renal Transplant	Germany	221	31 (14)	89 (40)	118 (53)	(Stark *et al.*, 1997)
Chronic liver disease	South Africa	98	12 (12.2)	32 (32.7)	33 (47.1)	(Sathar *et al.*, 1999a)

n- number; % - percent; + positive; IVDU-intravenous drug user; *RNA + and/or Anti-E2 +

1.8 Site(s) of GBV-C/HGV replication

A true hepatotropic virus replicates in the liver. GBV-C/HGV is a flavivirus with a positive stranded RNA genome that is similar to HCV, as such replication should proceed via a negative strand RNA intermediate, the detection of which should be possible in the liver.

GBV-C/HGV RNA has been detected by RT-PCR in washed hepatocytes in 9 of 58 (15%) children with chronic viral hepatitis (Lopez-Alcorocho *et al*, 1997). Madejon *et al* (1997) and Saito *et al* (1997) detected GBV-C/HGV antigenomic RNA in 12 of 13 livers and in peripheral blood mononuclear cells (PBMCs) of one of the same 13 patients examined. Because hepatocytes and PBMCs are bathed in blood, it is possible that the PCR signal noted may have been due to cell-bound virus rather than active replication occurring in these cells (Laras *et al*, 1999). Using RT-PCR with tagged primers and southern blot analysis, antigenomic GBV-C/HGV RNA was detected in 4 of 6 liver specimens; using *in situ* hybridisation in two such specimens GBV-C/HGV infection was restricted to hepatocytes (Seipp *et al*, 1999). Using RT-PCR with tagged primers and *in vitro* derived templates, Mellor *et al* (1998) was unable to detect antigenomic GBV-C/HGV RNA in the liver biopsies nor in the PBMCs of 20 GBV-C/HGV infected individuals. Radkowski *et al* (1998) suggested that PBMCs may not be the replication site of GBV-C/HGV. In 5 of 17 patients undergoing liver transplant, GBV-C/HGV RNA

was detected in sera and not in the liver on repeated testing for viral RNA from different portions of the liver (Fan *et al*, 1999). In patients co-infected with GBV-C/HGV and HCV, the hepatotropism of HCV and not GBV-C/HGV was consistently proven (Kudo *et al*, 1997; Laskus *et al*, 1997; Pessoa *et al*, 1998).

In vitro studies have provided evidence that GBV-C/HGV is able to infect and replicate in established cell lines of haematopoietic origin, vascular endothelial cells and human hepatoma cell lines (Ikeda *et al*, 1997; Seipp *et al*, 1999; Fogeda *et al*, 1999). In contrast, contradictory results have been reported with respect to the detection of GBV-C/HGV in liver and PMBC samples from chronically infected patients (Laskus *et al*, 1997; Pessoa *et al*, 1998; Fan *et al*, 1999). Some of the these discrepancies between different studies may be due to either the existence of GBV-C/HGV variants with different cell tropism (Fogeda *et al*, 2000) or differences in the inoculums used as a source of the virus. Patients with GBV-C/HGV are often co-infected with HCV, and since HCV infects and replicates in both the liver and PMBC, HCV may influence the tropism of GBV-C/HGV variants by facilitating the replication of some strains (Fogeda *et al*, 2000). Alternatively, the cell lines used for the study may have been co-infected with HCV (Ikeda *et al*, 1997; Shimizu *et al*, 1999).

Hepatocytes may not be the primary site of viral replication. Antigenomic GBV-C/HGV RNA was also detected in the mononuclear cell infiltrates in the portal areas of the liver

(Kobayashi *et al*, 1999;), in lymphoid and megakaryoctoid cell lines and primary vascular endothelial cells (Handa *et al*, 2000). The detection of replicating virus in hepatic tissue may reflect virus replication in haematopoietic cells and/or endothelial cells present in the liver.

In their study of 6 cadaver biopsies from one GBV-C/HGV positive patient co-infected with HIV, Mushahwar *et al* (1998) detected glyceraldehyde-3-phosphate dehydrogenase (GAP-DH) mRNA and GBV-C/HGV RNA only in the liver which was localised to individual hepatocytes. In multiple cadaver autopsies of 12 patients (4 with AIDS, 6 HIV positive and 2 with end stage liver disease), Laskus *et al*, (1998) and Radkowski *et al* (1999) consistently demonstrated GBV-C/HGV RNA intermediaries in the bone marrow and spleen. However, these results are difficult to interpret in immunocompromised patients. In a preliminary study of 23 cadaver biopsies from four GBV-C/HGV positive patients who were HIV negative, the spleen and bone marrow biopsies were uniformly positive for both negative-and positive strand GBV-C/HGV RNA (Tucker *et al*, 2000). The authors concluded that GBV-C/HGV is a lymphotropic virus that replicates primarily in the spleen and bone marrow (Tucker *et al*, 2000). These findings require confirmation using *in situ* hybridisation and immunohisochemical staining.

Strand-specific detection of RNA is fraught with problems such as false priming of the incorrect strands or self-priming related to RNA secondary structure. All of the strand

specific studies used methods to reduce false priming and self-priming events viz. chemical modification of the 3′ ends (Saito *et al*, 1997; Madejon *et al*, 1997); conducting cDNA synthesis at high temperature with the thermostable enzyme (rTth) (Laskus *et al*, 1997, Tucker *et al*, 2000); using *in vitro* derived templates (Laskus *et al*, 1997; Mellor *et al*,1998; Tucker *et al*, 2000); using "tagged" primers (Mellor *et al*,1998; Seipp *et al*, 1999); *in situ* hybridisation of liver biopsies (Seipp *et al*, 1999; Kobayashi *et al*, 1999) and *in vitro* infection of human hepatoma cells with GBV-C/HGV mono-infected serum (Seipp *et al*,1999). Only Laskus *et al* (1997) and Mellor *et al* (1998) qualified their reactions using *in vitro* derived templates and provided end point titration data. The site of GBV-C/HGV replication has been an area of intense interest and remains unresolved.

1.9 Disease association of GBV-C/HGV infection

1.9.1 Liver disease

Despite the accumulated body of knowledge after the discovery of GBV-C/HGV the clinical significance of GBV-C/HGV and whether it caused significant acute or chronic liver disease was controversial. Most GBV-C/HGV infections appear to be asymptomatic, transient, and self-limiting, with slight or no elevation of alanine aminotransferase (ALT) (Alter *et al*; 1997a,b). Co-infection with GBV-C/HGV does not alter the clinical course of community acquired Hepatitis A, B or C (Alter et al; 1997a,b).

Most of these sub-clinical cases resolve after loss of serum GBV-C/HGV RNA with a concomitant appearance of anti-E2 antibodies (Dille *et al*, 1997; Gutierrez *et al*, 1997). To evaluate the clinical course of GBV-C/HGV infection, patients infected with GBV-C/HGV only were studied by Wang *et al* (1996). Among 25 such patients who acquired their GBV-C/HGV infection by transfusion, twenty (20) patients who were followed up at 2-4 week intervals over six months maintained normal ALT activities (Wang *et al*, 1996); the other five patients showed only moderate elevations in ALT (< 124 IU/l) over the first six months, with no further elevations in the subsequent follow-up period of two years (Wang *et al*, 1996). In these five patients, there were no other clinical signs of liver disease. Jaundice was absent in the 25 patients, whereas it was present in two out of the seven patients with HCV co infection (Wang *et al*, 1996). GBV-C/HGV is capable of inducing persistent infection in about 5 -10% of infected individuals. In a retrospective study, Masuko *et al* (1996) followed eight haemodialysis patients with GBV-C/HGV infection for 7-16 years. In two patients, the virus was present at the start of haemodialysis. One had a history of transfusion, and GBV-C/HGV RNA persisted over a period of 16 years, the other cleared GBV-C/HGV RNA after 10 years. In five patients, GBV-C/HGV RNA was first detected 3-20 weeks after blood transfusion and persisted for up to 13 years. No elevations in serum ALT or signs of active liver disease were found in these patients. It would appear that in many patients infected with GBV-C/HGV, virus replication could occur without detectable damage to the liver. GBV-C/HGV transmission to chimpanzees and tamarins resulted in infection without elevation in ALT

(Bukh *et al*, 1998). On the contrary, GBV-C/HGV infection in macaques (Cheng *et al*, 2000) produced mildly elevated ALT levels with mild hepatitis and positive antigenic expression in hepatocytes, suggesting that GBV-C/HGV may be pathogenic to primates.

A strong association between GBV-C/HGV and fulminant hepatitis has been suggested (Yoshiba *et al*, 1995; Heringlake *et al*, 1996a) and may be associated with a specific strain of GBV-C/HGV (Heringlake *et al*, 1996a). However, these studies did not clearly define whether GBV-C/HGV was transmitted as a result of the transfusions they received prior to the onset of fulminant hepatitis. Additional studies by Yoshiba *et al* (1995) showed that only a few of the fulminant hepatitis patients studied had received blood transfusion prior to the onset of fulminant hepatitis. In a similar study, GBV-C/HGV RNA was detected in 3/15 (20%) patients with HBV infection and in 3/25 (12%) patients without markers of hepatitis A-E infection (Tameda *et al*, 1996). Of the six patients with GBV-C/HGV RNA, only three had a history of transfusion and all of these patients were co infected with HBV. According to Tameda *et al* (1996) these results support a role of GBV-C/HGV in inducing fulminant hepatitis either by itself or in concert with other hepatitis viruses. Using real time detection polymerase chain reaction (RTD-PCR), GBV-C/HGV RNA was measured serially in the sera of 3 Japanese patients with non A-E fulminant hepatitis, none of whom received any therapeutic transfusions before admission (Inoue *et al*, 1999). Serum ALT levels paralleled GBV-C/HGV RNA in all 3 cases and sequence analysis revealed that the same GBV-C/HGV strain infected the

patients during their entire clinical course, despite plasma exchange therapy. In one patient hepatocyte destruction continued with persistent viraemia, although ALT levels decreased. The authors (Inoue *et al*, 1999) concluded that their assumption of an association of GBV-C/HGV with fulminant hepatitis in these 3 patients was further strengthened by the disappearance or persistence of GBV-C/HGV RNA in serum which appeared to be linked to the prognosis. However, several other studies have provided no evidence of an association of GBV-C/HGV with fulminant hepatitis (Hadziyannis, 1997). The discrepancies in the association of GBV-C/HGV with fulminant hepatitis may be influenced by the sensitivity of the detection system and the differences in the GBV-C/HGV infection rates in the different populations studied. The role of GBV-C/HGV in the aetiology of fulminant hepatitis remains inconclusive.

Some investigators have reported histological features in liver biopsies of GBV-C/HGV infected individuals. Among six chronic hepatitis patients with GBV-C/HGV RNA only, the histology of the liver samples revealed chronic active hepatitis in one patient and chronic persistent hepatitis in five others (Fiordalisi *et al*, 1996). All patients with chronic hepatitis had elevated ALT levels between 89 and 478 U/L. In contrast, among the 11 acute hepatitis cases positive for GBV-C/HGV RNA, the ALT levels varied between 615 and 2477 U/L. Colombatto *et al* (1997) studied GBV-C/HGV in 67 patients with liver disease without any markers for hepatitis A-E. They reported an association between

non-specific inflammatory bile duct lesions and elevated cholestatic enzymes (gamma glutamyl trans peptidase and alkaline phosphatase) in 50% of patients. Ross *et al.* (1997) showed that GBV-C/HGV infection might affect the clinical course and outcome after orthotopic liver transplantation (OLT) by the development of severe cholestasis, which could result from bile duct damage and bile duct loss. In a preliminary study, an association between recurrent or *de novo* GBV-C/HGV infection and severe post-transplant cholestasis and ductopenia was also observed in the grafts of GBV-C/HGV-positive liver organ transplant patients (Dhillon *et al*, 1996). However, in many studies no correlation between GBV-C/HGV infection and elevation of cholestatic enzymes was noted. Further investigations are needed to substantiate these findings.

Manolakopoulos *et al* (1998) found an association between GBV-C/HGV and HCV-viremia and portal and periportal inflammation. They reported that the duration of HCV/GBV-C/HGV co-infection may be an important factor in the progression of liver disease and that inflammation with necrosis in the portal and periportal tracts was significantly higher in patients with combined viremia compared to those with HCV infection alone. The authors suggested that GBV-C/HGV in patients with HCV infection might accelerate liver injury toward more severe fibrosis in patients with dual infection. Diamantis *et al* (1997) reported that mild fibrosis correlated with GBV-C/HGV whilst Francesconi *et al* (1997) observed subtleties in histological appearance in HCV co-

infected patients. However, numerous studies have shown that in HCV co-infected individuals, GBV-C/HGV does not affect HCV replication, HCV RNA concentration or liver disease (Slimane *et al*, 2000; Enomoto *et al*, 1998; Pawlotsky *et al*, 1998; Petrik *et al*, 1998; Bralet *et al*, 1997; Tanka et *al*, 1996).

During OLT, pre-transplant GBV-C/HGV has been reported to be associated with post-transplant viraemia (Fried *et al*, 1997; Feucht *et al*, 1997b; Haagsma *et al*, 1997). In the absence of HBV or HCV in liver transplant recipients, the prevalence of GBV-C/HGV infection has no influence on the graft (Haagsma *et al*, 1997). Berg *et al* (1996b) found a significantly higher percentage of hepatocellular carcinoma in patients with pre-OLT GBV-C/HCV co infection compared with patients with HCV infection alone (5/6 vs. 16/68;$P<0.01$). Bizollon *et al* (1998) on the other hand showed that the prevalence of hepatocellular carcinoma was not different in patients with pre-transplantation GBV-C/HGV co infection or with HCV infection. In addition, GBV-C/HGV co-infection did not seem to have a significant impact on the course of HCV infection after transplantation.

1.9.2. Hepatocarcinogenicity

In a study in Japan. GBV-C/HGV RNA was detected in 11/111(10%) cases of hepatocellular carcinoma (HCC) (Kanda *et al*, 1997). The authors concluded that GBV-C/HGV was unlikely to be a major aetiological agent of non-B non-C HCC. In a large series of 503 patients with HCC in Europe, Brechot *et al* (1998) demonstrated a major impact of HBV (19% positive) and HCV (40%) but not GBV-C/HGV (7%) in HCC. In a study of 167 Black South Africans with HCC and 167 matched controls, Lightfoot *et al* (1997) showed that patients infected with GBV-C/HGV did not have an increased relative risk of developing HCC. In addition, co-infection with GBV-C/HGV did not further increase the risk of HCC in patients chronically infected with HBV and HCV. In a retrospective study of GBV-C/HGV in formalin-fixed, paraffin embedded (FFPE) tissues of HCC patients from various geographic areas (Japan, Spain, Korea, United States, Japanese Americans in Hawaii), GBV-C/HGV was neither detected nor was there any evidence of any association of GBV-C/HGV with HCC (Abe *et al*, 1998). In this study HCV genotype II/1b and HBV were significantly associated with HCC.

In a population based study of non-Asian patients with HCC and community controls in Los Angeles, California, Yuan *et al* (1999) concluded that GBV-C/HGV infection may account for approximately 8% of HCC. GBV-C/HGV RNA was detected in the sera of 12/144 (8.3%) non-Asian patients with HCC and 5/225 (2%) community controls. The

presence of GBV-C/HGV RNA was associated with a statistically significant 5.4 fold risk which was independent of the effects of HBV and HCV infections (Yuan *et al*, 1999). In a hospital based case controlled study in Brescia, Italy the relative risk factor suggested a fair association between GBV-C/HGV infection and HCC (Tagger *et al*, 1997). However, GBV-C/HGV did not seem to be a major aetiological agent of HCC because the population-attributable risk was lower (4%) than those for HBsAg (52%), HCV RNA (36%) and excessive alcohol intake (52%) (Tagger *et al*, 1977). Among subjects with GBV-C/HGV exposure (RNA and anti-E2 positive) a greater proportion of cases (40%) than controls (14%) had a transfusion history (Tagger *et al*, 1997). Hepatocarcinogenicity of GBV-C/HGV is an important question that remains to be conclusively demonstrated.

1.9.3 Extra hepatic manifestations

Hepatitis associated aplastic anaemia is a rare but well-documented phenomenon unlikely to be caused by any of the known hepatitis viruses (Brown *et al*, 1997a; Bymes *et al*, 1996). In some cases of hepatitis–associated aplastic anaemia GBV-C/HGV was the only aetiological agent detected, even if the patients had not received any transfusions before diagnosis (Zaidi *et al*, 1996; Kiem *et al*, 1997a,b; Crepo *et al*, 1999). Moriyama *et al* (1997) detected GBV-C/HGV RNA in 5/18 (27.7%) patients with aplastic anaemia who received blood transfusions before diagnosis but not in 8 patients who did not receive

transfusions. Similarly, Brown *et al* (1997a,b) detected GBV-C/HGV RNA in 26.3% and 23.1% of patients with aplastic anaemia and multitransfused control patients, respectively. Kiem *et al* (1997b) detected GBV-C/HGV RNA in 26.1% of patients with hepatitis-associated aplastic anaemia and idiopathic aplastic anemia who did not receive transfusions. The authors concluded that although transfusions are a major source of GBV-C/HGV infection, the high prevalence in those who did not receive transfusions suggests an association of GBV-C/HGV with aplastic anaemia, whether associated with hepatitis or not. Further studies in serial serum samples and meticulous evaluation of the disorders associated with the infection will be needed to prove or disprove a causal association of GBV-C/HGV and aplastic anaemia.

It is interesting to note that GBV-C/HGV replication has been consistently shown to occur in bone marrow and spleen, but not in the lymph nodes and tonsils (Laskus *et al*, 1998; Radkowski *et al*, 1999; Tucker *et al*, 2000) suggesting a haematological cell tropism. Because the genomic organisation, structural and biological characteristics of GBV-C/HGV are similar to that of HCV, GBV-C/HGV has been investigated as a possible aetiological agent in the development of haematological disorders. Ongoing GBV-C/HGV infection was detected in 29 of 60 (48%) multitransfused patients with hematological malignancies (Skidmore *et al*, 1997). GBV-C/HGV prevalence in patients with B-cell non-Hodgkin's lymphoma was significantly higher than in healthy controls (1-5%) (Ellenrieder *et al*, 1998). All patients were asymptomatic and without clinical or

sonographic signs of chronic liver disease (Ellenrieder *et al*, 1998). GBV-C/HGV prevalence in lymphoma or cryoglobulinemia patients do not support the hypothesis that this virus also may play a major role in lymphomagenesis or in the production of mixed cryoglobulinemia (Cacoub *et al*, 1997; Nakamura *et al*, 1997; Ellenrieder *et al*, 1998). Pavlova *et al* (1999) investigated two groups of patients, one with clonal stem cell disease with long latency period (myelodysplasia, myeloproliferative disease) and one with malignant haematological diseases (Hodgkin's lymphoma, non-Hodgkin's lymphoma, acute leukemia, multiple myeloma). The prevalence of GBV-C/HGV RNA in the group of oncological cases (72%) was significantly higher (P= .02) than in the patients with clonal stem cell diseases (28%). A correlation could not be confirmed between GBV-C/HGV and liver enzyme levels, blood transfusions, chemotherapy, or viral co infection (Pavlova *et al*, 1999). GBV-C/HGV infection in these patients is most likely to have originated from exposure to blood products, and to persist because of deficient immune surveillance. However, the clinical significance of these findings with respect to liver dysfunction is not yet clear. The pathogenetic consequences of GBV-C/HGV infection in lympoproliferative disorders has not been conclusively proven.

Viral infections are presumed to trigger autoimmune processes. Although Heringlake *et al* (1996b) reported a higher prevalence of GBV-C/HGV in autoimmune hepatitis (AIH) type I-III (9.8%). It was not statistically significant compared to blood donors (4.7%). In contrast, Tribl *et al* (1999) found a significantly increased prevalence of GBV-C/HGV in

patients with AIH (11%) than in healthy controls(2%). However, it remains unclear whether infection with GBV-C/HGV has an impact on the course of disease in patients with AIH. Persistent GBV-C/HGV RNA was detected in 7/36 (19.4%) thalassemic patients but was not associated with significant biochemical evidence of liver damage (Zemel *et al*, 1998). Patients with common variable immunodeficiency (CVID) are prone to unexplained chronic hepatitis whilst patients with X-linked agammaglobulinemia (XLA) who have a similar primary antibody deficiency are not prone to hepatitis (Morris *et al*, 1998). In their study of 78 CVID and 28 XLA patients, Morris *et al* (1998) concluded that the high prevalence of GBV-C/HGV viraemia is due to the long-term exposure to blood products and that GBV-C/HGV does not cause chronic hepatitis in immuno-compromised XLA patients. In addition, the authors (Morris *et al*, 1998) suggested that in the majority of CVID patients GBV-C/HGV is not the cause of chronic non-B or -C hepatitis. In Japanese leprous patients the prevalence of GBV-C/HGV was higher (5.2%) than in blood donors (1%) (Egawa *et al*, 1996). Tucker *et al* (1998) suggested an association with glomerulonephritis, hinting that virus replication may occur in the kidney.

1.10 GBV-C/HGV infection in patients infected with HIV

Based on the detection of GBV-C/HGV RNA and/or anti-E2, several studies have reported a high frequency (9-66%) of GBV-C/HGV infection in HIV-infected risk groups

(Nubling and Lower, 1996; Feucht *et al*, 1996; Jarvis *et al*, 1996; Kinoshita *et al*, 1997; Nubling *et al*, 1997; Toyoda *et al*, 1998; Woolley *et al*, 1998; Ibanez *et al*, 1998; Scallan *et al*, 1998; Nerurkar *et al*, 1998; Rey *et al*, 1999; Bourlet *et al*, 1999), but few studies have performed a detailed analysis of the clinical significance of GBV-C/HGV in HIV infected patients. In their study of high risk groups, Rey *et al* (1999) reported that the high prevalence of GBV-C/HGV was not influenced by the clinical status of the patient, but by the immune (CD4+) status. However, Rey *et al* (1999) did not compare their results to a non-HIV infected cohort. Goubau *et al* (1999) observed no significant difference in the CD4+ lymphocyte counts between RNA and anti-E2 positive patients. This finding contrasts with the study by Heringlake *et al* (1998), who found higher CD4+ lymphocyte counts in GBV-C/HGV RNA positive patients than in E2 antibody carriers. Wooley *et al* (1998) on the other hand observed no significant difference in the mean CD4+ counts between GBV-C/HGV positive and GBV-C/HGV negative patients, however, the mean CD8+ count in GBV-C/HGV negative group was significantly lower than the mean CD8+ count in the GBV-C/HGV positive group. The authors (Wooley *et al*, 1998) concluded that this may reflect an expression of CD8 lymphocytes in response to GBV-C/HGV infection, but this relationship requires further confirmation.

Some researchers have suggested that GBV-C/HGV may be a favourable prognostic factor in HIV infected patients (Toyaoda *et al*, 1998; Heringlake *et al;* 1998; Lefrere *et al*, 1999; Yeo *et al*, 2000). Yeo *et al* (2000) and Heriglake *et a* (1998) reported higher

CD4 + lymphocyte counts and better AIDS-free survival rate in HIV infected patients co-infected with GBV-C/HGV, an association that was independent of age and CCR5 genotype (Yeo *et al*, 2000). Toyoda *et al* (1998) reported lower HIV viral load and AIDS incidence with the detection of GBV-C/HGV RNA in serum. Conversely, Sabin *et al* (1998) found an increased risk for AIDS and death with detection of GBV-C/HGV RNA or anti-E2 antibodies. The difference was not statistically significant in either of these studies. Lefrere *et al* (1999) noted significantly lower HIV viral load, higher CD4+ Lymphocyte count, and better AIDS-free survival in patients with GBV-C/HGV RNA than in those without GBV-C/HGV RNA or anti-E2 antibodies.

Xiang *et al* (2000) constructed a full length cDNA from the plasma of a patient with chronic GBV-C/HGV viraemia. PBMC transfected with these transcripts resulted in viral replication, primarily in CD4+ T cells (Xiang *et al*, 2000). Since GBV-C/HGV replication has been demonstrated in CD+ T cells *in vitro* (Xiang *et al*, 2000), it is tempting to speculate that GBV-C/HGV may cause viral interference with HIV leading to delayed disease progression (Toyaoda *et al*, 1998; Heringlake *et al*, 1998; Lefrere *et al*, 1999; Yeo *et al*, 2000).

1.11 Interferon (IFN) treatment of and immunity to GBV-C/HGV infection

There are conflicting reports concerning the sensitivity of GBV-C/HGV to interferon (IFN) therapy. In some studies it seems to be similar to HCV (Tanka *et al*, 1996; Berg *et al*, 1996b; Orito *et al*, 1997 Jarvis *et al*, 1999), in other studies it appears to be independent (McHutchison *et al*, 1997; Umlauft *et al*, 1997; Nagayama *et al*, 1997), however, the response may be different (Saiz *et al*, 1997). During IFN-α therapy, serum GBV-C /HGV RNA levels decrease in most patients treated, and it may become undetectable (Nagayama *et al*, 1997; Saiz *et al*, 1997; Jarvis *et al*, 1999; Martinot *et al*, 1997). In only a small percentage of patients the response is sustained, and in most cases the GBV-C/HGV RNA concentration returned to pretreatment levels after therapy was stopped. Predictors for the efficacy of IFN therapy on HCV infection are genotype, viral load, IFN dose and the amino acid substitutions in the NS5A region, designated as the interferon sensitivity determining region (ISDR) (Shiratori *et al*, 1997). However, most researchers detect no influence of GBV-C/HGV infection in response to IFN-α in patients with chronic HCV (Tanaka *et al*, 1996; Orito *et al*, 1997; Saiz *et al*, 1997; Martinot *et al*, 1997; Kato *et al*, 1999;). No correlation between the amino acid sequence in the GBV-C/HGV NS5A region and response to IFN therapy was found, indicating that the GBV-C/HGV NS5A region does not act as the ISDR (Kato *et al*, 1999; Fujisawa *et al*, 2000). A GBV-C/HGV sustained response is predictable in patients with a low

pretreatment GBV-C/HGV viral load (Saiz *et al*, 1997; Nagayama *et al*, 1997; Orito *et al*, 1997; Enomoto *et al*, 1998; Jarvis *et al*, 1999).

Little is known about the mechanisms that enable GBV-C/HGV to establish persistent infection. Although HCV and GBV-C/HGV belong to the same Flaviviridae family, these viruses have different pathogenicity with the host. Analyses of GBV-C/HGV isolates of diverse origin have shown a different evolutionary pattern for these viruses (Erker *et al*, 1996; Muerhoff *et al*, 1996; Pickering *et al*, 1997; Wang *et al*, 1997b; Gimenez-Barcons, *et al*, 1998). Neither a viral hypervariable region, nor putative immune escape variants have been detected in chronic GBV-C/HGV infection (Erker *et al*, 1996; Muerhoff *et al*, 1996; Wang *et al*, 1997b; Bukh *et al*, 1998; Kato *et al*, 1998). Recently, Giminez-Barcons *et al* (2000) analysing the temporal nucleotide sequence changes in 5′ NCR and NS3 regions of GBV-C/HGV isolates from patients with chronic hepatitis co-infected with HCV and who received a single course of IFN therapy, showed to a limited extent that positive selection may act over the GBV-C/HGV genome during interferon therapy, and contribute to the persistence of infection with this virus.

Little is known about the intrinsic immunogenecity of the GBV-C/HGV envelope E2 protein. Humoral immune response to viral proteins is usually associated with clearance of GBV-C/HGV RNA (Feucht *et al*, 1997a; Tacke *et al*, 1997a,b; Dille *et al*, 1997). Little or no information concerning the cellular immune responses has been reported.

Sequence differences between viral strains and genotypes of many viruses can affect antibody and T cell recognition (Zhang *et al*, 1997). Immune responses to two recombinant E2 proteins, representing Genotypes 1 and 2 of GBV-C/HGV were studied in mice and in individuals with or without chronic or past infection with GBV-C/HGV (Lazdina *et al*, 2000). The authors (Lazdina *et al*, 2000) observed that immunised mice developed E2-specific antibodies, recognising linear antigenic regions and IL-2, IL-6 and γ IFN cytokine responses regardless of the viral genotype (Lazdina *et al*, 2000). In addition individuals with past GBV-C/HGV infection had E2 antibody titres that did not recognise *E.coli* derived E2 protein or linear antigenic regions. Proliferative E2-specific responses were detected in peripheral PBMC from patients with and without GBV-C/HGV markers (Lazdina *et al*, 2000). Whist the study only included 2 of the 4 (Smith *et al* 2000) current genotypes, immune responses to GBV-C/HGV E2 protein appears to cross react with different variants of genotypes of GBV-C/HGV. Proliferative responses appear to be rare and the E2 protein appears to be more immunogenic in immunised mice than in infected humans. It is generally accepted that the host immune system is a major cause for the destruction of liver cells and the development of liver disease in most infection caused by non-cytolytic hepatotropic viruses. Basic studies on the immune responses to GBV-C/HGV proteins and GBV-C/HGV infections may help to understand the natural course and pathogenesis, if any, of this infection.

Since 1992 alarm over emerging and re-emerging diseases has resulted in a number of national and international initiatives to restore and improve surveillance for infectious diseases in order to detect emerging diseases promptly and to identify new infectious diseases. At the time of gestation of this dissertation (1996-1997) nothing was known about the prevalence and pathogenic role of GBV-C/HGV in Southern Africa, especially in Kwa Zulu Natal (KZN), South Africa. In an endemic area where HBV is known to be associated with chronic liver disease, GBV-C/HGV may be an important commensal in patients with liver disease due to chronic HBV and/or HCV.

South Africa is considered to have the fastest-growing HIV positive population in the world, and Durban in KZN, is the epicentre of the HIV epidemic. The impact of co-infection with HBV or HCV on the outcome of AIDS, although still controversial, is associated with an impaired survival. Most of these patients often die from liver failure instead of AIDS. Thus one would presume that since GBV-C/HGV is considered to be a "hepatitis" virus, similar results would be expected in the interaction between GBV-C/HGV and HIV. To what extent GBV-C/HGV persistence leads to chronic liver disease is unclear nor is it known whether GBV-C/HGV persistence affects the rate of progression to AIDS. Long lasting diseases such as chronic viral hepatitis may become more relevant in these patients, especially in an environment where hepatitis is endemic.

Initially, systematic analysis of six complete GBV-C/HGV genome sequences revealed that congruent phylogenetic relationships were obtained for only a minority of fragments, and that the optimal region was all or part of the 5' NCR. At present 33 epidemiologically unrelated GBV-C/HGV complete virus genome sequences are available in the Genbank database. A re-analysis of the phylogenetic relationships of GBV-C/HGV complete genome sequences and the extent to which these can be reproduced by analysis of sub genomic regions is essential.

The routine screening of donated blood will be difficult to implement until the clinical significance and pathogenicity of GBV-C/HGV are established. Epidemiological studies based on immune and molecular assays are required to establish whether GBV-C/HGV is a true pathogen. The prevalence data, pathogenesis and possible genotypes of the virus may have important implications especially in blood donors which may influence Blood Bank policies on an already limited resource, blood and blood products.

Objectives of the study:

This dissertation will attempt to define the role of GBV-C/HGV infection in KZN, South Africa with the following aims in mind:

1. To assess and optimise methods that can be used to diagnose GBV-C/HGV infection so that the situation of GBV-C/HGV in KZN can be determined.

Nucleic acid amplification by PCR remains the method of choice. A protocol using published primer sequences will be used to detect GBV-C/HGV in sera. The in-house assay will be compared with a prototype commercial automated RT-PCR assay. Testing of GBV-C/HGV by RT-PCR is time consuming and expensive. An alternative to RT-PCR will be the detection of antibodies to GBV-C/HGV. A prototype serological assay will be used to detect GBV-C/HGV antibodies and compared to RT-PCR. The detection methods will be used to determine the prevalence and transmission of GBV-C/HGV in blood donors, patients with liver disease and high-risk groups.

2. To determine an association, if any, of GBV-C/HGV to liver disease

The study group will include blood donors (from all four racial groups); patients with chronic liver disease; patients on maintenance haemodialysis; alcoholic subjects with and

without liver disease and HIV seropositive patients. Detailed demographic, clinical, hisological and biochemical data will be obtained from all study groups where possible.

3. To establish the molecular epidemiology of GBV-C/HGV in KZN.

Virus phylogeny will be performed using sub genomic regions of local isolates of GBV-C/HGV. To determine the phylogenetic relationship of local isolates, sequence data from various regions of the GBV-C/HGV genome will be compared with sequence data available in the Genbank database from various geographic regions of the world. A re-analysis of the phylogenetic relationships of GBV-C/HGV complete genome sequences and the extent to which these can be reproduced by analysis of sub genomic regions will be undertaken.

CHAPTER 2

CHAPTER 2

DIAGNOSIS AND PREVALENCE OF GBV-C/HGV INECTION IN KWAZULU NATAL, SOUTH AFRICA

2.1 INTRODUCTION

A sensitive technique for the specific detection of GBV-C/HGV without cross reactivity to HCV is essential in order to study the epidemiology of GBV-C/HGV infection. Thus, the identification of viraemic samples has relied on the detection of viral RNA. Several nucleic acid-based assays have been developed for this purpose. Although labour-intensive, many of these assays are sensitive and specific. The second generation RT-PCR assays target highly conserved regions within the 5' NCR (Schlueter *et al*, 1996) that appears to be more sensitive and more widely used (Kao *et al*, 1997a). Kao *et al* (1997a) compared the sensitivity and specificity of PCR assays using primers from different regions of the viral genome viz. 5' NCR, E2 and NS3. The positive rates by 5' NCR, NS3 and E2 primers were 100%, 98% and 84%, respectively, and the sensitivity of the PCR assays using 5' NCR primers were 10-100 times more likely to detect GBV-C/HGV RNA than that of NS3 and E2 primers (Kao *et al*, 1997a). These results were confirmed by other investigators (Bhardwaj *et al*, 1997; Cantaloube *et al*, 1997). In this dissertation, nested primers from the 5' NCR (Jarvis *et al*, 1996) of the genome will be used to detect GBV-C/HGV RNA.

Because GBV-C/HGV RNA was detected in blood donors (Table 1.3), the question arose as to whether blood banks should include the detection of GBV-C/HGV in their routine screening algorithm. The screening of blood donations is mostly performed with serological tests. In contrast to what is found for HCV, the identification of serological markers indicative of ongoing GBV-C/HGV infection needs to be established. Anti-E2 antibody detection is not associated with active replication of the virus, and these antibodies are detectable after RNA clearance. It therefore seems logical to use anti-E2 to study the epidemiology and effectiveness of treatment (should the virus be found to be associated with clinical disease) of GBV-C/HGV infection. Third generation RT-PCR assays have been developed which permit a higher level of through-put in conjunction with ease of use. Very little is known about the epidemiology of GBV-\C/HGV in African countries and much less information is available on the prevalence and preferred transmission routes of GBV-C/HGV.

2.2 METHODS

2.2.1 Source of Samples

2.2.1.1 Collection of Sera

Venous blood was collected in a 6ml sterile Vacutainer® SST®Gel and Clot Activator tubes (Becton Dickenson, UK). Blood samples were transported in crushed ice to the laboratory. All blood samples were processed within 2 hours after bleeding. Vacutainer® Tubes were spun for 5 minutes (min) at 2500 rpm

(revolutions per minute) at 4°C in a Beckman Model J6B - Centrifuge. Sera were serially aliquoted and stored at - 85°C. Sera were thawed once only for any given assay.

2.2.1.2 Patients

Sera were collected from a high-risk group of 70 patients with chronic renal failure who were undergoing maintenance haemodialysis, 98 consecutive patients with chronic liver disease and 50 patients with biopsy proven alcoholic liver disease (Table 2.1). Diagnosis of liver disease was based on clinical findings and laboratory data and confirmed on liver biopsy. Histological examinations were done by the same pathologist who was not aware of the clinical and laboratory findings.

GBV-C/HGV infection was also studied in a "convenience" sample of 75 HIV positive mothers and their infants who participated in an HIV prospective clinical investigation on the perinatal transmission of HIV in KZN (Coutsoudis *et al*, 1999) (Table 2.1). Sera were collected from the mothers at 33-38 weeks of gestation. The sera were stored under coded identifier numbers in aliquots at – 85°C. To investigate mother-to-infant transmission of HIV, babies were followed up for 18 months (Coutsoudis *et al*, 1999). To identify mother-to-infant transmission of GBV-C/HGV, serum samples were only available for 20 mothers and their babies at 1 week and at 3-monthly intervals for 9 months. Serum samples were not available for all 20 babies.

2.2.1.3 Control groups

The control groups included sera obtained from 232 adult volunteer blood donors from the four racial groups (Africans [n=76], Whites [n=49], "Coloureds" {mixed origin} [n=54], and Indians [n=52] (Table 2.1). Thirty-five (35) individuals who did not present to hospitals for signs or symptoms of liver disease were referred to the local center for alcohol detoxification (Alcoholics Anonymous) were recruited (Table 2.1). The latter group was studied after at least 2 weeks of alcohol withdrawal (healthy alcoholic subjects without liver disease).

2.2.2. Extraction of viral RNA and DNA from sera

2.2.2.1 GBV-C/HGV and HCV RNA

GBV-C/HGV and HCV viral RNA were extracted from serum using the QIAamp® Viral RNA Kit (Qiagen, Gmbh, Germany). The manufacture's recommended procedure was modified (Marshall *et al*, 1998). Carrier RNA (poly A) (6.7 ug/ml) was used at a reduced concentration in AVL Buffer. For the 50 prepration QIAamp Kit® 1.5ml of buffer AVL was added to the lyophilised carrier RNA tube (825ug) provided. The RNA was dissolved thoroughly. Three hundred and seventy-five microlitres (375ul) of the solution was transferred to the Buffer AVL and thoroughly mixed. For the 250 preparation QIAamp Kit® 3.0ml of buffer AVL was added to the lyophilised carrier RNA tube (4200ug) provided. Seven hundred and

fifty microliters (750ul) of the solution was transferred to the Buffer AVL and thoroughly mixed. Buffer AVL/carrier RNA was stored at 4°C in 7.0ml aliquots (each 7.0ml aliquot was enough for 24 specimens). Ethanol (96-100%) as recommended by the manufacture was added to the Buffer AW before use. Aliquots of 1ml of 0.1% (v/v) diethylpyrocarbonate (DEPC) treated water was dispensed into 1.5 ml sterile RNAase free microfuge tubes. Each 1ml aliquot contained enough water to elute approximately 9-0 specimens. The aliquots of water were equilibrated at 80°C in a water bath. For each serum specimen 280ul of Buffer AVL/carrier RNA was dispensed into a sterile RNAase-free 1.5ml microfuge tube. The microfuge tubes were transferred to a biosafety hood approved for working with biohazardous material. Serum specimens were thawed at room temperature (15-30°C) and stored on wet ice immediately upon thaw. Each specimen was vortexed for 2-5 seconds (sec) and then centrifuged (Eppendorf Microcentrifuge Model 5415C) at 14 000 rpm for 5 min at room temperature (15-30°C) to clear any precipitate which may be present. Twenty-five microlitres (25ul) of serum was added to 280ul of AVL buffer/carrier RNA. Tube was vortexed for 2-5 sec and allowed to incubate at room temperature for 10 min to complete the lysis process. The tubes were pulse centrifuged at 14 000 rpm for 2-5 sec to remove any buffer from the cap of the microfuge tubes. Ethanol (280 ul) was then added to the mixture which was vortexed for 2-5 sec. The tubes were pulse centrifuged at 14 000 rpm for 2-5 sec to remove any buffer from the cap of the microfuge tubes. The entire volume (585 ul) was applied to the QIAamp spin column without moistening the rim. The cap of the microfuge tube was closed and

the tubes centrifuged at 8 000 rpm for 1 min. The QIAamp spin column was transferred to a clean 2ml collection tube and the tube containing the filtrate was discarded. The QIAamp spin column was carefully opened to prevent aerosol formation and 500ul of Buffer AW was added without moistening the rim, the cap was closed and the tube centrifuged at 8000 rpm for 1 min. The QIAamp spin column was transferred to a clean 2ml collection tube and the filtrate discarded. Five hundred microliters (500ul) of Buffer AW was added without moistening the rim, the cap closed and the tube centrifuged at 8000rpm for 1 min and at 14 000 rpm for an additional 2 min. The QIAamp spin column was transferred to a sterile RNase free 1.5 ml microfuge tube and the filtrate discarded. The QIAamp spin column was carefully opened and 100ul of DEPC-treated water preheated to 80°C in a water bath was added directly onto the filter matrix, being careful not to run the water down the side of the column. The tube was centrifuged for 1 min at 8 000 rpm. The column was discarded and the microfuge tube with the purified RNA stored on ice. Purified RNA was either used immediately within 4 hr or stored at -70°C. Upon thaw the purified RNA was vortexed for 2-5 sec prior to use and pulsed centrifuged to remove any residual solution from the cap of the microfuge tubes. The extracted RNA was used to detect GBV-C/HGV by RT-PCR and by LCx® system and HCV.

2.2.2.2 HBV DNA

HBV viral DNA was extracted from sera using the InstaGene™ Matrix (Bio-Rad Laboratories, CA, USA) according to the manufacturer's instructions.

2.2.3 Diagnostic Assays

The serological and molecular assays performed on the sera are illustrated in (Table 2.1).

Table 2.1 A table summarising the tests that were performed on the samples. Serological (GBV-C/HGV Anti-E2 and HBeAg, HBsAg, anti-HBs, anti-HBc) and or molecular (PCR, LCx) assays used to diagnose HBV, HCV and GBV-C/HGV infections in blood donors, patients with liver disease (ALD and CLD), alcoholics without liver disease and high risk groups (HAEM, HIV positive)

		TEST PERFORMED								
Study group	**No.**	**HBV**					**HCV**	**GBV-C/HGV**		
		eAg	**sAg**	**sAb**	**cAb**	**PCR**	**PCR**	**E2**	**PCR**	**LCx**
HAEM	70						✓	✓	✓	
CLD	98	✓	✓				✓	✓	✓	✓
ALD	50	✓	✓	✓	✓	✓	✓	✓	✓	
HIV+ mothers	75							✓	✓	
Blood donors										
Africans	76							✓	✓	
Whites	49							✓	✓	
Indians	52							✓	✓	
"Coloureds"	54							✓	✓	
Alcoholic controls	35	✓	✓	✓	✓	✓	✓	✓	✓	
Total	504									

ALD-alcoholic liver disease; CLD-chronic liver disease; HAEM-haemodialysis; GBV-C/HGV-GB Virus C/Hepatitis G Virus; HCV-hepatitis C virus; HB/HBV-hepatitis B virus; HIV-human immunodeficiency virus; Ag-antigen; Ab/anti- antibody; c-core; s-surface; LCx-ligase chain reaction; + positive; "Coloureds"- mixed origin; ✓ - test performed

2.2.3.1 Molecular based assays

2.2.3.1.1 Reverse Transcription

Using a modified method of Brown *et al* (1992) complementary DNA (cDNA) was synthesized from 10ul of extracted RNA (GBV-C/HGV and HCV) melted at 70°C for 10 min and added to 15ul of a mix containing 1.5uM pd$(N)_6$ random hexamers (Pharmacia Biotech, Uppsala, Sweden), 5 x First Strand Buffer [50mM Tris-HCl [pH 8.3]; 40mM KCL; 6mM $MgCl_2$) [Gibco-BRL, Life Technologies, Paisley, UK); 250U Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Gibco-RL, Life Technologies, Paisley, UK), 0.5mM (dNTPs) (Promega Corporation, Madison, USA) and 0.1% (v/v) DEPC treated-H_2O and incubated at 37°C for 1 hr. The reaction was terminated by incubation at 95°C for 10 min.

2.2.3.1.2 PCR assay

Highly conserved nested primers (Oswald DNA Services, Southampton, UK) designed from published sequences from the 5′ NCR of the GBV-C/HGV (Table 2.2) of isolates PNF2161 (U44402), R10291 (U45966) and GBV-C (U36380) [Appendix A] and HCV (Table 2.3) genomes were used to perform the nested PCR.

Table 2.2 Sequence and location of primers used for amplification of the 5′ NCR of the GBV-C/HGV genome (Jarvis *et al*, 1996)

Location	Region	Polarity*	Sequence 5′ to 3′
108	5′ NCR	+	AGGTGGTGGATGGGTGAT
531	5′ NCR	-	TGCCACCCGCCCTCACCCGAA
134	5′ NCR	+	TGGTAGGTCGTAAATCCCGGT
476	5′ NCR	-	GGRGCTGGGTGGCCYCATGCW

Orientation of primer sequence (+ sense, - anti-sense)

Table 2.3 Sequence and location of primers used for amplification of 5′NCR of the HCV genome

Name	Region	Polarity*	Sequence 5′ to 3′
209	5′ NCR	+	ATACTCGAGGTGCACGGTCTACGAGACCT
939	5′ NCR	-	CTGTGAGGAACTACTGTCTT
211	5′ NCR	+	CACTCTCGAGCACCCTATCAGGCAGT
940	5′ NCR	-	TTCACGCAGAAAGCGTCTAG

* Orientation of primer sequence (+ sense, - anti-sense)

In the first round of the PCR reaction, 5ul of the cDNA was added to 95ul of a master mix containing a final concentration of 0.2mM dNTPs (Promega Corporation, Madison, USA); l0xPCR Buffer (1.5mM $MgCl_2$, 20mM $[NH_4]_2SO_4$; 75mM Tris-HCl [pH 9.0]; 0.01%(w/v) Tween 20) (Advanced Biotechnologies,Surrey, UK); 0.1% (v/v) DEPC treated - H_2O, 2.5U Thermoprine ^{plus} DNA Polymerase (Advanced Biotechnologies, Surrey, UK) and 50pmol of each primer located at positions 108 and 531 (Table 2.1). Two microliters (2ul) of the

first round PCR product was added to 48 ul of a PCR master mix containing 0.2mM dNTPs; 10 x PCR buffer [1.5mM Mg_2Cl, 20mM $[NH_4]_2SO_4$; 75mM Tris-HCl (pH 9.0), 0.01% Tween 20] and 50 pmol of each primer located at positions 134 and 476 (Table 2.2). Amplification was over 25 cycles in a Progene thermocycler (Progene, Techne, Cambridge, UK) for both first and second rounds of PCR, using the following temperatures, 96°C for 1 min, 50°C for 1 min, 72°C for 2.5 min with a final extension at 72°C for 10 min followed by a 4°C soak. Standard precautions for avoiding contamination for PCR were observed. Known positive and negative GBV-C/HGV sera together with DEPC-H_2O were included in each run to ensure specificity.

The amplification of HBV DNA was performed according to the method described by Wright *et al* (1992) using the primers targeting the surface antigen (Table 2.4).

Table 2.4 Sequence and location of primers used for the detection of HBV DNA (Wright *et al*, 1992)

HBV	Polarity*	Sequence
Outer	+	5′-GTTAGGGTTTAAATGTATACCC-3′
primers	-	5′-CATCTTCTTATTGGTTCTTCTGG-3′
Inner	+	5′-CATCTTCTTATTGGTTCTTCTGG-3′
primers	-	5′-AATGGCACTAGTAAACTGAGCC-3′

* Orientation of primer sequence (+ sense, - anti-sense)

2.2.3.1.3 Agarose Gel Electrophoresis

A 2% agarose gel was poured by melting 2.0g of agarose in 100mls of 1x TBE buffer. Five microliters (5ul) of Ethidium bromide (50ug/ml) was added to the gel before it was poured. Five microlitres (5ul) of each PCR product was added to 5ul of Bromophenol blue gel loading buffer (50% glycerol; 1x TAE buffer; 1% bromophenol blue : 1% Xylene cyanole ff). Two microliters (2ul) of molecular weight marker (1Kb DNA Ladder, (Gibco-RL, Life Technologies, Paisley, UK), was diluted in 3 ul of sterile DEPC-H_2O and added to 5ul of loading buffer. Ten microliters (10ul) of each reaction along with the molecular weight marker was loaded into the appropriate wells in the gel. Electrophoresis was performed at a constant 100V for approximately 1 hr. The gel was visualised on a UV transilluminator and photographed.

2.2.3.1.4 Detection of GBV-C/HGV by the Abbott LCx® system

The Abbott LCx® GBV-C Assay uses the nucleic acid amplification method of RT-PCR to generate amplified product material from GBV-C/HGV RNA in clinical specimens and uses the LCx® Probe System for the qualitative detection of RNA from the GBV-C/HGV in human serum or plasma in a single tube format. This material is hybridized to a GBV-C/HGV specific oligonucleotide probe and hybrids are detected in an LCx® Analyzer. The LCx® GBV-C Assay includes all of the necessary components to generate a detectable GBV-C/HGV reaction product in

the presence of the GBV-C/HGV target. The assay utilizes a primer/probe set that is specific for the 5' NCR region of the GBV-C/HGV genome (Table 2. 5) The first primer (cDNA/PCR primer) is complementary to the RNA genome and serves as both the cDNA primer and one of the PCR primers (S1). The second primer (AS2) is the same sense as the genome and acts as the second PCR primer. A third oligonucleotide, the probe, is present in the reaction mix and is complementary to one of the strands of the PCR product (3FS3). In the presence of GBV-C/HGV amplified product the probe/PCR strand complex serves as the detection product in the LCx® Analyzer.

Table 2.5 Primer and Probe sequences used in the LCx® GBV-C Assay (Marshall *et al*, 1998)

Assay	Oligo name	Sequence
LCx®	S1	5′-CACTGGGTGCAAGCCCCAGAA-3′
LCx®	AS2	5′-ATTTACGACCTACCAACCCTG-3′
LCx®	3FS3	5′-AAAGGTGGTGGATGG-3′

The assay was carried out according to the manufacturer's instructions. Briefly, 20ul of activation solution (32.5 mM manganese) were added to amplification vials containing ready to use master mix, and 20ul of extracted RNA were then added to a "unit dose" tube containing the rest of the reagents (180ul) for RT-PCR, which included a thermostable combined reverse transcriptase/DNA polymerase. After vortexing, tubes were transferred to the amplification area and were placed in LCx/Perkin Elmer Model 480 thermal cycler, where the following cycles were

performed: a reverse transcription step of 30 min at 60°C, followed by 35 PCR cycles of 94°C for 40 sec and 63°C for 1 minute. Subsequently the PCR products were denatured (97°C for 5 min) and rapidly cooled to 15°C for 5 min. After amplification the vials were allowed to remain at 15°C in the Thermal Cycler for up to 24 hr prior to detection.

Within four hours following amplification, tubes were centrifuged and removed to the detection system (LCx®), which is based on the use of microparticle enzyme immunoassay (MEIA) technology. A total of 24 samples may be analysed in one hr, with the presence of GBV-C/HGV indicated by a positive rate of production for the fluorescent product, 4-Methylumbelliferone. The rate of product generation was automatically monitored and reported as counts per second per second (c/s/s). The cut-off rate for positivity was predetermined by Abbott Laboratories. Any sample falling within an intermediate zone was re-tested in duplicate. The LCx® GBV-C Assay reduces the potential of PCR product contamination in a number of ways. Two high positive, two low positive and two negative controls were included in each PCR and detection assay. To reduce the risk of amplification product contamination, at the end of the LCx® GBV-C Assay, amplification product is automatically inactivated using a two-reagent chemical inactivation system. Both reagents are delivered into the LCx® Reaction Cells by the LCx® Analyzer after the amplification product has been detected. The ensuing reaction results in the nearly complete destruction of any nucleic acid present. This effectively reduces the risk of contamination of the laboratory by amplification product.

2.2.3.1.5 Purification of the GBV-C/HGV PCR product

The 344 bp PCR product generated by RT-PCR was purified using the QIA quick PCR purification kit (Qiagen, GmbH, Germany) according to the manufacturer's instruction.

2.2.3.1.6 Sequencing reaction

Both strands of the 344 bp PCR product were sequenced by Direct cycle sequencing using the Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Biosystems, CA, USA). Briefly, for each reaction 4.0ul of terminator ready reaction mix, 90ng of the PCR product (DNA), 1.5ul (5pmol) of either reverse or forward primer in a total volume of 20 ul of sterile deinosied water was added to a 0.2 ml PCR tube. The reagents were mixed, briefly spun and cycle sequenced in the Perkin Elmer GeneAmp `2400 thermal cycler (Perkin Elmer, Biosystems, CA, USA) initially at 96°C for 30 sec, followed by 96°C for 10 sec, 50°C for 10 sec, 60°C for 4 min for 25 cycles followed by a 4°C soak. The tubes were spun briefly. The extension product was purified in microfuge tubes .The entire contents of each extension reaction was transferred to a sterile 1.5ml microfuge tube, to which was added 16ul of sterile deionised water and 64ul of 95% ethanol. The contents of the tube were vortexed, allowed to stand for 10-15 minutes in the dark to precipitate the extension products, and the tubes were centrifuged at 14 000 rpm 15 min. The supernatants were carefully aspirated

without disturbing the "pellet". One hundred microliters (100ul) of 70% ethanol was added to the microfuge tube and briefly vortexed to mix. The tubes were spun for 10 minutes at 14 000rpm. The supernatant was carefully aspirated without disturbing the "pellet". The samples were dried on a heating block for 2 min at 90°C. Twenty microliters (20ul) of template suppression reagent was added to the tube and mixed well. The tubes were heated for 2 min at 100°C and thereafter held on ice for 1 min. The product was transferred to a sequencing tube and kept in the dark at 4°C.

2.2.3.1.7 Sequence analysis

The sequences were determined using an Applied Biosynthesis Prism 310 Automated Genetic Analyser (Perkin Elmer, Biosystems, CA, USA). The sequences were assemble and analysed using the Staden Package (http://www.mrc-lmb.cam.ac.uk/pubseq/). Alignment of sequences was done using Clustal W with minor manual adjustments.

2.2.3.1.8 Phylogenetic analysis

Phylogenetic analysis of GBV-C/HGV sequences was carried out on a 310 base pair fragment in the 5' NCR region (positions 160-470) in HGV type2a clone, PNF2161 (Muerhoff *et al*, 1996). Sequences were compared using a distance-based method (p-distance at all sites followed by neighbor joining) as implemented in

Phylip package (Joseph Felsenstein 1980, http:// evolution. genetics Washington. edu/phylip.html) Robustness of grouping was assessed by bootstrap re-sampling; numbers on branches indicate the percentage of 500 bootstrap replicates that supported the observed phylogeny.

Sequences compared include those of designated genotype for which complete genomic sequences are available. These include sequences from Group 1-4 described by Smith *et al* (2000).

2.2.3.2 Serology based assays

2.2.3.2.1 Detection of GBV-C/HGV Anti-E2 antibodies

Anti-E2 of GBV-C/HGV were determined by ELISA "μ Plate Anti-HG_{env}" (Boehringher Mannheim, Germany) according to the manufacturer's instructions. Briefly, viral E2 protein used in this assay was expressed within Chinese ovary hamster cells and was bound to a streptavidin-coated microtitre plate via a monoclonal mouse GBV-C/HGV E2 antibody (Tacke *et al*, 1997a). Calculation of the cut-off value was performed according to manufacturer's recommendations ($A_{cut\text{-}off} = 0.2 \times A_{positive\ control} + A_{negative\ control}$; A:absorbance). Samples with a borderline absorbance of the cut-off value were subjected to a second run, where the E2 antigen was omitted to exclude unspecific binding. A specimen was

considered negative for Anti-E2 whenever $A_{\text{sample with E2 antigen}}/A_{\text{sample without E2 antigen}}$ was < 1.5.

2.2.3.2.2 Detection of HBV and HIV

Sera were tested for serological markers for HBV and HIV by commercial microparticle enzyme immunoassays (MEIA) (IMx AUSAB assay, Abbott Laboratories, North Chicago, IL) and (AxSYM® HIV 1/2gO; Abbott Laboratories, Chicago, IL, USA), respectively. The assays were performed by routine multianalysis systems in the central laboratory at King Edward VIII Hospital, Durban.

2.2.3.2.3 Carbohydrate Deficient Transferrin (CDTect®)

Regular high consumption of alcohol results in the appearance of serum transferrin deficient in carbohydrate (CDT) (Allen *et al*, 1994). Analysis of the CDT value, provides a valuable tool to diagnose and manage alcohol-related diseases (Allen *et al*, 1994). CDT was measured in sera of alcoholics with and without liver disease by a commercially available CDTect® competitive enzyme immunoassay (EIA) kit (CDTect®, Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden). The assay was performed according to the manufacturer's instructions.

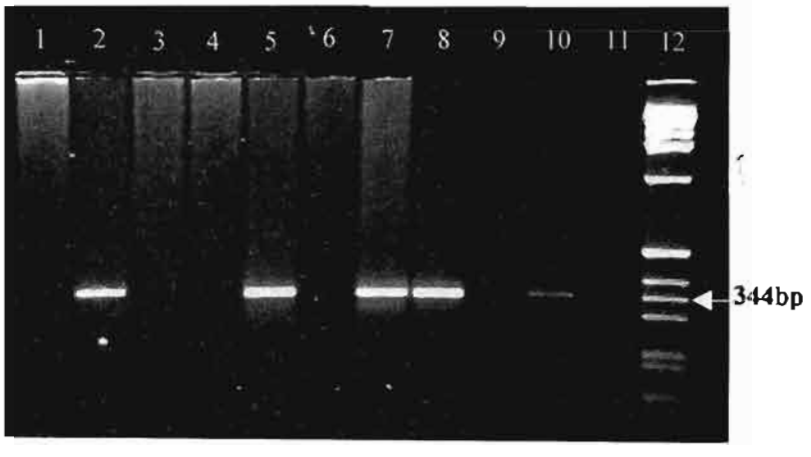
2.3 STATISTICAL ANALYSIS

The data of blood donors, haemodialysis patients (HAEM), patients with chronic liver disease (CLD) and patients with alcoholic liver disease (ALD) were analysed separately. Chi-squared test was used to compare groups (GBV-C/HGV positive versus GBV-C/HGV negative) with respect to categorical data, while Student's t-test was applied to continuous data. Analysis of covariance was used to compare GBV-C/HGV positive and GBV-C/HGV negative patients with respect to liver function test, adjusting for age, sex and disease group. Multiple logistic regression was used to determine any confounding influence of age, sex and disease group on GBV-C/HGV. Blood donors were compared to CLD and HAEM and ALD patients with regard to demographic parameters using Student's t-test or Chi-squared test.

2.4 RESULTS

2.4.1 Comparison of the GBV-C LCx, the "in-house" RT-PCR and Anti-E2

GBV-C/HGV RNA was extracted from a known GBV-C/HGV positive sample and diluted serially four-fold. A commercial (GBV-C LCx, Abbott Laboratories) and an "in-house" RT-PCR assays were used for the detection of GBV-C/HGV RNA in 98 patients with chronic liver disease. The RT-PCR assays were compared to serology (anti-E2). Fig. 2.1 shows a typical agarose gel stained with ethidium bromide and photographed whilst Fig.2.2 shows the results of the GBV-C/HGV dilutions.



. **Fig. 2.1** Agarose gel demonstrating the results of RT-PCR for GBV-C/HGV. Lanes 1 to 8 are extracted sera. Lane 9 is the DEPC H_2O blank. Lane 10 is a known GBV-C/HGV positive control serum. Lane 11 is a known GBV-C/HGV negative control serum. Lane 12 shows the size distribution of the 1 kilobase molecular weight marker. The approximate size of the PCR product (344 bp) is indicated by the arrow

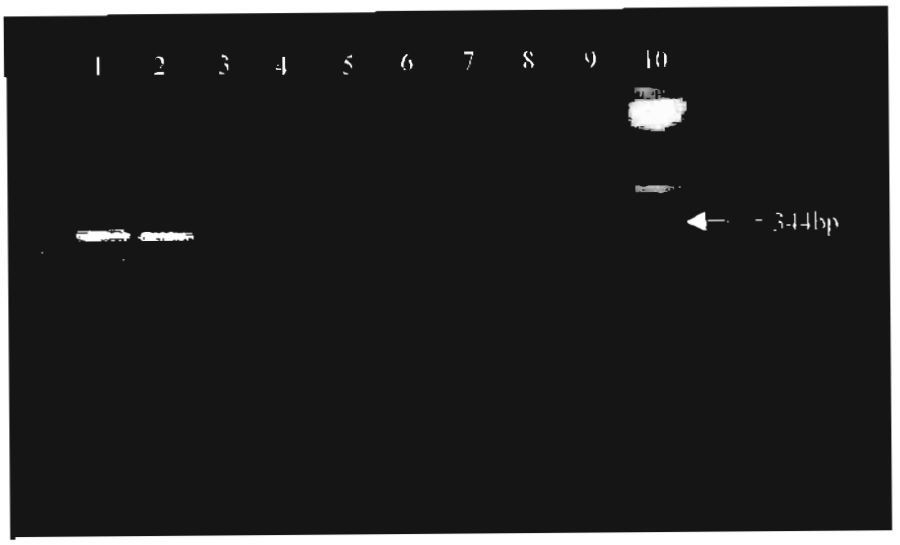


Fig. 2.2. Agarose gel demostrating the results of GBV-C/HGV RNA dilutions. Lanes 1-6 have the PCR products in serial four fold dilutions from undiluted to 1:1024. Lane 7 is a negative control serum. Lanes 8-9 are DEPC-H_2O blanks. Lane 10 shows the molecular size distribution of the 1 kilobase molecular weight marker. The approximate size of the PCR product (344bp) is indicated by the arrow.

A comparison of the test results for the detection of GBV-C/HGV using each of the three assays is shown in Table 2.6.

Table 2.6. Comparative analysis of the detection of GBV-C/HGV in 98 patients with chronic liver disease by the "in-house" RT-PCR, the Abbott LCx® GBV-C and the anti-E2 assays

RT-PCR + (%)	LCx + (%)	Anti-E2 + (%)	RNA & anti-E2 + (%)
12/98(12.2%)	27/98(27.5%)	32/98(32.7%)	2/27(7.4%)

%-per cent; +-positive; - negative; Anti- antibody

GBV-C/HGV was detected in 27/98(27.5%) serum samples by at least one of the two RT-PCR assays. All twelve (12) of 98 (12.2%) patients detected by the "in-house" RT-PCR were also detected by the LCx assay. Fifteen (15) additional samples were positive by the LCx assay. These 15 discordant samples were retested by the "in-house" RT-PCR with identical results. The samples were not retested by the LCx assay. There was concordance between the "in –house" RT-PCR and LCx in 83/98 (84.7%) samples. Two of 27 (7%) RNA positive (LCx and/"in-house" RT-PCR) samples were also positive for anti-E2 GBV-C/HGV antibodies. Anti E2 was mutually exclusive in 93%(25/27) of RNA positive samples. A summary of the precision of the LCx assay is presented in Table 2.7. System variability was determined by percent coefficient of variation (%CV=S.D./Mean) × 100).

Table 2.7 Variability of the LCx rates (counts/sec/sec) of control specimens supplied in lots of the GBV-C LCx kits (Abbott)

Lot No.	Neg. Control	Pos. Control	Calibrator	No. of tests
0019302F9	9.6	402.4	354.0	17
	9.1	419.4	76.2	
0019302F9	9.9	247.8	35.3	11
	10.2	532.3	380.7	
0019302F9	16.5	440.3	177.3	18
	9.8	372.1	293.3	
0019302F9	9.7	458.0	428.1	18
	9.7	392.0	411.2	
0019302F9	9.4	25.7	59.5	18
	9.0	9.8	9.1	
0019302F9	10.0	10.7	197.4	16
	9.5	350.9	210.3	
Mean	**10.2**	**305.12**	**219.37**	
SD	**2.0**	**187.12**	**152.69**	
%CV	**19.6**	**61.32**	**69.60**	

Neg,negative; Pos,positive; No, number, SD,standard deviation; %, per cent; CV, coefficient of variation

2.4.2 Prevalence of GBV-C/HGV RNA in blood donors

Of the 232 unpaid volunteer blood donors 76 (32.8%) were African, 49 (21.1%) Whites, 52 (22.4%) Indians and 55 (23.7%) "Coloureds."(mixed race group) All the blood donors were negative for HCV, HBV and HIV. None of the blood donors had a history or clinical evidence of liver disease. Overall, GBV-C/HGV RNA was detected in 44 of 232 (18.9%) blood donors [29/76 (38.2%) African, 2/52 (3.8%) Indians, 2/55 (3.6%) "Coloured" and 11/49 (22.4%) White] (Table 2.8). There was

no significant difference in the prevalence of GBV-C/HGV RNA between Indians and "Coloured" blood donors ($p = 0.81$) and between African and White blood donors ($p = 0.07$). However, there was a significant difference in the prevalence of GBV-C/HGV RNA between the other racial groups (Indians vs Whites [$p < 0.005$]; Asians vs Africans [$p < 0,00001$]; "Coloureds" vs Whites [$p < 0.005$] and "Coloureds" vs Africans [$p < 0.00001$]).

2.4.3 Prevalence of GBV-C/HGV RNA in high risk groups and patients with liver disease

2.4.3.1 Haemodialysis group

Among the 70 haemodialysis patients there were 30 (42.9%) Africans, 29 (42.4%) Indians, 3 (4.3%) "Coloureds" and 8 (11.4%) Whites. Seventeen of 70 (24.3%) haemodialysis patients (5/30) [16.7%] Africans; 8/29 [27.6%] Indians; 1/3 [33%] "Coloureds" and 3/8 [11.43%] Whites) were infected with GBV-C/HGV [95% CI (5.7%; 18.7%)] (Table 2.8). GBV-C/HGV positive patients tended to have a longer duration of dialysis (not significant) and had more transfusions ($p = 0.03$) than non-infected patients. Four of 70 patients (5.7%) were infected with HCV but only 2/17 (11.76%) GBV-C/HGV positive patients were co-infected with HCV.

2.4.3.2 Chronic Liver Disease group

The majority of patients with chronic liver disease were Black [88 Africans (90%), 7 Indians (7%), 2 "Coloureds" [2%] and 1 White (1%)]. The diagnosis of chronic liver disease was confirmed by histology in 81 patients and by peritoneoscopy alone in 17 patients. The etiology of chronic liver disease in the majority of patients was alcohol abuse, viral or a combination of the two (Appendix B). In five patients, chronic liver disease was due to autoimmune liver disease and in seven it was cryptogenic (Appendix B). None of these patients gave a history of blood transfusions or intra-venous drug abuse. The prevalence of GBV-C/HGV RNA in

patients with chronic liver disease was 12.2% (12/98) [95% CI (5.7%; 18.7%)] (Table 2.8). Only 2 of 10 patients with HBV were co-infected with GBV-C/HGV. GBV-C/HGV RNA was detected more frequently in patients with an alcoholic etiology alone (6/56 [11%]) or in combination with alcohol and HBV (1/10 [10%]) or alcohol and HCV (1/4 [25%]). Two of seven (29%) patients with cryptogenic liver disease were GBV-C/HGV RNA positive.

There was no significant difference in the prevalence of GBV-C/HGV RNA between blood donors and patients. There was a significant difference in the prevalence of GBV-C/HGV RNA between patients with chronic liver disease (12/98; 12.2%) and haemodialysis patients (17/70; 24.2%) [p = 0.04].

2.4.3.3 Alcoholic Liver Disease group

All the patients with ALD were Black Africans (31 males and 19 females). The diagnosis of ALD was assessed by history and laboratory markers. This included a questionnaire on alcohol consumption, lab-markers (Appendix C) and histological findings. The prevalence of GBV-C/HGV RNA in alcoholics without liver disease was 14% (5/35) [Table 2.8]. Two of the 5 (40%) GBV-C/HGV RNA positive subjects had a past exposure to HBV (HBc). HCV infection was detected in 1 (2.9%) alcoholic without liver disease. This patient was positive for Anti-HBc and anti-E2 to HBV and GBV-C/HGV infections, respectively. The prevalence of GBV-C/HGV RNA in ALD patients was 28% (14/50) [Table 2.8]. Eleven of 33 (33%)

ALD patients with HBV infection were co-infected with GBV-C/HGV RNA. Of the two patients (2/50; 4%) with HCV infection, one had past exposure to HBV (Anti-HBc) and GBV-C/HGV (anti-E2). Although the prevalence of GBV-C/HGV RNA in ALD patients was twice (28%; 14/50) that of alcoholics without liver disease (14%; 5/35), this difference was not significant ($p = 0.153$). There was no significant difference in the prevalence of GBV-C/HGV RNA between African blood donors and ALD patients. There was a significant difference in GBV-C/HGV RNA prevalence between African blood donors and alcoholics without liver disease ($p = 011$).

2.4.4 Prevalence of Anti-E2

There was a higher prevalence of Anti-E2 in African blood donors (21/76; 27.6%) than White (14/49; 8.2%), "Coloured" (7/55; 12.7%) or Indian (3/52; 5.7%) blood donors [Table 2.9] and this difference was significant ($p < 0.05$). There was a significant difference ($p < 0.05$) in the prevalence of Anti-E2 in chronic liver disease [32/98 (32.7%)], and haemodialysis patients [18/70 (25.7%)] compared to blood donors [35/232 (15.1%)] (Table 2.8). There was no significant difference in Anti-E2 seroprevalence between chronic liver disease and haemodialysis patients ($p = 0.33$). There was a higher prevalence of Anti-E2 in alcoholics without liver disease (54.3%; 19/35) than ALD patients (42%; 21/50), this difference was not significant (Table 2.8). There was no significant difference in Anti-E2 seroprevalence between African blood donors and ALD patients (Table 2.8).

African blood donors (45/76; 59.2%) had a greater exposure to GBV-C/HGV infection (RNA and/or Anti-E2 positive) compared to White (15/49; 30.6%) [p = 0.002], "Coloured" (9/55; 16.4%) [p < 0.00001] and Indian (5/52; 9.6%) [p < 0.00001] blood donors. There was a significant difference (P < 0.05) in the overall exposure to GBV-C/HGV (RNA and/or Anti-E2 positive) between blood donors [31.9% (74/232)] and patients [43.9%(43/98) vs 47.1% (33/70), respectively [Table 2.8]. Alcoholics with or without liver disease had greater exposure (60%) [Table 2.8] to GBV-C/HGV (RNA and/or Anti-E2) infection. The prevalence was similar to that in African blood donors (59.2%) [Table 2.8]. GBV-C/HGV RNA and Anti-E2 were mutually exclusive in almost all (91-100%) patients and blood donors. GBV-C/HGV RNA and Anti-E2 was simultaneously positive in only 1/98 (1.1%) patients with chronic liver disease, 6/50 (12%) ALD patients, 2/70 (2.9%) haemodialysis patients, 5/232 (2.2%) blood donors and 3/35 (8.6%) alcoholics without liver disease (Table 2.8).

Table 2.8 GBV-C/HGV RNA and Anti-E2 prevalence among Blood donors, patients on maintenance haemodialysis, chronic liver disease and alcoholics with and without liver disease

Study population	n	GBV-C/HGV RNA + (%)	GBV-C/HGV Anti-E2 + (%)	GBV-C/HGV RNA+ & Anti-E2 + (%)	GBV-C/HGV Exposure RNA+ &/or Anti-E2 + (%)
Blood Donors					
African	76	29 (38.2)	21 (27.6)	5 (6.5)	45 (59.2)
White	49	11 (22.4)	4 (8.2)	0 (0)	15 (30.6)
Indians	52	2 (3.8)	3 (5.7)	0 (0)	5 (9.6)
"Coloured"	55	2 (3.6)	7 (12.7)	0 (0)	9 (16.4)
Total	**232**	**44 (18.9)**	**35 (15.1)**	**5 (2.1)**	**74 (31.9)**
Chronic liver disease	98	12 (12.2)	32 (32.7)	1 (1.0%)	43 (43.9)
Haemodialysis	70	17 (24.3)	18 (25.7)	1 (2.9)	33 (47.1)
Alcoholic liver disease	50	14 (28)	21 (42)	6 (12)	30 (60)
Alcoholics without liver disease	35	5 (14.2)	19 (54.3)	3 (8.6)	21 (60)

n-number; +-positive; %-percent; "Coloured"- mixed origin

2.4.5 GBV-C/HGV transmission studies

GBV-C/HGV RNA was detected in 27 of 75 (36%) HIV positive pregnant African mothers. Of the 20 mothers studied for the transmission of GBV-C/HGV, 15 (75%) mothers initiated breast feeding (12 having breast fed their infants for ≥ 3 months), 5 mothers did not breast feed their infants. Sera were available for 15 infants (3 were not breast fed) at 6 weeks after birth, none had detectable GBV-C/HGV RNA in their sera (Fig 2.2), implying that there was no evidence for *in utero* and/or *intrapaturm* transmission of GBV-C/HGV. At 3 months after birth, sera were available for 18 infants, GBV-C/HGV RNA was detected in 2 infants whose mothers were GBV-C/HGV RNA negative of whom one infant was never breast fed (Fig.2.2). Of the 5 GBV-C/HGV RNA positive mothers, GBV-C/HGV RNA was detected in only two infants (40%) 3 to 9 months after birth. In one such infant (1021), viraemia persisted from 3 to 9 months. In the remaining 3/5 (60%) GBV-C/HGV RNA positive mothers, no viral RNA was detected in the sera of their infants till 3 months of life. In two of 20 (10%) GBV-C/HGV RNA negative mothers, GBV-C/HGV RNA was detected in their infants at 3 months after birth. Of the five GBV-C/HGV RNA positive mothers, the transmission of GBV-C/HGV to two infants at nine months of age (Fig 2.2) was confirmed by nucleotide sequences The homologies for the sequenced fragments in the mother-infant pairs were, 97.6% for Mother - Child 1021 and 100% for Mother – Child 1035 (Fig.2.3). One infant (1021) was never breast-fed and was HIV negative whilst the other (1035) was breast fed for at least nine months and was HIV positive. By 9 months only 3/20 infants tested positive for HIV, one defaulted and no results were available in two. The phylogenetic tree of the sequenced fragment is depicted in

Fig. 2.4. Phylogenetic analysis of these isolates showed a close genetic relatedness to Group 5 isolates from KZN as expected (Fig. 2.4).

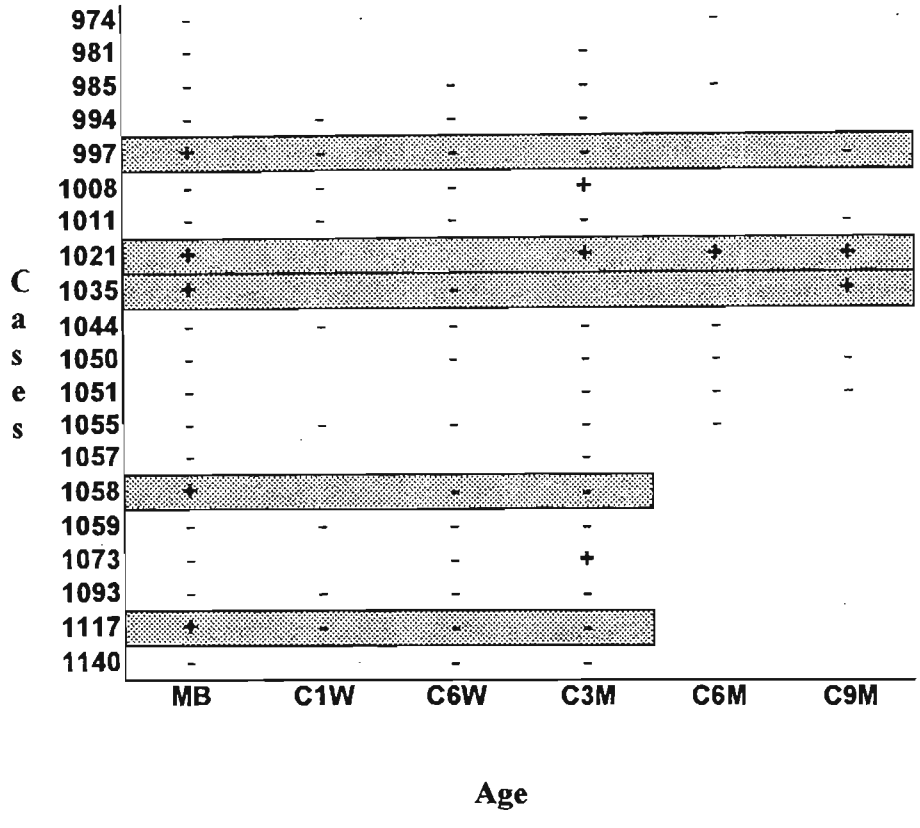


Fig. 2.3 Detection of GBV-C/HGV in HIV infected mothers and their infants during follow-up. The grey bars indicate the duration of the follow-up of infants born of GBV-C/HGV positive mothers. Plus (+) and minus (-) signs indicate positive and negative results of GBV-C/HGV RNA. MB = mother; C = child; W = week; M = month

```
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        10         20         30         40         50         60

AF398330        TGGTAGCCAC TATAGGTGGG TCTTAAGGGG AGGCTACAGT CCCTCTAGTG CCTGTGGAGA
AF398331        TGGTAGCCAC TATAGGTGGG TCTTAAGGGG AGGCTACAGT CCCTCTAATG CCCGTGGCGA
AF398332        TGGTAGCCAC TATAGGTGGG TCTTAAGGGG AGGCTACAGT CCCTCTAATG CCCGTGGCGA
AF398333        TGGTAGCCAC TATAGGTGGG TCTTAAGGGG AGGCTACAGT CCCTCTAGTG CCTGTGGAGA

AF398330        GAAAGCGCAC GGTCCACAGG TGTTGGCCCT ACCGGTGTGA ATAAGGGCCC GACGTCAGGC
AF398331        GAAAGCGCAC GGTCCACAGG TGTTGGTCCT ACCGGTGT-A ATAAGGACCC GACGTCAGGC
AF398332        GAAAGCGCAC GGTCCACAGG TGTTGGTCCT ACCGGTGT-A ATAAGGACCC GACGTCAGGC
AF398333        GAAAGCGCAC GGTCCACAGG TGTTGGCCCT ACCGGTGTGA ATAAGGGCCC GACGTCAGGC

AF398330        TCGTCGTTAA ACCGAGCCCG TTAACCCCCT GGGCAAACGA CGCCCACGTA CGGCCCACGT
AF398331        TCGTCGTTAA ACCGAGCCCA TTATCCCCCT GGGCAAACGA CGCCCATGTA CGGTCTACGT
AF398332        TCGTCGTTAA ACCGAGCCCA TTATCCCCCT GGGCAAACGA CGCCCATGTA CGGTCTACGT
AF398333        TCGTCGTTAA ACCGAGCCCG TTAACCCCCT GGGCAAACGA CGCCCACGTA CGGCCCACGT

AF398330        CGCCCTTCAA TGTCTCTCTT GACCAATAGG CA-ATGCCGG CGAGTTGGCA AGGGCCAGTG
AF398331        CGCCCTTCAA TGTCTCTCTT GACCAATAGG CATATGCCGG CGAGTTGGCA AAGACCAGTG
AF398332        CGCCCTTCAA TGTCTCTCTT GACCAATAGG CATATGCCGG CGAGTTGGCA AAGACCAGTG
AF398333        CGCCCTTCAA TGTCTCTCTT GACCAATAGG CA-ATGCCGG CGAGTTGGCA AGGGCCAGTG

AF398330        GGGGCCGGGG GCAGGGGGAA GGACCCCCTG TCTCGCCCTT CCCGGGGGTG CGGGAAATGC
AF398331        GGGGCCGGGG GTGGGGGGAA GGACCCCCCA TCCTGCCCGT TCCGGGGGAG TGGGAAATGC
AF398332        GGGGCCGGGG GTGGGGGGAA GGACCCCCCA TCCTGCCCGT TCCGGGGGAG TGGGAAATGC
AF398333        GGGGCCGGGG GCAGGGGGAA GGACCCCCTG TCTCGCCCTT CCCGGGGGTG CGGGAAATGC

AF398330        A-------
AF398331        ATGAGGCC
AF398332        ATGAGGCC
AF398333        ATGAGGCC
```

Fig. 2.4 Alignment of nucleotide sequences from the 5′ NCR of GBV-C/HGV isolates from mother infant pairs (AF398330 and AF398333; 1035 and AF398331 and AF398332; 1021), respectively. The sequences were determined using an Applied Biosynthesis Prism 310 Automated Genetic Analyser (Perkin Elmer, Biosystems, CA, USA). The sequences were assemble and analysed using the Staden Package (http://www.mrc-lmb.cam.ac.uk/pubseq/). Alignment of sequences was done using Clustal W with minor manual adjustments.

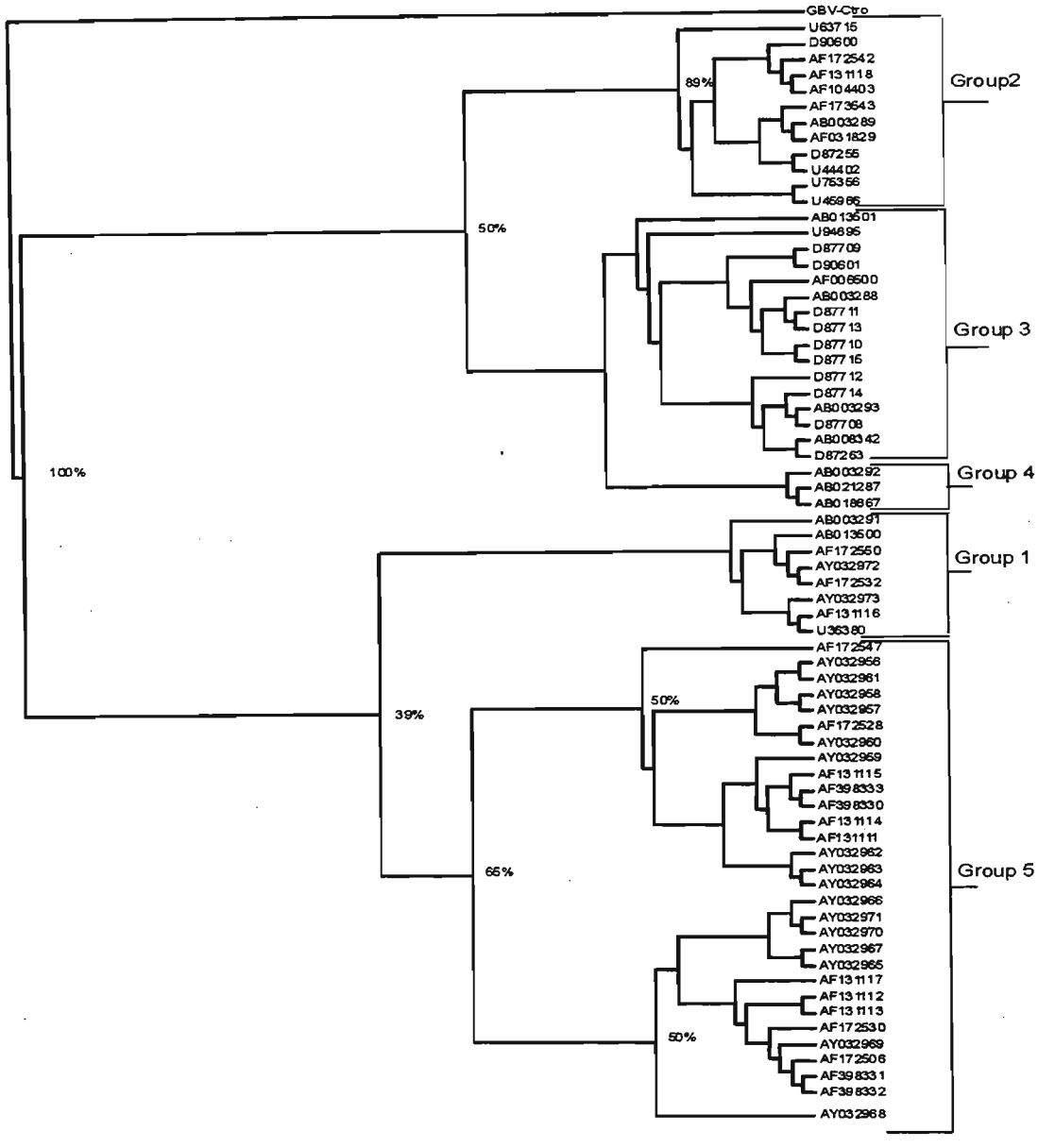


Fig. 2.4. Phylogenetic analysis of GBV-C/HGV sequences of mother infant pairs (AF398330-AF398333) were carried out on a 310 base pair fragment in the 5′ NCR region (positions 160-470) in HGV type2a clone, PNF2161 (Muerhoff *et al*, 1996). Sequences were compared using a distance-based method followed by neighbor joining. Robustness of groups were assessed by bootstrap re-sampling; numbers on branches indicate the percentage of 500 bootstrap replicates that supported the observed pylogeny. Sequences were compared with designated genotypes 1 to 4 (identified by Genbank acession numbers) for which complete genomic sequences are available.

2.5 DISCUSSION

The design, optimization and performance of the LCx assay for the Abbott LCx system has been described by Marshall *et al* (1997). The authors reported a sensitivity of 96.04% and a specificity of 99.76% (Marshall *et al*, 1997). Although both LCx and the "in-house" PCR assays are based on RT-PCR technology, there are several differences in the design of the assays that may contribute to the different detection sensitivities observed. When identical RNA isolation procedures were used upon the 98 serum samples, the LCx and the "in-house" PCR assays detected 12 and 27 GBV-C/HGV positive samples, respectively. In the LCx assay, the input of serum was reduced (25ul) and the volume of RNA eluate was increased (100ul) in contrast to the standard Qiagen virus RNA isolation protocol, input of 140ul serum and elution of RNA in 50ul of water. The format of the LCx assay with the use of a combined reverse transcriptase and DNA polymerase enzyme allows input of large volumes of RNA (20ul) giving a very sensitive assay. In comparison, the input of RNA in the "in-house" RT-PCR was smaller (10ul) and only 1/20 of the RT reaction products were inputs in the first round of amplifications. Thus in the "in-house" RT-PCR only 0.52 ul RNA solution was analysed. Thus the higher detection sensitivity in the LCx assay most probably is due to the larger RNA input. GBV-C/HGV is present in sera in very low titers and most assays rely on the RT-PCR to demonstrate viraemia or active infection. The detection limit of the RT-PCR has been projected to be as low as 500 genome eqivalents per ml (Fanson *et al*, 2000).

Fanson *et al* (2000) using a similar extraction method and RT-PCR assay also demonstrated that GBV-C/HGV RNA was detected at a dilution of 1:16. Fanson *et al* (2000) showed that using the phenol-chloroform extraction method increased the sensitivity of the RT-PCR assay 16 fold compared to the QIAamp extraction method. However, the latter method is harzardous (phenol), complicated, time consuming and labour intensive and not amenable to large sample throughputs. Whilst it is important to determine HCV RNA titers in order to determine chronicity of infection and to monitor treatment, GBV-C/HGV is not associated with any liver disease (as discussed in Chapter 3). Perhaps determining GBVC-C/HGV RNA titers may be important when studying the potentially protective role of GBVC-C-HGV in HIV infection. Under such circumstances the use of newer technologies such as branched chain DNA assay and real time PCR will be important (This is discussed in Chapter 5). However, no difference in the amplification signal on agarose gel electrophoresis was observed when either the modified (Marshall *et al,* 1998) or the manufacturer's method of RNA extraction was used in the "in-house" RT-PCR. To allow for conservation of the sample and reagents, the modified method of extraction (Marshall *et al,* 1998) was used in all RT-PCR.

A 311 base pair fragment in the 5′ NCR region (positions 160-470) in the prototype isolates GBV-C (U36380) and HGV (U44420) was compared with the sequences of the 12 GBV-C/HGV isolates from CLD patients (AF 172501-AF172512) detected by the "in-house" RT-PCR. Sequencing of these 12 GBV-C/HGV isolates confirmed that the PCR products were

GBV-C/HGV. Phylogenetic analysis of these isolates is discussed in detail in Chapter 4. Sequence analysis of the 15 additional GBV-C/HGV isolates was not possible as the final product in the LCx assay is "inactivated." The 15 discordant samples were retested by the "in-house" RT-PCR with identical results. The samples were not retested by the LCx assay. Discrepancies in the LCx rates between positive and negative controls and the LCx calibration controls were observed (Table 2.7.). In addition, 2 runs performed by the LCx had to be invalidated because controls were out of range (Table 2.7), meaning that 34 samples would have had to be retested at an additional cost. However, these samples were not retested by the LCx. The use of internal controls in the "in-house" RT-PCR to expose potential false negative results contributed to the quality assurance of the outer amplification and detection step. Laboratories performing PCR should participate in repeated quality control studies that include a common panel of GBV-C/HGV RNA-positive and –negative samples (Kunkel *et al*, 1998; Lefrere *et al*, 2000a). The inclusion of internal control/s in the LCx assay might be of great value for identification of false positive results.

The performance of each of the primers is decisive for the PCR reaction. Although the primers used in both assays were from the 5′ NCR region of the GBV-C/HGV genome there were differences in the amplification targets and conditions. The reverse transcriptase and amplification enzymes used may have also contributed to differences in sensitivities. The primers chosen for the "in-house" RT-PCR did not participate in primer-dimer formation. The LCx assay involves the use of primers labelled with haptens allowing detection of the

amplified products in an LCx analyser based on MEIA technology; while the "in-house" PCR products were visualised by the ethidium bromide staining in agarose gels. "Carry-over" contamination is a substantial concern in nested PCR which includes several manual transfers of PCR product and gel electrophoresis that might occasionally be performed erroneously. Guidelines to avoid contamination (Kwok and Higuchi 1989) were strictly followed, which included separate areas for RNA extraction, reagent preparation, PCR and detection; positive and negative controls were included in every PCR reaction. The LCx assay is a one tube RT-PCR assay. The use of "unit doses" in the LCx assay reduces the risk of operational or technical errors in the testing and reduces the risk of contamination causing false positive results. The LCx workflow is higher than the "in-house" RT-PCR, although the same extraction protocol was used, cDNA synthesis, amplification and detection are more time consuming in the case of the "in-house" RT-PCR. The LCx is easier to perform and suits better the requirements of a clinical microbiology diagnostic laboratory.

The overall prevalence of GBV-C/HGV RNA in blood donors in the province of KZN (18.9%) is higher than that of blood donors in Gauteng (11.1%) (Castelling *et al*, 1998) with the prevalence being higher in African (38%) and White (22%) blood donors in KZN compared to African (29%) and White (8,5%) blood donors in Gauteng (Castelling *et al*, 1998). Tucker *et al.* (1997) reported a GBV-C/HGV RNA prevalence of 6.3% in a predominantly "Coloured" rural community in the Eastern Cape which is higher than the 4% of "Coloured" blood donors infected with GBV-C/HGV in KZN. However, volunteer blood

donors probably represent a healthier population than rural communities. The prevalence of anti-E2 in healthy blood donors ranges from 2.7% to 20.3% in different parts of the world (Dille *et al*, 1997; Ross *et al*, 1998; Tacke *et al*, 1997b). The overall anti-E2 prevalence in our blood donor population (14.9%) was lower than the 20.3% reported by Ross *et al.* (1998) for South African blood donors. However, Ross *et al.* (1998) did not define the racial breakdown of the blood donor population, and our results indicate a racial difference in anti-E2 prevalence. The lower viraemia (3.8%) and anti-E2 response (5.7%) in the Indian blood donors compared to other racial groups is in agreement with the low prevalence of Anti-E2 (2.7%-6.3%) in Asian countries (Ross *et al*, 1998) and reflects the infrequent exposure to GBV-C/HGV in this community. Anti-E2 prevalence in potential risk groups (haemodialysis vs chronic liver disease) was higher (25.7% vs 32.7%) than in blood donors (15.1%) [$p <$ 0,05]. Concurrent detection of viraemia and seropositivity was seen in 1-12% of our study population. This probably indicates an overlap between E2 sero conversion and presence of viraemia (Tacke *et al*, 1997b; Dille *et al*, 1997). It may be possible that at the time of testing these patients were still viraemic and in the process of seroconverting. Viraemia and sero reactivity was almost mutually exclusive. The combined overall exposure of GBV-C/HGV infection in African blood donors (59.2%) was higher compared to that reported in a West African population (46.7% (PCR + Anti-E2) [Dille *et al*, 1997]. GBV-C/HGV infection appears to be a common infection in Kwa Zulu Natal, South Africa.

There is a higher prevalence of GBV-C/HGV infection in apparently healthy individuals in African countries (10-40%) (Dawson *et al*, 1996; Dille *et al*, 1997; Tucker *et al*, 1997; Castelling *et al*, 1998; Mphahlele *et al*, 1999;) with infection occurring more commonly during childhood (Mphahlele *et al*, 1999). compared to blood donor groups in non-African countries (1-4%) [Linnen *et al*, 1996a; Masuko *et al*, 1996; Moaven *et al*, 1996; Alter *et al*, 1997b; Wang *et al*, 1997a; Roth *et al*, 1997). The reason for the high prevalence of GBV-C/HGV in blood donors worldwide and the basis for the racial differences in GBV-C/HGV infection in the South African blood donor populations is not known. Racial differences in the prevalence of HAV (Sathar *et al*, 1994), HBV (Dusheiko *et al*, 1989) and HCV (Soni *et al*, 1993) infections due to differences in socio-economic factors in South Africa is well documented, whether this is true for GBV-C/HGV is not known, although a relationship was noted between GBV-C/HGV infection and the lack of water borne sewage (Tucker *et al*, 1997).

Generally, infection with GBV-C/HGV is significantly associated with a history of IVDA, exposure to blood transfusions, dialysis and with HCV and HBV infections (Tables 1.3-1.5). The high prevalence in commercial blood donors (5-26%) is probably due to the increased risk of parenteral acquisition in this group (Pilot-Matias *et al*, 1996b; Roth *et al*, 1997; Dille *et al*, 1997; Gutierrez *et al*, 1997). Prior to this no studies have dealt with GBV-C/HGV positivity in alcoholic patients with liver disease in South Africa. In limited studies in non-African countries, GBV-C/HGV RNA positivity rates of 2 -14% have been reported (Linnen *et al*,

1996a; Lampe *et al*, 1997; Guilera *et al*, 1998; Tran *et al*, 1998; Sobue *et al*, 1998; Shimanaka *et al*, 1999). In African ALD patients 14% were GBV-C/RNA positive with 60% of patients having had some experience with GBV-C/HGV (Table 2.8). Tran *et al* (1998) reported that 38.8% (9.3% GBV-C/HGV RNA and 29.5% Anti E2) of ALD patients had past or present infection with GBV-C/HGV. Most of the patients were heavy drinkers. The present study showed that the prevalence of GBV-C/HGV RNA was twice as high as that in alcoholics with liver disease than in alcoholics who were abstaining. The overall exposure in both groups was higher though not significant. Reasons for the higher prevalence of GBV-C/HGV may be similar to that for HCV, viz. that excessive alcohol consumption diminishes the immune function and that cellular immunity is impaired, but the precise mechanism remains obscure, even in the case of HCV.

The prevalence of GBV-C/HGV infection in patients with HIV infection has been assessed in non-African countries. Based on the detection of GBV-C/HGV RNA and/or anti-E2, several studies have reported frequencies as low as 9% and as high as 66% in HIV-infected risk groups (Feucht *et al*, 1996; Jarvis *et al*, 1996; Nubling and Lower, 1996; Kinoshita *et al*, 1997; Nubling *et al*, 1997; Ibanez *et al*, 1998; Scallan *et al*, 1998; Nerurkar *et al*, 1998; Toyoda *et al*, 1998; Woolley *et al*, 1998; Rey *et al*, 1999; Bourlet *et al*, 1999). The primary difference between these studies was the number of individuals in each study who were in primarily high risk groups. Although the current study is biased by a small study group of HIV positive African females, the prevalence of GBV-C/HGV (36%) was similar to that of African blood donors (38.2%) [Sathar *et al*, 1999a] and HIV positive females (39.8%) [Xiang *et al*, 2001].

Since the discovery of GBV-C/HGV, attempts have been made to clarify its principle mode of transmission. In non - African countries transmission of GBV-C/HGV occurs parenterally and is considered to be similar to HCV (Linnen *et al*, 1996b). In this study GBV-C/HGV infected dialysis patients tended to have had more transfusions and a longer duration of dialysis than non infected patients. The majority of patients on maintenance haemodialysis appear to acquire their GBV-C/HGV infection through the transfusions they receive (De Lamballerie *et al*, 1996; Linnen *et al*, 1996b; Masuko *et al*, 1996; Tsuda *et al*, 1996; Wang *et al*, 1997a; Lampe *et al*, 1997; Shrestha *et al*, 1997; Castelling *et al*, 1998; Murthy *et al*, 1998). The absence of a history of blood transfusion and intravenous drug abuse in patients with chronic liver disease, in addition to the high prevalence of GBV-C/HGV in blood donors and in rural communities, especially in African children (Mphahlele *et al*, 1999), tends to suggest that GBV-C/HGV is being transmitted by as yet undefined non-parenteral routes in our environment. It has been suggested that perinatal transmission may be an important source of infection in blood donors lacking parenteral risk factors (Lefrere *et al*, 2000b).

In non-African countries the transmission of GBV-C/HGV from mother-to-infant varies from 30%-89% (Feucht *et al*, 1996; Viazov *et al*, 1997b; Zanetti *et al*, 1997; Hino *et al*, 1998; Wejstal *et al*, 1999; Lefrere *et al*, 2000b). Based on their prevalence study, Mphahlele *et al* (1999) reported a GBV-C/HGV prevalence of 2.9% in Black South African neonates less than 4 months old. In the absence of a serial follow-up study, the authors concluded (Mphahlele *et al*, 1999) that vertical transmission of GBV-C/HGV is not common in South Africa.

Mother-to-infant transmission rates of 10%-60% have been reported in newborns (1-5 days old) (Viazov *et al*, 1997b; Wejstal *et al*, 1999; Lefrere *et al*, 2000b). The mother-to-infant transmission rate of GBV-C/HGV in Black South Africans in KZN is 40% (2/5). This is close to the 38.2% prevalence of GBV-C/HGV reported in African blood donors in KZN (Sathar *et al*, 1999a). In this study GBV-C/HGV RNA was not detected in infants 1-6 weeks old. The lower rate in infants perhaps may be due to the lower viral load which may be below the detection level of the "in-house" PCR assay.

Although the current study may be biased by a small select study group (HIV positive pregnant mothers), the data suggests that some infants (40%; 2/5) acquire their infection between 3-9 months after birth from their infected mothers, as confirmed by the sequence data, whilst other infants acquire their infections by routes independent of mother-to-infant transmission. Similar observations have been reported by others (Zanetti *et al*, 1997; Viazov *et al*, 1997b; Wejstal *et al*, 1999; Lefrere *et al*, 2000b). There is a higher risk of mother-to-infant transmission in high risk groups (Feucht *et al*, 1996; Zanetti *et al*, 1997; Fischler *et al*, 1997; Viazov *et al*, 1997b; Wejstal *et al*, 1999). However, it is not clear whether co-infection with HCV, HIV-1 or both or IVDU are the underlying cause for transmission of GBV-C/HGV from mother to infant. GBV-C/HGV has not been detected in breast milk to-date (Schroter *et al*, 2000) and it is not clear as to whether transmission of GBV-C/HGV is influenced by breast feeding or by the mode of delivery (Viazov *et al*, 1997b; Zanetti *et al*, 1997; Wejstal *et al*, 1999). It is therefore possible to formally conclude that there is some mother-to-infant

transmission of GBV-C/HGV in KZN, though it is very probable that infants, or at least some of them could be infected by as yet undefined non-parenteral routes.

HBV infection is endemic amongst the African population of South Africa and the risk of horizontal transmission of HBV is well recognised (Dusheiko *et al*, 1989). Whether the association of GBV-C/HGV with HBV implies that GBV-C/HGV might spread by sexual routes can not be determined by this study. Various studies have pointed to the important role of sexual exposure as a likely route of transmission of GBV-C/HGV, in both non-HIV infected subjects without the risk for parenteral transmission (IVDU and multitransfused individuals including haemophiliacs) [Kao *et al*, 1997b,c; Scallan *et al*, 1998; Sawayama *et al*, 1999) and HIV infected individuals with the risk for parenteral and sexual transmission (homosexuals, heterosexual and prostitutes) [Nubling *et al*, 1997; Nerurkar *et al*, 1998; Ibanez *et al*, 1998; Bourlet *et al*, 1999]. Infection with GBV-C/HGV appears to be more frequent in patients with a sexual risk than those with parenteral exposure (Ibanez *et al*, 1998; Bourlet *et al*, 1999). The role of semen in the transmission of GBV-C/HGV is controversial (Chen *et al*, 1997; Seemyer *et al*, 1998; Eugenia *et al*, 2001). Recent reports have suggested that human saliva may contribute to the spread of GBV-C/HGV RNA (Chen *et al*, 1997; Seemyer *et al*, 1998; Eugenia *et al*, 2001). Tucker *et al* (2000) did not detect GBV-C/HGV replicative intermediaries in the cadaver biopsies of salivary glands and the gonads of GBV-C/HGV positive patients. The results imply that the virus may be present in the saliva and semen of infected individuals, but not be transmitted via these routes. Despite the evidence for increased

frequencies of GBV-C/HGV infection in association with sexual exposure, the mechanism of transmission remains unclear.

The frequency of positivity for RNA or Anti-E2 varies among groups, depending on the subjects' origins and the methods used to detect GBV-C/HGV markers. The differences in the prevalence of detecting GBV-C/HGV infection may be due to the differences in the sensitivity of the various PCR protocols and primers (derived from various regions of the genome) used by various investigators. The nested PCR used in this study is likely to contribute to an increase in the sensitivity of the assay as compared to the one step PCR procedures. In addition the testing of Anti-E2 greatly extends the ability of RT-PCR to define the epidemiology of GBV-C/HGV. Although the study population may not be representative of the overall prevalence of GBV-C/HGV in KZN, the results indicate that (1) RT-PCR using nested primers from the 5´NCR of the GBV-C/HGV genome is sensitive and specific, (2) there is a high prevalence of GBV-C/HGV infection in KZN, South Africa, (3) there are racial differences in the prevalence of GBV-C/HGV infection, and (4) that there is some mother-to-infant transmission of GBV-C/HGV in KZN, though it is very probable that transmission does occur by as yet undefined non-parenteral routes. In an environment where HBV is endemic and HIV is considered to be pandemic, the disease potential, if any, of GBV-C/HGV infection needs to be determined.

CHAPTER 3

CHAPTER 3

CLINICAL SIGNIFICANCE OF GBV-C/HGV INFECTION IN KWAZULU NATAL, SOUTH AFRICA

3.1 INTRODUCTION

In South Africa, the prevalence of hepatitis viruses ranges from 8 -15%, depending on whether the patient originates from an urban or rural environment (Kew, 1996). In addition, there is a high prevalence of GBV-C/HGV infection in blood donors, high risk groups and patients with liver disease in KZN, South Africa (Sathar *et al*, 1999a). Whilst in non-African countries the clinical association of GBV-C/HGV with liver damage is still debatable (Mushahwar and Zuckerman, 1998), its association with liver disease in Africa has not been defined. Alcohol-related medical diseases, including chronic liver disease, are common in the local hospital population. It has always been questionable whether alcohol alone is a common cause of cirrhosis in African patients. Lelbach *et al* (1975) have shown that a history of alcohol consumption of more than 80 grams per day to is the minimum amount necessary to cause alcoholic liver disease. It is doubtful whether many of the African patients labeled as having alcohol-related liver disease are able to afford the large quantity of alcohol required to produce hepatic cirrhosis (160 gm alcohol per day for 5 years in males) (Wands and Blum, 1991). Therefore, one wonders whether alcohol alone is able to produce the amount of hepatic injury that is attributed to it, or whether viral infections serve as a cofactor for the hepatic injury (Brechot *et al*, 1996), especially in an area where both contributions are endemic.

South Africa has the fastest-growing HIV AIDS population in the world. Official Department of Health figures compiled from antenatal clinics across the country show a significant increase in the infection rate among pregnant women in most provinces, the highest figures (35%) being reported for the province of KZN, which is considered to be the epicentre of the HIV pandemic in South Africa. To what extend GBV-C/HGV persistence affects the rate of progression to AIDS in African countries is not known. Whether GBV-C/HGV infection is associated with any abnormality in HIV infected patients in KZN is not known. GBV-C/HGV is a flavivirus with some homology to HCV, and often co-existing with GBV-C/HGV. HIV infection like HBV is a serious health problem in KZN. AIDS patients infected with HBV or HCV often die from liver failure instead of AIDS (Ockenga *et al*, 1997) With the high prevalence of GBV-C/HGV in KZN (Sathar *et al* 1999a) one assumes that GBV-C/HGV (if it does cause hepatitis) would have similar interactions in HIV infected patients.

This chapter will attempt to determine the clinical association, if any, that GBV-C/HGV has with liver disease and HIV in KZN, South Africa.

3.2 METHODS

3.2.1. Source of Samples

3.2.1.1 Collection of Sera

Venous blood was collected and stored as described in 2.2.1.1

3.2.1.2 Patients

The study population comprised the same patients studied in Chapter 2.2.1.2 viz.70 patients with chronic renal failure who were undergoing maintenance haemodialysis (HAEM), 98 consecutive patients with chronic liver disease (CLD) and 50 patients with biopsy proven alcoholic liver disease (ALD). In addition, sera were collected from 56 HIV positive mothers, recruited at 33-38 weeks gestation from a randomized placebo-controlled clinical trial on Vitamin A supplementation (Coutsoudis *et al*, 1999) and stored at – 85°C under coded identifier numbers. Serum samples were obtained at baseline for immunophenotyping. HIV infection was diagnosed by western blot analysis/serology and PCR if required.

3.2.1.3 Control groups

The control groups included those studied in Chapter 2.2.1 3 viz. 232 adult volunteer blood donors from the four racial groups and 35 individuals who did not present to hospitals for signs or symptoms of liver disease and attended local center for alcohol detoxification (Alcoholic Anonymous).

3.2.2 Diagnostic assays

3.2.2.1 Detection of GBV-C/HGV RNA

The RT-PCR assay has been described in detail in Chapters 2.2.3.1.1 – 2.2.3.1.3

3.2.2.2 Detection of GBV-C/HGV Anti-E2 antibodies

The assay has been described in Chapters 2.2.3.2.1

3.2.2.3 Carbohydrate Deficient Transferrin (CDTect®)

The assay has been described in Chapter 2.2.3.2.2.

3.2.3 Liver function tests

Raised liver enzymes (alanine aminotransferase [ALT]; aspartate aminotransferase (AST); alkaline phosphatase [ALP]; gamma-glutamyltransferase [GGT] and bilirubin) are suggestive of acute or chronic liver disease (Craxi and Almasio, 1996). The most frequent cause of high ALT levels (≥ 10 times upper normal limits) [Table 3.1], with or without symptoms, is an acute hepatitis of viral etiology. Liver enzymes and/or liver function tests were performed by routine multianalysis systems in the central laboratory at King Edward VIII Hospital, Durban, KZN.

Table 3.1 Tests used to determine liver function and normal ranges of liver enzymes for King Edward VIII Hospital, Durban, Kwa Zulu Natal, South Africa

Parameters	Normal Ranges
ALB (g/L)	32-50
GLOB (g/L)	20-32
T-Bil (umol/L)	0-17
ALT (U/L)	10-45
AST (U/L)	15-45
GGT (U/L)	10-42
ALP (U/L)	10-60

ALB-albumin; GLOB-globulin;
T-Bil-Total bilirubin;
ALT- alanine aminotransferase;
AST- aspartate aminotransferase;
GGT-gamma-glutamyltransferase ;
ALP-alkaline phosphatase;
g/l-grams per litre; umol/L-micromoles per litre;
U/L-units per litre

3.2.4 Immunophenotyping

Immunophenotyping of lymphocytes was performed using specific monoclonal antibodies on the Becton Dickinson Facs Vantage flowcytometer (Becton Dickinson Immunocytometry Systems, San Jose, Cal, USA) using a CellQuest software programme. Briefly 100*u*l of whole blood sample was added to each Falcon tube. 10*u*l of each monoclonal antibody was added to each respective tube according to the panel set-up. Each tube was agitated at medium speed on a vortex and the cells were allowed to incubate for 30 minutes. The peripheral blood samples were lysed with a Coulter Q-Prep.

The samples were read using CellQuest program on the Facs Vantage flowcytometer. To acquire data on the Facs Vantage flowcytometer, the amplifiers, PMT voltage, compensation and threshold were adjusted for each sample type and fluorochrome that was used. Those adjustments positioned the cells of interest within the scatter and fluorescence channels. Collectively this process was known as sample optimisation. The Facs Vantage instrument settings were optimised using the Cellquest software. Cellquest software was a general program for acquiring and analysing data from a flowcytometer. It was also used for adjusting electronics and setting up conditions for data acquisition.

3.3 STATISTICAL ANALYSIS

The data of blood donors, HAEM, CLD and alcoholics with and without liver disease were analysed separately. Chi-squared analysis was used to compare groups (GBV-C/HGV) positive versus GBV-C/HGV negative) with respect to categorical data, while Student's t-test was applied to continuous data. Analysis of covariance was used to compare GBV-C/HGV positive and GBV-C/HGV negative patients with respect to liver function test, adjusting for age, sex and disease group. Multiple logistic regression was used to determine any confounding influence of age, sex and disease group on GBV-C/HGV. Blood donors were compared to CLD and HD patients with regard to demographic parameters using Student's t-test or Chi-squared test.

Descriptive statistics were calculated by group: means and standard deviations for continuous and frequencies and percentages for categorical data. ALD patients were compared with alcoholic controls using Student's t-test for continuous data. Two-way analysis of variance was used for comparison of ALD and controls, and for GBV-C/HGV positive and GBV-C/HGV negative patients, and to assess the interactions between these groups of patients. Chi-squared tests were used to compare groups with relation to categorical data.

HIV positive patients' data were calculated using descriptive statistics (means and standard deviation). In HIV positive patients GBV-C/HGV positive and negative patients were compared with relation to all parameters using non-parametric Wilcoxon 2-sample tests.

3.4 RESULTS

3.4.1 Alcoholic liver disease

Of the 50 ALD patients 27 of 39 (69.2%) were anti-HBc positive (Table 3.2), 14/27 (52.1%) were anti-HBs positive. Seven of 50 (14%) ALD patients were positive for HBsAg (Table 3.2). These seven ALD patients were tested for additional HBV viral markers to assess whether viral replication persisted (Table 3.3). HBV DNA was detected in 2/7 (28.6%) ALD patients (Table 3.3). Anti HBs and anti-HBc were detected in 2/7(28.6%) (patient nos. 41 & 131) and HBeAg detected only in one (patient no.131) (Table 3.3). Both these patients (41 & 131) were co-infected with GBV-C/HGV. HBsAg was detected in one alcoholic without liver disease [1/35 (2.9%)], this subject had no other viral markers for HBV infection. Thirty - three of 50 (66%) ALD patients had one or more viral markers for HBV infection. There was a higher prevalence of HBV and HCV in ALD patients than in alcoholics without liver disease, this difference was not significant (Table 3.2). Twenty of 35 (57.1%) alcoholics without liver disease were anti-HBc positive, suggestive of ongoing or previous infection (Table 3.4). Although not significant, there was a higher viral persistence and replication of HBV in ALD patients than alcoholics without liver disease.

Table 3.2 Markers for HBV and HCV infection in alcoholics with alcoholic liver disease and alcoholics without liver disease

PARAMETER	ALCOHOLIC LIVER DISEASE n (%)	ALCOHOLICS WITHOUT LIVER DISEASE n (%)	p-value
HBV			
Sag	7/50 (14)	1/35 (2.9)	ns
Cab	27/39 (69.2)	20/35 (57.1)	ns
DNA	3/50 (6)	1/35 (2.9)	ns
HCV RNA	2/50 (4)	1/35 (2.9)	ns

HBV-Hepatitis B Virus; HCV-Hepatitis C Virus; sAg-surface antigen; cAb-core antibody; DNA-deoxiribonucleic acid, RNA-ribonucleic acid; no-number; %-per cent; ns-not statistically significant

Table 3.3 HBV and GBV-C/HGV markers in alcoholic patients with liver disease (ALD) who were HBsAg positive

Patient I.D. No.	Anti-HBs	HBeAg	Anti-HBc	DNA	GBV-C/HGV RNA	GBV-C/HGV anti-E2
8	-	-	-	-	+	-
28	-	-	-	-	+	-
41	+	-	+	-	-	+
131	-	+	+	+	+	+
197	-	-	-	+	+	-
304	-	-	-	-	+	+
307	-	-	-	-	-	+

HB-hepatitis B virus; sAg-surface antigen; eAg-envelope antigen; anti- HBs or HBc-antibody surface or core antigen; E2- enevelope; nd-not done; DNA-deoxiribonuleic acid

3.4.2 Liver biochemistry in patients and control groups

Raised ALT levels were observed in 6 of 232 (2.6%) blood donors (Table 3.4). Only 1/44 (2.3%) GBV-C/HGV positive donors had a raised ALT level (69 U/L) (Table 3.4). Raised ALT levels were observed in 2/70 (2.85%) dialysis patients (Table 3.5); 17/35 (48.6%) alcoholics without liver disease and 4/50 (8%) ALD patients (Table 3.6). In 3 ALD patients ALT levels were greater than 10 times the normal range (Table 3.4), [472 U/L; 756 U/L and 1476 U/L]. All three patients were infected with HBV; one (1476u/l) was co-infected with HCV (HCV RNA positive) and the other (472U/L) was co-infected with GBV-C/HGV RNA. Although ALT and GGT levels were higher in ALD patients compared to the control group, this difference was not significant (Table 3.6). The ALP levels in ALD patients were significantly higher than the control groups ($p = 0.0001$) (Table 3.6). In contrast, CDTect levels were significantly higher in the control group compared to ALD patients ($p = 0.0005$) (Table 3.6).

Using multivariate analysis and controlling for the influence of confounding factors (age, sex and disease groups) no significant differences were observed between GBV-C/HGV infected and non-infected blood donors in each of the four racial groups (Table 3.4). No significant differences in liver biochemistry were observed in CLD patients, HAEM patients (Table 3.5) and alcoholic subjects with and without liver disease patients (Table 3.6).

Table 3.4 Demographic and Biochemical features of Blood donors (n = 232) with and without GBV-C/HGV Infection

Blood donors	GBV-C/HGV+ Mean ± SD (Range)	GBV-C/HGV – Mean ± SD (Range)	p-value
Africans (n = 76)	**(n=29)**	**(n=47)**	Ns
ALT(U/L)	17.24 ± 11.40 (9-69)	15.68 ± 6.6 (5-45)	Ns
Age (yrs)	32.10 ± 10.06 (19-48)	28.38 ± 10.53 (18-59)	Ns
Sex (M:F)	8.6:1	4.2:1	ns
Indians (n = 52)	**(n=2)**	**(n=50)**	ns
ALT(U/L)	16.5 ±2.12 (15-18)	23.48± 16.82 (7-92)	ns
Age (yrs)	47.0±7.07 (42-52)	30.68± 12.09 (17-67)	ns
Sex (M:F)	1:1	11.5:1	ns
Whites (n = 49)	**(n=11)**	**(n=38)**	ns
ALT(U/L)	24.45 ±11.64 (11-54)	23.76± 13 83 (8-63)	ns
Age (yrs)	40.27± 9.71 (19-53)	39.32 ±15.19 (17-67)	ns
Sex (M:F)	2:0	2.25:1	ns
Coloureds (n = 55)	**(n=2)**	**(n=53)**	ns
ALT (U/L)	12.5 ±2.12 (11-14)	19.06± 12.51 (5-73)	ns
Age (yrs)	19.0 ±1.41 (18-20)	31.23 ±12.46 (17-68)	ns
Sex (M:F)	2.67:1	1.9:1	ns

SD-standard deviation; n-number; M-male; F-female; ALT-alanine aminotransferase; ns-not statistically significant; U/L-units per litre; + positive; - negative

Table 3.5 Demographic and Biochemical features of patients with chronic liver disease (n = 98) and Haemodialysis (n = 70) patients with and without GBV-C/HGV Infection

	GBV-C/HGV + Mean ± SD (Range)	GBV-C/HGV – Mean ± SD(Range)	p-value
Chronic liver disease	**(n=12)**	**(n=86)**	
Age (yrs)	45.7 ± 15.9 (18-75)	44.8 ± 16.8 (2-81)	ns
Sex (M:F)	1.4:1	1.2:1	ns
T- Bil (umol/l)	68.6 ± 73.4 (7-235)	63.3 ± 83.3 (7-506)	ns
ALP(U/L)	197.0 ± 163.29 (18-576)	173.8 ± 143.41 (3-838)	ns
AST (U/L)	92.2 ± 48.4 (23-173)	154.6 ± 350.6 (14-2454)	ns
Haemodialysis	**(n=17)**	**(n=53)**	
Age (yrs)	38.17± 12.06 (17-65)	39.57± 12.76 (15-67)	ns
Sex(M:F)	1:0.8	1: 0.8	ns
Dialysis(mths)	76.23 ± 50.90 (12-180)	59.60 ± 54.82 (3-216)	ns
TRANSF(no.)	12.58 ± 7.79 (5-31)	8.07 ± 7.17 (2-30)	**0.03**
AST (U/L)	14.88 ± 5.33 (6-28)	18.60 ± 13.22 (6-90)	ns
ALT (U/L)	12.58 ± 6.97 (5-32)	17.92 ± 18.24 (4-101)	ns
ALP (U/L)	132.58 ± 146.39 (36-667)	107.11 ± 131.50 (5-920)	ns
T-BIL (umol/L)	11.94 ± 2.22 (8-17)	11.32 ± 2.25 (5-17)	ns

T-Bil-Total bilirubin; ALT-alanine aminotransferase; GGT-gamma-glutamyltransferase; ALP-alkaline phosphatase; TRANS-transfusion; umol/L-micromoles per litre; U/L-units per litre; no-number; mths-months; M-male; F-female;-yrs-years; SD-standard deviation; + positive; - negative.

Table 3.6 Biochemical features of alcoholic patients with and without liver disease with and without GBV-C/HGV infection

	GBV-C/HGV + Mean ± SD	GBV-C/HGV – Mean ± SD	p-value
Alcoholics with liver disease	**(n=14)**	**(n=36)**	
ALB (g/L)	20.71 ± 6.88	24.80 ± 7.08	ns
GLOB (g/L)	57.00 ± 17.21	51.80 ± 12.66	ns
T-Bil (umol/L)	67.14 ± 54.53	58.94 ± 97.13	ns
ALT (U/L)	14.65 ± 117.95	100.00 ± 271.05	ns
GGT (U/L)	241.57 ± 188.91	222.44 ± 222.76	ns
ALP (U/L)	283 43 ± 229.45	213.39 ± 160.35	ns
CDTect (U/L)	22.01 ± 9.721	30.70 ± 21.57	ns
Alcoholics without liver disease	**(n=5)**	**(n=30)**	
ALB (g/L)	40.40 ± 8.67	41.14 ± 6.64	ns
GLOB (g/L)	41.80 ± 10.66	42.51 ± 8.17	ns
T-Bil (umol/L)	9.00 ± 1.87	13.51 ± 6.33	ns
ALT (U/L)	85.40 ± 22.09	54.51 ± 35.14	ns
GGT (U/L)	104.00 ± 101.07	163.41 ± 229.89	ns
ALP (U/L)	51.00 ± 19.46	77.37 ± 46.53	ns
CDTect (U/L)	89.09 ± 34.51	50.99 ± 37.20	ns

ALB-albumin; T-Bil-Total bilirubin, ALT-alanine aminotransferase; GGT-gamma-glutamyltransferase; ALP-alkaline phosphatase, CDTect-carbohydrate transferin test; n- number; umol/L-micromoles per litre; U/L-units per litre; SD-standard deviation; +-positive; - - negative.

3.4.3 HIV infected patients

In 55 HIV positive mothers the mean haematological laboratory values in patients with and without GBV-C/HGV infection were not significantly different (Table 3.7). With the exception of relative rather than absolute differences in CD3, CD30 and gammadelta T cells (γδT) [TGD], immune parameters were not significantly different in both groups. There was no significant differences in CD4 (461.12 ± 163.28 vs 478.42 ± 181.22) and CD8 (680.83 ± 320.36 vs 862.52 ± 354.48) absolute cell counts between HIV positive mothers co-infected with GBV-C/HGV and those not infected with GBV-C/HGV, respectively (Table 3.8). However, significantly higher CD3 % [80.0 ± 4.17 vs 70.99 ± 19.79] (p = 0.015), gammadelta T cells (γδT %) [3.22± 1.30 vs 2.15 ± 29.12] (p=0.052) and lower CD 30 % [35.45 ± 17.86 vs 50.59 ± 9.20] (P= 0.041) status were observed in GBV-C/HGV positive compared to GBV-C/HGV negative HIV infected patients, respectively (Table 3.8).

Table 3.7 Comparison of mean haematological indices in HIV infected patients with and without GBV-C/HGV infection

Haemotological Indices	GBV-C/HGV + Mean ± SD [n=22]	GBV-C/HGV – Mean ± SD [n=33]	p-value
Haemoglobin (g/dl)	10.57 ± 1.32	10.65 ± 2.24	ns
Haematocrit (%)	30.59 ± 3.47	31.81 ± 3.26 (%)*	ns
Mean cell volume (fl)	86.25 ± 8.41	88.02 ± 5.82 (fl)*	ns
RDW (%)	13.58 ± 2.07	13.58 ± 2.48 (%)*	ns
Reticulocytes (%)	2.2 ± 1.26	2.49 ± 1.10	ns
RETCORR (%)	1.52 ± 0.89	1.77 ± 1.04	ns
RET ABS (%)	81.01 ± 50.90	87.31 ± 43.32	ns
Platelets ($\times 10^9$ / l)	268.59 ± 81.13	250.16 ± 69.12 ($\times 10^9$ / l)*	ns
White cell count ($\times 10^9$ / l)	7.7 ± 2.2	7.44 ± 2.09	ns
Neutrophils ($\times 10^9$ / l)	5.03 ± 1.99	4.83 ± 1.65	ns
Lymphocytes ($\times 10^9$ / l)	1.74 ± 0.44	1.73 ± 0.63	ns
Monocytes ($\times 10^9$ / l)	0.62 ± 0.19	0.66 ± 0,63	ns
Eosinophils ($\times 10^9$ / l)	0.29 ± 0.21	0.29 ± 0.27 ($\times 10^9$ / l)*	ns

g/dl- grams per decilitre; fl- femto litres; l- liter; n- number; *n = 32; ns- not statistically significant; + positive; - negative

Table 3.8 Comparison of mean lymphocyte subset indices in HIV infected patients with and without GBV-C/HGV Infection

Lymphocyte subsets	GBV-C/HGV + Mean ± SD [n=22]	GBV-C/HGV - Mean ± SD [n=33]	p-value
CD4 (%)	26.70 ± 8.30	26.64 ± 8.67	ns
CD4(cells/ul)	461.12 ± 163.28	478.42 ± 181.22	ns
CD8 (%)	48.47 ± 10.08	48.70 ± 10.78	ns
CD8(cells/ul)	680.83 ± 320.36	862.52 ± 354.48	ns
CD4/CD8 Ratio	0.58 ± 0.25	0.59 ± 0.28	ns
CD3 (%)	80.0 ± 4.17	70.99 ± 19.76	**0.015**
CD3(cells/ul)	1395.45 ± 366.79	1451.29 ± 571.37	ns
CD30 %	35.45 ± 17.86	50.59 ± 9.20	**0.041**
CD30(cells/ul)	577.75 ± 307.64	823.88 ± 392.00	ns
γδT(%)	3.22 ± 1.30	2.15 ± 1.08	**0.052**
γδT	53.11 ± 18.35	39.15 ± 29.12	ns

CD-cluster of differentiation; γδT- gamma delta T cells; n- number; SD- standard deviation, ns-not statistically significant; + positive; - negative

3.5 DISCUSSION

The majority of haemodialysis patients and blood donors, including those with GBV-C/HGV infection, had normal liver enzymes (Tables 3.4 – 3.6). The high prevalence of GBV-C/HGV in blood donors and in haemodialysis patients (Sathar *et al*, 1999a) without biochemical evidence of liver damage suggests that many patients are viraemic in the absence of liver disease. In positive cases of GBV-C/HGV RNA persistence where transient elevated levels of ALT have been observed, these levels subsequently returned to normal (Wang *et al*, 1996; Masuko *et al*, 1996; Linnen *et al*, 1996a,b; Alter *et al*, 1997a,b). Although persistent GBV-C/HGV infection may not cause liver damage or dysfunction to a greater extent than HCV infection, there are a few reports on histology in GBV-C/HGV infection (Dhillon *et al*, 1996; Fiordalis *et al*, 1996; Colombatto *et al*, 1997; Ross *et al*, 1997). However, normal ALT levels do not definitely prove normal histology as shown for HCV infection (Alberti *et al*,1991). Numerous studies have shown that in HCV co-infection, GBV-C/HGV does not influence the liver histology (Tanaka *et al*, 1996; Bralet *et al*, 1997; Enomoto *et al*, 1998; Pawlotsky *et al*, 1998). On the other hand, some investigators have suggested that GBV-C/HGV in HCV infected patients might accelerate liver injury (Diamantis *et al*, 1997; Francesconi *et al*, 1997; Manolakopoulos *et al*, 1998). However, in the absence of liver histology to detect GBV-C/HGV no significant differences were observed in the liver biochemistry of patients with liver disease and control groups who were infected with GBV-C/HGV as compared to those not infected with GBV-C/HGV.

Obtaining an accurate history of the amount of alcohol ingested by a patient is often difficult because of the unreliability of the patient report (Orrego *et al*, 1979). The CDTect™ considered to be the most accurate biochemical marker of alcohol consumption currently available (Salapro *et al*, 1999), was used to assess if there was a discrepancy in the history of alcohol consumption. Most of the patients in the study did not attempt to conceal their alcohol consumption. The greater spread of CDTect™ values (6.08-166.7U/L) in the former group suggests that bloods were taken from these alcoholics at different times/points of entry into the local center for alcohol detoxification (Alcoholic Anonymous). In addition, it also reflects the degree of abstinence. The shorter spread of CDTect™ values of ALD patients (9.52-49.22 U/L) except one (143.22u/l) suggests that because these patients are very sick when admitted into hospital. They were already abstaining and during their period of hospitalisation do not have access to alcohol. Despite favourable reports concerning the CDTect™, a variety of important issues on the value and applicability of the test in clinical and research situations remain inadequately explored. There are still some uncertainties, regarding the sensitivity and specificity of the tests, which probably depends on the cut-off point for CDTect™, ethnicity of the population studied, control groups and time of blood taking in relation to abstinence (Salapuro *et al*, 1999). CDTect™ is a good marker of alcoholism similar to the commonly used markers. It may be useful in the control of alcohol consumption in patients with liver disease. The results also suggest that the CDTect ™was a significantly better marker in

detecting high levels of alcohol consumption than the GGT. Similar observations were reported by Salaspuro *et al* (1999) in his review of the CDTect™

Several factors, in addition to alcohol intake, have been implicated to explain different evolutions of liver damage observed in alcoholic patients (Nei et al, 1983; Sorenson *et al*, 1984; Diehl, 1989; Zetterman,1990;). Among these, HBV infection has received special consideration (Mills *et al*, 1979; Nalpas *et al*, 1985; Esteban *et al*, 1989). HBV infection may be present despite the absence of serological markers of persistent viral infection (Brechot *et al*, 1982; 1985; Nalpas *et al*, 1985). It is now known that HBV can exist in serum and liver at very low levels, even when serological markers such as HBsAg and HBV DNA are not identifiable with the usual methods (Theirs *et al*, 1988; Feray *et al*, 1990). Antibodies against HCV antigens (anti-HCV) have been found in several series with alcoholic liver disease, suggesting a high prevalence of HCV infection, especially in patients with severe liver injury. This has been confirmed by identification of HCV RNA, leading to the hypothesis that chronic liver disease in alcoholic patients may only have a viral pathogenesis (Brillanti *et al*, 1989; Esteban *et al*, 1989; Pares *et al*, 1990).

The role of viral infections in alcoholic liver disease varies with the stage of liver disease and with the race and country of origin of the patients (Brechot *et al*, 1996). Several studies have reported an association of alcoholism with HBV and HCV, suggesting that alcohol accelerates liver damage in subjects with HBV and HCV infection (Mills *et al*, 1979; Brechot *et al*, 1982;

1985; Nalpas *et al*, 1985; Esteban *et al*, 1989; Brillanti *et al*, 1989; Pares *et al*, 1990). A large proportion of these patients have serological evidence of past exposure to HBV (anti-HBc positive), and are thus not considered to harbour the virus in liver or serum (Brechot *et al*, 1996). Approximately, 60% of alcoholics in the current study with and without liver disease had serological markers indicating previous HBV infection. In contrast HBV PCR showed HBV replication in only 3-6% of alcoholic patients. This would tend to suggest that inapparent active HBV infection plays a marginal role in the pathogenesis of liver disease in the group of alcoholic patients studied. HBV DNA was detected in 2.9% (1/34) and 9.3% (4/43) of HBsAg - negative alcoholics without and with liver disease, respectively. In several series of HBsAg-negative alcoholic patients, the prevalence of HBV DNA approached 10% (Nalpas *et al*, 1985; Zignego *et al*, 1994). These results demonstrate that HBV serology alone does not accurately reflect the frequency of infection among alcoholics. The detection of HBV DNA in the sera of HBsAg negative alcoholic patients may be related to the production of low amounts of HBsAg. Alternatively it may reflect the production of variants not detected by currently available serological tests. When the results of classic markers are combined with those of HBV DNA, the overall prevalence of HBV in alcoholics is > 60%.

Cirrhosis due to both alcohol and the hepatitis viruses is a common occurrence (21% HBsAg+ and 23%anti-HCV +) in KZN (Soni *et al*, 1996). Whilst the prevalence of anti-HCV is much lower in our local population (0.75% in African blood donors in Natal), this virus has a greater propensity to cause chronic hepatitis and cirrhosis as it is not efficiently cleared by the immune system (Soni *et al*, 1993). In KZN, HCV is prevalent in almost one quarter of patients

with cirrhosis (Soni *et al*, 1993). Using HCV PCR the prevalence of HCV in haemodialysis patients (4/70; 5.7%); in patients with chronic liver disease (6/98; 6.1%) and in alcoholic patients with (2/50; 4%) and without (1/35; 2.9%) liver disease is similar to that reported previously (4.8%) and confirms the low prevalence of HCV in KZN (Soni *et al*, 1993). Of the three ALD patients with abnormal ALT levels one was positive for HCV RNA, another was HBsAg-positive and GBV-C/HGV viraemic and the third had no other viral markers except anti-HBc.

Although an association between excessive alcohol intake and GBV-C/HGV infection has been reported in South Africa (Tucker *et al*, 1997), no studies have dealt with GBV-C/HGV positivity in alcoholic patients with liver disease in African countries. In limited studies GBV-C/HGV RNA positivity rates of 2 - 14% have been reported (Linnen *et al*, 1996a; Lampe *et al*, 1997; Sobue *et al*, 1998; Tran *et al*, 1998; Guilera *et al*, 1998; Shimanaka *et al*, 1999) in non-African countries. Tran *et al* (1998) reported that 38.8% (9.3% GBV-C/HGV RNA and 29.5% anti E2) of ALD patients had past or present infection with GBV-C/HGV. Most of the patients were heavy drinkers. In the present study the prevalence of GBV-C/HGV RNA was twice that in alcoholics with liver disease than in alcoholics who were abstaining (28% vs 14%, respectively) The reasons proposed for the higher prevalence of GBV-C/HGV may be similar to that for HCV, viz. that excessive alcohol consumption diminishes the immune function and that cellular immunity is impaired, but the precise mechanism remains obscure, even in the case of HCV.

In South African Blacks HBV has the greater propensity for establishing liver disease, because of its endemic nature. Although the prevalence of HCV infection is low, it is now accepted that both HBV and HCV are carcinogenic in humans. In South African Blacks with HBsAg the relative risk for HCC is 23.3% (Kew *et al*, 1997; 2001). The two viruses almost certainly interact in HCC, and each probably interacts with alcohol in inducing chronic hepatic parenchymal disease that in turn is complicated by malignant transformation (Kew *et al*, 1997; 2001). However,no association was reported between GBV-C/HGV and hepatocellular carcinoma in South Africans Blacks (Lightfoot *et al*, 1997).

Some studies have suggested that GBV-C/HGV infection did not affect the clinical characteristics and histological severity of liver injury (Tran *et al*, 1998; Sobue *et al*, 1998; Shimanaka *et al*, 1999). In the current study no significant association was observed between GBV-C/HGV and ALD.

Only a few studies have investigated the clinical consequences of GBV-C/HGV co – infection in HIV positive patients, and they confirm the absence of liver disease (Stark *et al*, 1999; Lau *et al*, 1999; Tillman *et a*l, 2001). Like Woolley *et al* (1998) no association was observed between GBV-C/HGV infection and haematological abnormalities in the current study.

Recently, researchers have suggested that GBV-C/HGV may be a favourable prognostic factor in HIV patients, delaying the progression of AIDS, resulting in prolonged survival (Heringlake *et al*, 1998; Toyoda *et al*, 1998; Lefrere *et al*, 1999; Yeo *et al*, 2000; Tillman *et al*, 2001; Xiang *et al*, 2001). Tillman *et al*, (20001), Yeo *et al* (2000) and Heriglake *et al* (1998) reported higher CD4 + lymphocyte counts and better AIDS-free survival rate in HIV patients infected with GBV-C/HGV Toyoda *et al* (1998) reported lower HIV viral load and AIDS incidence with the detection of GBV-C/HGV RNA in serum. Conversely, Sabin *et al* (1998) found an increased risk for AIDS and death with detection of GBV-C/HGV RNA or anti-E2 antibodies. The differences were not statistically significant in either of these studies. Lefrere *et al* (1999) noted significantly lower HIV viral load, higher CD4+ Lymphocyte count, and better AIDS-free survival in patients with GBV-C/HGV RNA than in those without GBV-C/HGV RNA or anti-E2 antibodies. In this dissertation no association was observed between GBV-C/HGV infection and "immune abnormalities" specifically, CD4 and CD8 counts, when the means of GBV-C/HGV positive and negative groups were compared. Wooley *et al*, (1998) and Goubau *et al*, (1999) showed similar results. In addition, Woolley *et al* (1998) reported a mild CD8 lymphocytosis associated with GBV-C/HGV infection. Some studies selected their patients based on CD4 counts (Lau *et al*, 1999; Yeo *et al*, 2000); like Goubau et al,(1999) there were no specific inclusion criteria for the study population in this dissertation apart from being HIV positive.

Reported for the first time, significantly higher CD3 and T cell receptor gamma/delta expression ($\gamma\delta$ TCR) and lower CD 30 status were observed in GBV-C/HGV positive

compared to GBV-C/HGV negative HIV positive patients. T lymphocytes recognise antigen via CD3/TCR complex (T cell receptor complex) which is expressed on mature T cells in the periphery. Less than 10% of human peripheral T cells express the γδ TCR complex. γδ T cells are often found in increasing numbers during the course of several viral infections in humans and they seem to provide natural immunity at the early stages of the second virus infection by having a significant impact on virus replication (Sciammas and Bluestone, 1999; Kabelitz and Wesch, 2001). GBV-C/HGV viraemia occurs in 35-40% of human HIV-infected individuals (Nerurkar *et al*, 1998). It is well established that GBV-C/HGV maintains a persistent viraemia for many years (Masuko *et al*, 1996), which suggests effective replication of GBV-C/HGV. Replication of GBV-C/HGV has been demonstrated in CD4 cells and the inhibitory effect of GBV-C/HGV infection on HIV replication leading to delayed disease progression was demonstrated *in vitro* recently (Xiang *et al*, 2001). Significant alterations in the peripheral blood γδ TCR repertoire occur in HIV infected individuals which is thought to be due to "superantigens / phosphoantigens" derived from pathogenic bacteria, parasites or viruses (Kabelitz and Wesch, 2001). Human γδ T cells possess potent cytotoxic activity and are capable of producing a large array β-chemokines and pro-inflammatory cytokines which are known to interfere with HIV replication (Kabelitz and Wesch, 2001). The relatively higher CD3 (due to an increase in T cell status) and an increase in γδT expression, together with a decrease in CD 30 (reflects a decrease in activation status and an increase in T helper Type 1 response) would tend to suggest an association with protection and or delayed progression of HIV disease in GBV-C/HGV infected patients. It is tempting to speculate that GBV-C/HGV

could indirectly affect AIDS risk through the expression of γδT cells; induction of various chemokines and other soluble factors; and altered expression of chemokine receptors essential for HIV co-receptors (Woitas *et al*, 2001). CD30 marker is expressed on all T-cell lineages; CD3+ T cells can be separated into two lymphocyte subsets bearing hetero-dimeric TCR composed of either alphabeta (αβ) or gammadelta (γδ) chains. While the role of TCR αβ cells is well understood, that of γδ is recently being unraveled. In part they function by modulating CD4+αβ TCR responses (especially in the gut and lung, and in response to viral infection). CD30 is a member of the TNF receptor family, (includingTNF-R1, TNF-R2, Fas-R, CD40, CD27, and TRAIL-R) [Bengtsson, 2001]. Members have pleiotropic effects depending on the context of activation, causing proliferation of activated T cells and T-cell-like Hodgkin cell lines but growth inhibition in Ki-1 lymphoma cell lines [Bengtsson, 2001]. There is evidence that CD30 is involved in negative selection of thymocytes as well as antigen withdrawal-induced apoptosis [Bengtsson, 2001]. CD30 expression in normal tissue is limited to activated T cells, activated B cells, and some vascular beds. It is highly expressed in Hodgkin disease, and some cutaneous lymphomas [Bengtsson, 2001]. There are some anti-CD30 antibody-based therapies for carcinoma. In terms of limiting a viral response, CD30 seems to act as an 'immune-brake', as knock-out animals do not clear autoreactive thymocytes [Bengtsson, 2001]." GBV-C/HGV is a Flavivirus with some homology (25%) to HCV, and often the two viruses co-exist. AIDS patients infected with HBV or HCV often die from liver failure instead of AIDS (Ockenga *et al*, 1998).

It has been suggested that HCV may reduce HBV replication (Zarski *et al*, 1998). If GBV-C/HGV has a core protein like HCV, as has been suggested (Xiang *et al*, 1998), it may interfere with HIV replication, leading to a favorable prognosis in HIV infected patients. Co-infection with HCV has been associated with faster progression to AIDS and higher mortality rates (Mohsen *et al*, 2002). In co-infected mothers, there is evidence to suggest that mother to child transmission of HCV is facilitated by HIV infection (Moshen *et al*, 2002), which may be associated with higher HCV viral load, mode of delivery and perhaps breastfeeding (Moshen *et al*, 2002). However, few studies have addressed the impact of HCV on HIV transmission in co-infected mothers. The host immune response and the repertoire of lymphocytes expressed to HCV antigen is thought to determine whether viral clearance or chronicity occurs. Recent studies have high lighted the essential roles of CD 30, NK cells, γδT cells and NKR^{+} T cells in immunity in hepatotropic viruses in immunopathology (Fattovich *et al*, 1996; Woitas *et al*, 1997; Kakimi *et al*, 2000; Liu *et al*, 2000; Tseng *et al*, 2002). Whether, the above phenomenon is characteristic of GBV-C/HGV is unknown at this stage.

Recently, Mosier and Chisari (2002) suggested that the cytotoxic T lymphocyte response to GBV-C /HGV co-infection may be responsible for the delayed progression of HIV. In addition, Nunnari *et al* (2002) reported that T-helper-1 (Th1) cytokine (IL-2 and IL-12) levels were significantly higher in HIV positive patients co-infected with GBV-C/HGV, while a predominant T-helper-2 (Th2) pattern was found in the GBV-C/HGV negative group.

Nunnari *et al* (2002) has suggested that GBV-C/HGV could immunologically interfere with HIV progression to AIDS by maintaining an intact T-helper 1 cytokine profile. Although cytokine profiles were not measured in the current study, the data supports the findings of Mosier and Chisari (2002).

In the absence of histochemical studies of liver biopsies to detect GBV-C/HGV, the higher prevalence of GBV-C/HGV (in blood donors, HIV positive patients and patients with chronic liver disease), the lack of elevated liver enzyme values as well as clinical hepatitis in blood donors and haemodialysis patients would tend to suggest that GBV-C/HGV is not associated with liver disease. It is obscure whether alcohol intake plays a role in the development of liver injury in patients with GBV-C/HGV infection. In KZN HBV and not HCV or GBV-C/HGV is associated with alcoholic liver disease. This has important public health implications that could influence national policies on vaccination against HBV in this endemic area. Even though GBV-C/HGV infection is common in HIV patients in KZN, it is not associated with an identifiable clinical syndrome. There is evidence for a beneficial influence of GBV-C/HGV infection in HIV positive patients. To further understand and determine the impact of GBV-C/HGV infection on the natural course of HIV infection in KZN, South Africa, the observed phenomena will require more detailed *in vivo* and *in vitro* longitudinal studies Until then, GBV-C/HGV is a virus still looking for a disease (Sathar *et al*, 2000).

CHAPTER 4

CHAPTER 4

PHYLOGENETIC ANALYSIS OF GBV-C/HGV ISOLATES FROM KWAZULU NATAL, SOUTH AFRICA

4.1 INTRODUCTION

A high prevalence of GBV-C/HGV has been documented in KZN (Sathar *et al*, 1999a), however, very little is known about the strains of GBV-C/HGV that are infecting or circulating in the local population. The best approach is to analyse the genetics of each strain. Sequence and phylogenetic analysis of complete genome is expensive and labour intensive. HCV genotypes can be distinguished by phylogenetic analysis of a variety of subgenomic regions as small as 200 nucleotide (nt) that reproduce the phylogenetic relationships of complete genomes (Simmonds *et al*, 1994). It was anticipated that studies of GBV-C/HGV from different geographical regions of the world might reveal the existence of phylogenetic groups (i.e. genotypes) and that different groups/genotypes may display specific biological properties and clinical manifestations similar to that found with distinct HCV genotypes. Attempts to genotype GBV-C/HGV based on sequence analysis of the NS3 (Kao *et al*, 1996; Pickering *et al*, 1997) and NS5A (Viazov *et al*, 1997a) genomic regions have been unsuccessful. Systematic analysis of six complete GBV-C/HGV genome sequences revealed that congruent phylogenetic relationships were obtained for only a minority of 300, 600 and 1200 nucleotide (nt) fragments, and that the optimal region was all or part of the of 5′non-

coding region (5′ NCR) (Muerhoff *et al*, 1997; Smith *et al*, 1997a). Sequence analysis of the 5′ NCR provided some evidence that GBV-C/HGV can be separated into three major groups that correlate with the geographic origin of the isolates (Muerhoff *et al*, 1996; 1997; Smith *et al*, 1997a). In addition, sequence and phylogenetic analysis of the 5′ NCR was considered to be predictive of the sequence analysis of the complete genome (Muerhoff *et al*, 1996; 1997; Smith *et al*, 1997a).

At present 33 epidemiologically unrelated GBV-C/HGV complete virus genome sequences are available in the Genbank database. The recent discovery of closely related chimpanzee viruses (Adams *et al*, 1998) and the availability of a complete genome sequence (GBV-C_{tro}) (Birkenmeyer *et al*, 1998) allows phylogenetic trees to be constructed using a more appropriate outgroup. Based on the assumption that sequence and phylogenetic analysis of the 5′ NCR was predictive of the sequence analysis of the complete genome (Muerhoff *et al*, 1996; 1997; Smith *et al*, 1997a), the phylogenetic relationship and the nucleotide sequences of the 5′ NCR of GBV-C/HGV isolates from KZN were compared with published sequences of GBV-C/HGV isolates from other geographical regions of the world. In addition, a re-analysis of the phylogenetic relationships of GBV-C/HGV complete genome sequences was undertaken to determine the extent to which these can be reproduced by analysis of subgenomic regions.

4.2 METHODS

4.2.1. Source of samples

Sera from individuals who were previously shown to be infected with GBV-C/HGV were used as a source of viral RNA [Chapters 2.2.1 and 3.2.1] (Sathar *et al*, 1999a). They included 17/70 (23%) patients with chronic renal failure who were undergoing maintenance haemodialysis, 12/98 (12.2%) consecutive patients with chronic liver disease, 44/232 (18.9%) adult blood donors from the four racial groups (Africans, Indians, Whites and "Coloureds" [individuals of mixed origin] and 22/40 (55%) African female patients attending antenatal clinics in the eight geographical health regions of KZN (Fig.4.1).

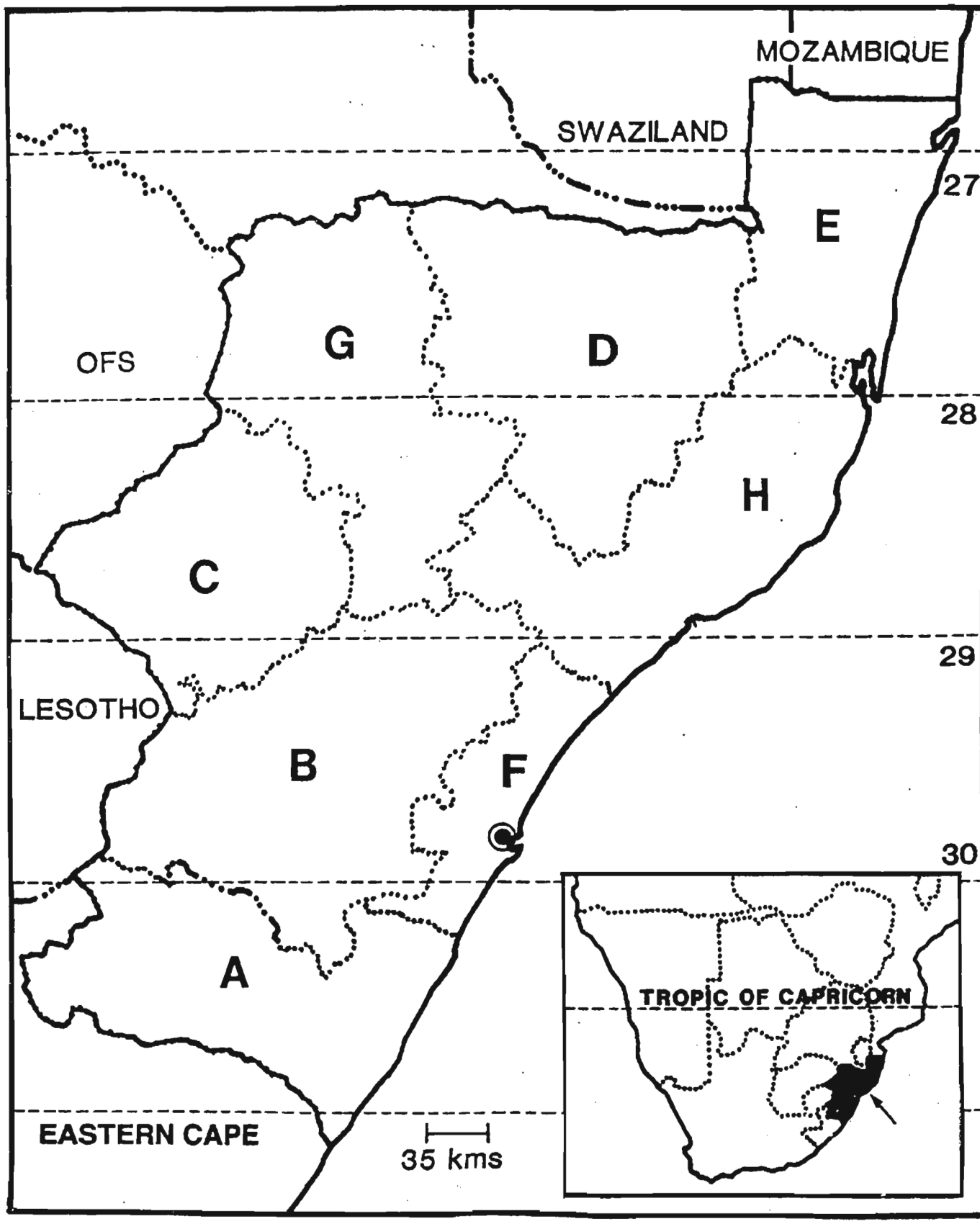


Fig 4.1 Map of KwaZulu Natal (KZN) relative to South Africa (insert) showing the location of the eight health regions (A-H)

4.2.2 Purification of the PCR product

The RT-PCR used to detect GBV-C/HGV RNA has been described in detail in Chapter 2.2.2-2.2.3. The 344 bp PCR product generated by RT-PCR was purified using the High Pure PCR Product Purification Kit (Boehringer Mannheim, RSA) according to the manufacturer's instruction with slight modifications. Precautions were taken at every step to prevent contamination. Two hundred microliters (200ul) of binding buffer (3M guanidine-thiocynate, 10mM Tris-HCl, 5% ethanol (v/v), pH 6.6) was added to 40 ul of the PCR product and vortexed to mix. The total volume (240ul) was pipetted into the High Pure filter tube without touching the rim. The tube was centrifuged for 30 seconds (sec) at 14 000 rpm in a micro-centrifuge (Eppendorf Microcentrifuge Model 5415C, Merck, RSA). The flow through was discarded and the filtered tube transferred to the collection tube. Three hundred (300ul) microliters of wash buffer (20mM NaCl and 2mM Tris-HCl pH 7.5) was pipetted into the upper reservoir and centrifuged for 30sec at 14 000 rpm. The wash buffer flow through was discarded and the filter tube transferred to the collection tube. The previous step was repeated with 200ul of wash buffer. The collection with the filtrate was discarded and the filter tube transferred to a sterile 1.5ml microcentrifuge tube. Forty microliters (40ul) of elution buffer (10mM Tris-HCl and 1Mm EDTA, pH 8.5) was added directly over the membrane and the tube centrifuged for 30 sec at 14 000 rpm. The filter tube was discarded and the filtrate containing the DNA was used in the sequencing reaction.

4.2.3. Sequencing reaction

4.2.3.1 ABI sequencing

Both strands of the 344 bp PCR product were sequenced by Direct cycle sequencing using the Perkin Elmer dRhodamine Terminator Cycle Sequencing Kit (Perkin Elmer, Biosystems, RSA). Briefly, for each reaction, 8.0ul of terminator ready reaction mix, 90 ng of PCR product (DNA), 1.3ul (3.2 pmol) of either reverse or forward primer in a total volume of 20 ul of sterile deionised water was added to a 0.2 ml PCR tube. The reagents were mixed, briefly spun, and cycle sequenced in the Perkin Elmer GeneAmp 2400 thermal cycler (Perkin Elmer, Biosystems, and RSA) at 96°C for 10 sec, 50°C for 5 sec, 60°C for 4 minutes (min) for 25 cycles followed by a 4°C soak. The tubes were spun briefly. The extension product was purified from incorporated nucleotides by precipitation. The entire contents of each extension reaction was transferred to a sterile 1.5ml microfuge tube. To which was added 16ul of sterile deionised water and 64ul of 95% ethanol. The contents of the tube were briefly vortexed. The tubes were allowed to stand for 10-15 min to precipitate the extension products and microfuged for 15 min. The supernatants were carefully aspirated off without disturbing the "pellet." The "pellets" were then washed with 70% ethanol (100ul) and microfuged for 10 min. The supernatant was carefully aspirated off without disturbing the "pellet." The samples were dried under vacuum for 10-15 min. Twenty-five microliters (25ul) of template suppression reagent was added to the tube and mixed well. The tubes were heated for 3 min at 95°C and thereafter held on ice.

4.2.3.2 Amersham sequencing

The amplification of the E2 gene of KZN GBV-C/HGV isolates and its subsequent sequencing were performed by Dr Smith and his colleagues (Smith *et al*, 2000) and are not described in any detail in the dissertation. The E2 gene was sequenced using the Thermosequenase Kit (Amersham, UK) in reactions containing ^{33}P-labelled dideoxynucleotides.

4.2.4. Sequence analysis of 5′ NCR

4.2.4.1 GBV-C/HGV isolates from blood donors and patients

The sequences of GBV-C/HGV isolates from Blood Donors and patients (chronic liver disease and haemodialysis) were determined using an Applied Biosystems Prism 310 Automated Genetic Analyser (Perkin Elmer, Biosystems, RSA). The sequences were analysed using the Sequence Navigator Software (V.1.0.1). (Perkin Elmer, Biosystems, RSA). Nucleotide sequences were aligned manually using the Simmonic - 2000 sequence editor package supplied by Dr Simmonds of the University of Edinburgh (P. Simmonds, unpublished).

4.2.4.2 GBV-C/HGV isolates from the eight geographical health regions of KZN

Nucleotide sequences of GBV-C/HGV isolates from the eight geographical health regions of KZN were assembled and analyzed using the Staden Package (http://www.mrc.lmb.ac.uk/pubseq/). Sequences were aligned with 28 unrelated sequences from each of the four major groups (Smith *et al*, 2000) using Genetic Data Environment Software (GDE) and Clustal W programmes (Higgins *et al*, 1992) with minor visual adjustments.

4.2.5. Sequence analysis of complete genome sequences of GBV-C/HGV isolates

Nucleotide sequences of 33 epidemiologically unrelated complete genome sequences were obtained from Genbank and manipulated and aligned using Simmonic-2000 software (P. Simmonds, unpublished) by Dr Smith and his colleagues (Smith *et al*, 2000). Nucleotide positions of aligned sequences were numbered relative to the AUG codon at the beginning of the E1 gene of the prototype isolate GBV-C (U36380). The 33 epidemiologically unrelated complete genome sequences were AB003288-93, AB013500, AF104403, AB013501, AF031829, D87255, D90600-1, U44402, U45966, U63715, AB008342, AF006500, D87263, D87708-15, U75356, U94695, AB018667 and AB021287 together with the outgroup AF070476. The partial sequence (positions -396 to 6118) of an additional isolate from Thailand (K-10) was made available by Sirirrug Songsivilai to Dr Smith (Smith *et al*, 2000).

4.2.6 Phylogenetic analysis of 5′ NCR

4.2.6.1 GBV-C/HGV isolates from blood donors and patients

Phylogenetic analysis of GBV-C/HGV sequences from Blood donors, haemodialysis and patients with chronic liver disease were carried out on a 311 base pair fragment in the 5′ NCR region (positions 160-470 in the HGV type 2a clone, PNF2161 (Muerhoff *et al*, 1997). Sequences were compared using a distance based method (p-distance at all sites followed by neighbor joining) as implemented on the Molecular Evolutionary Genetic Analysis (MEGA) package (Kumar *et al*, 1993). Robustness of grouping was assessed by bootstrap re-sampling; numbers on branches indicate the percentage of 500 bootstrap replicates that supported the observed pylogeny (restricted to values of 75% or greater). P - distances are indicated on the scale bar. Sequences compared included those of designated genotype for which complete genomic sequences were available at the time (Genbank/EMBL accession numbers in parentheses). These include the Group1 sequences, GBV-C (U36380) and CG12LC (AB003291) which corresponds to variants recently found in Central African Pygmy populations, which are characterised by an insertion in the NS5 region (Tanaka *et al*, 1998); Group 2a sequences, PNF2161 (U44402) and R10291 (U45966); Group 2b sequence GBV-C (EA) (U63715); Group 3 sequences GT230 (D90601) and GSI85 (D87262). The following sequences of unclassified genotypes were also included: HGVC-964 (U75356) from China and G05BD (AB003292) from Japan.

4.2.6.2 GBV-C/HGV isolates from the eight geographical health regions of KZN

Phylogenetic analysis of GBV-C/HGV sequences of isolates from the eight geographical health regions of KZN was carried out on a 311 base pair fragment in the 5′ NCR region (positions 143 – 442) in the GBV-C prototype isolate (U36380). The reliability of grouping was assessed by bootstrap analysis using 500 repeats. Phylogenetic analysis was performed using the Maximum likelihood method contained in the Phylogeny Inference Package (PHYLIP) version 3.5c (Felsentein, 1993) and the F-84 model of substitution (Tamura and Nei, 1993) in the Puzzle program (Strimmer and von Haeseler, 1999).The final phylogenetic trees were visualized using the TREEVIEW program VERSION 1,5 (IBLS, University of Glasgow). Sequences of the 5′ NCR of KZN isolates were compared with sequences of 28 geographically distinct isolates whose full genomic sequences are available at the time in the Genbank. The 28 unrelated sequences were (Genbank/EMBL accession numbers in parentheses): U36380, AB003291, AB013500 (Group 1); AF104403, AF031829, AB003289, D87255, D90600, U44402, U45966 and U63715 (Group 2); AB003288, AB003293, AB008342, AF006500, D87708 –15, D90601 and U94695 (Group 3); AB003292, AB018667 and AB021287 (Group 4); and the outgroup GBV-C_{tro} (AF070476). Included in the analysis were 13 sequences of GBV-C/HGV isolates from the western and eastern Cape provinces, South Africa (AF13111 – AF13123) (Tucker *et al*, 1999).

4.2.7 Phylogenetic analysis of the complete genome sequences of GBV-C/HGV isolates

Complete coding region sequences of the 33 complete genome sequences were analysed by Dr Smith and his colleagues (Smith *et al*, 2000) and are not described in detail in the dissertation.

4.3 RESULTS

4.3.1 Sequence analysis of 5′ NCR of GBV-C/HGV isolates from KZN

Fifty-four (54) of 73 (74%) GBV-C/HGV isolates from blood donors and patients and 18 of 22 (82%) isolates from the eight geographical health regions of KZN were considered suitable for sequence analysis of the 5′ NCR of the viral genome (Appendices D and E). The remainder giving rise to either faint bands or poor nucleotide sequences. The 5′ NCR sequences of the 54 GBV-C/HGV isolates from blood donors (Fig 4.2 a-c) and 18 isolates from the eight geographical health regions (Appendix F) of KZN were aligned for phylogenetic analysis with representative isolates from each group.

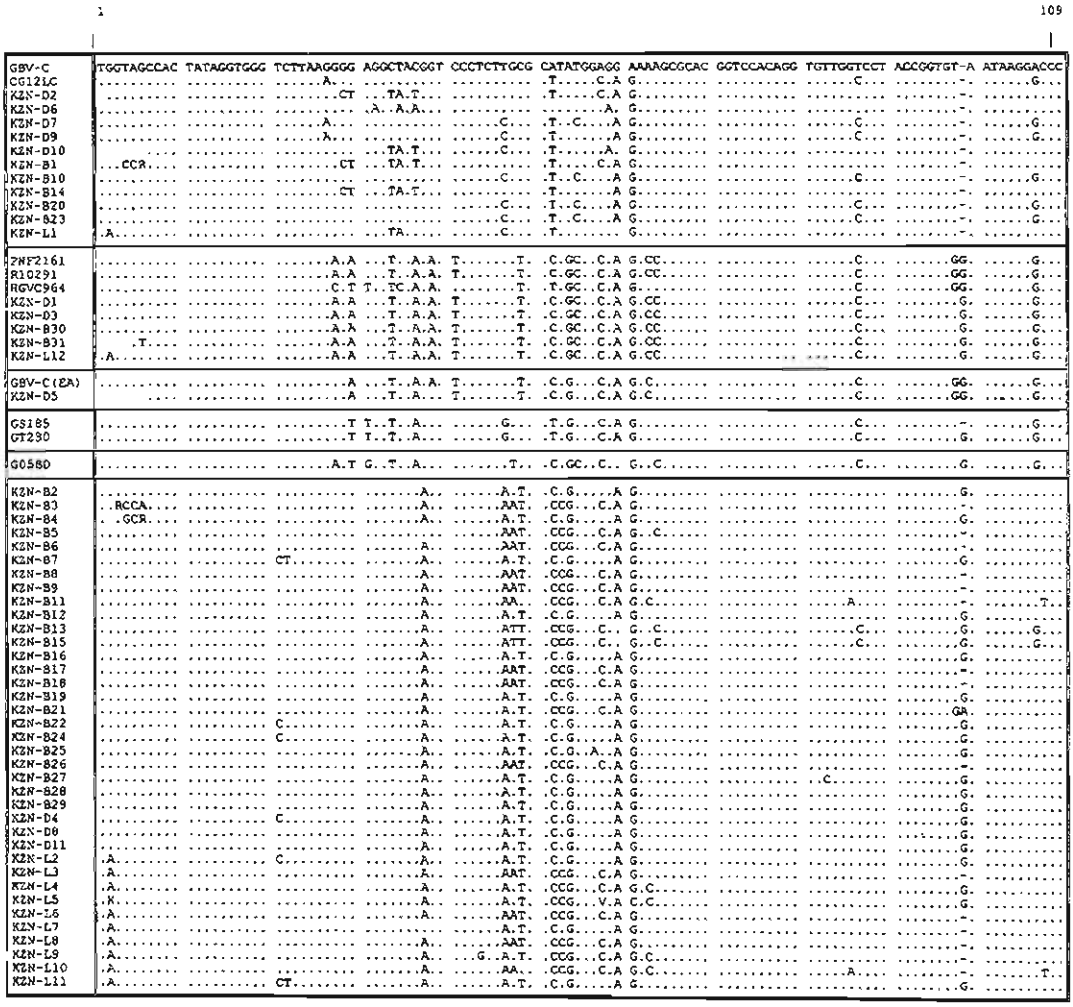


Fig.4.2a Alignment of 54 nucleotide sequences from the 5′ NCR of GBV-C/HGV isolates blood donors (B), haemodialysis (D) and patients with chronic liver disease (L) from the province of KZN (KwaZulu Natal), South Africa. Sequences were compared with GBV-C/HGV or representative variants of GBV-C/HGV (sources listed in Chapter 4.1.5). Symbols: '.', similar nucleotide to GBV-C; '', nucleotide not determined; and '-', gap introduced to preserve alignment (Sathar *et al*, 1999b)

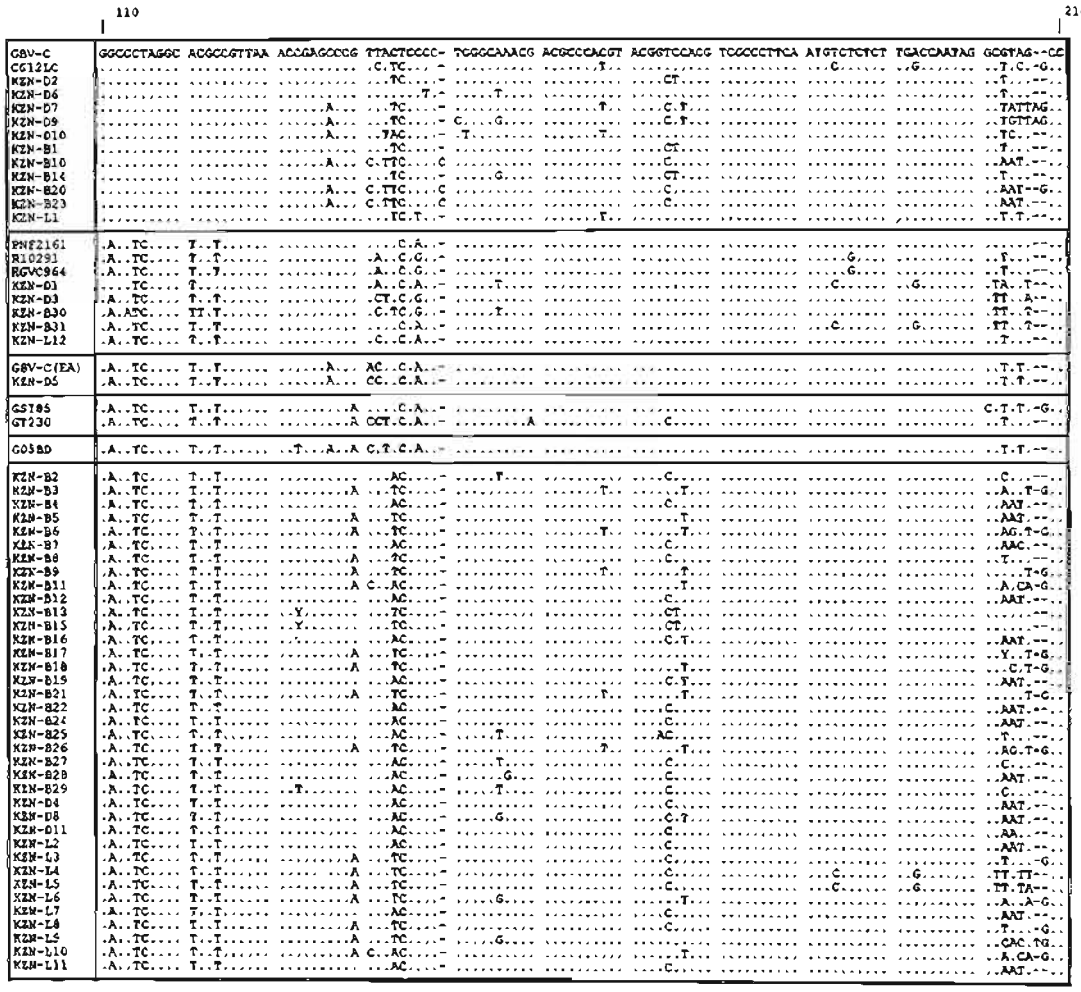


Fig.4.2b Alignment of 54 nucleotide sequences from the 5′ NCR of GBV-C/HGV isolates from blood donors (B), haemodialysis (D) and patients with chronic liver disease (L) from the province of KZN (KwaZulu Natal), South Africa. Sequences were compared with GBV-C/HGV or representative variants of GBV-C/HGV (sources listed in Chapter 4.1.5). Symbols: '.', similar nucleotide to GBV-C; ", nucleotide not determined; and '-', gap introduced to preserve alignment (Sathar *et al*, 1999b)

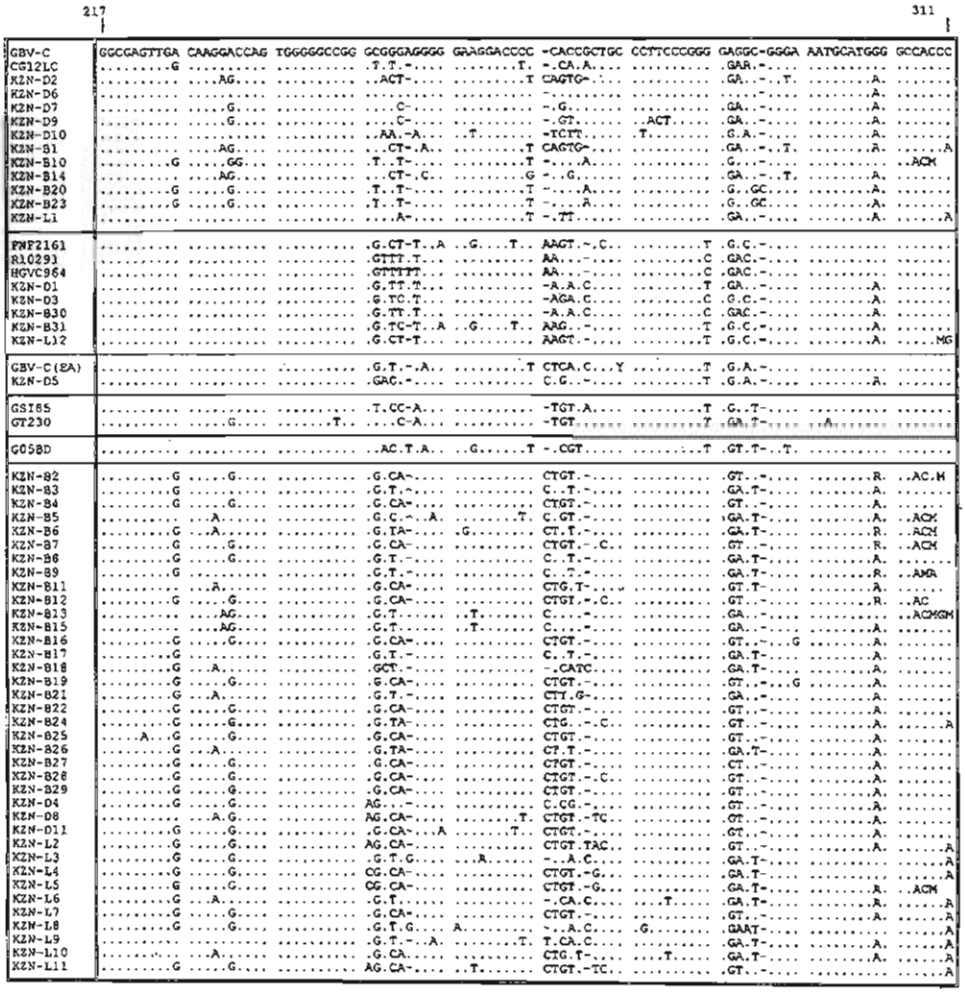


Fig.4.2c Alignment of 54 nucleotide sequences from the 5′ NCR of GBV-C/HGV isolates from blood donors (B), haemodialysis (D) and patients with chronic liver disease (L) from the province of KZN Kwa Zulu Natal), South Africa. Sequences were compared with GBV-C/HGV or representative variants of GBV-C/HGV (sources listed in Chapter 4.1.5). Symbols: '.', similar nucleotide to GBV-C; '', nucleotide not determined; and '-', gap introduced to preserve alignment (Sathar *et al*, 1999b)

4.3.2. Phylogenetic analysis of 5′ NCR

4.3.2.1 GBV-C/HGV isolates from blood donors and patients

The phylogenetic tree comparing sequences from the 5′ NCR of the 54 isolates (31 blood donors [KZN B1-31]; 11 haemodialysis patients [KZN D1-11] and 12 patients with chronic liver disease [KZN 1-12]) with isolates from other geographic regions is shown in Fig.4. 3. Using either the full sequence, or a portion excluding regions that are variable but do not correlate with the geographical origin of the isolates (Smith *et al*, 1997a). GBV-C/HGV isolates segregated into 3 distinct phyologenetic groups (Groups 1,2 and a New group) [Fig. 4.3].

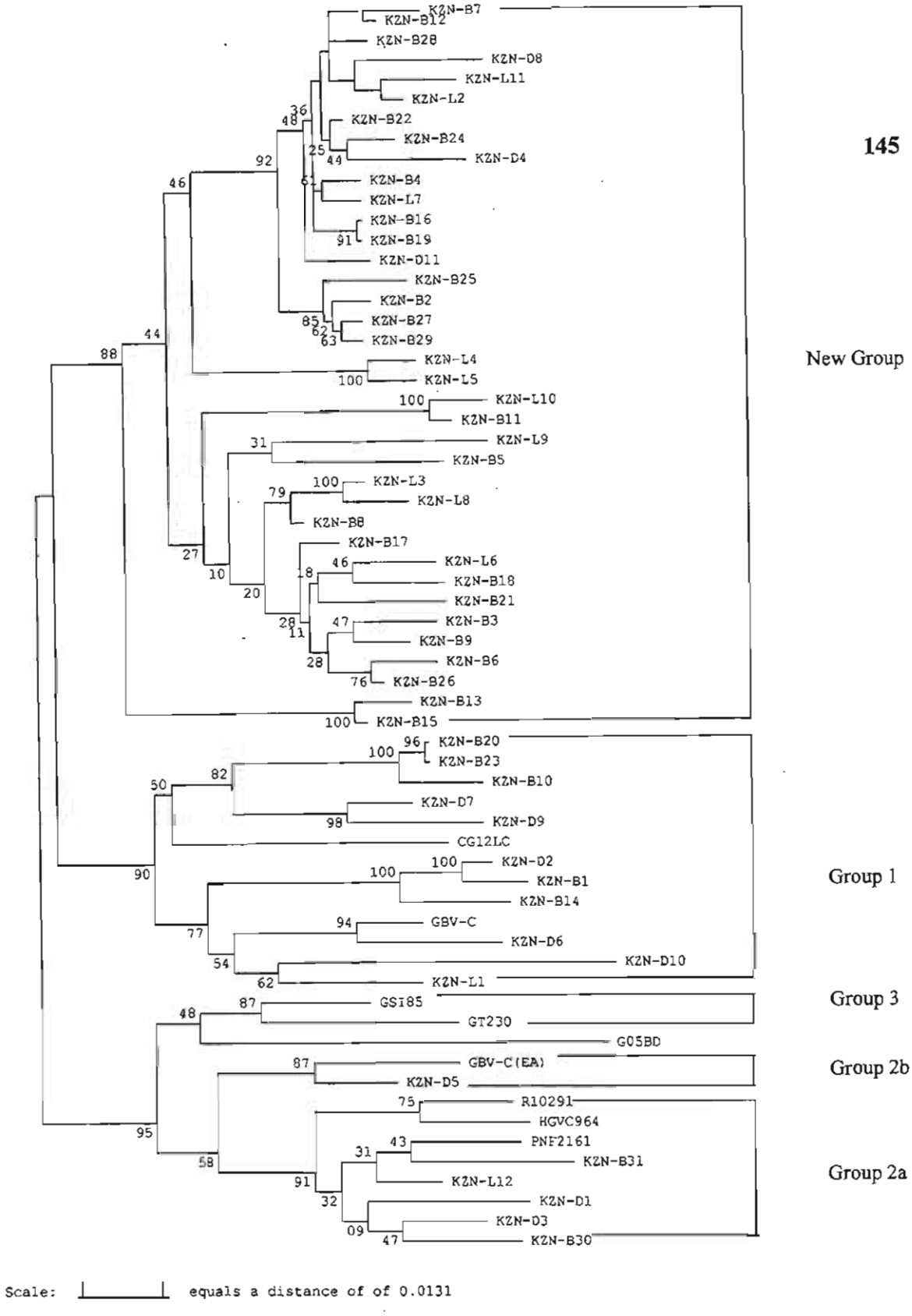


Fig. 4.3 Phylogenetic tree of GBV-C/HGV 5′ NCR sequences from blood donors and patients. A neighbour joining tree was constructed from p-distances between representative published nucleotide sequences (GenBank/EMBL; accession numbers in parentheses) and from KZN isolates (KZN-KwaZulu Natal, South Africa; KZN D1-D11 for haemodialysis patients, KZN L1-L12 for CLD patients, KZN B1-B31 for blood donors). Bootstrap values greater than 75% are indicated (500 replicates) (Sathar *et al.* 1999b).

The majority (37/54) of GBV-C/HGV sequences formed a cluster which was distinct from all other known GBV-C/HGV sequences, supported by 88% of bootstrap replicates (Fig 4.3). Eleven (11) isolates clustered as group 1, with a bootstrap support of 90% (Fig 4.3). Six additional isolates clustered as group 2 variants, 5 of which grouped with 2a (bootstrap support of 91%) and one (KZN D5) with 2b (bootstrap support of 87%) (Fig 4.3). Although this analysis did not strongly support the groupings of group 2a and 2b sequences (58% of replicates), this analysis included the group 3 isolate HGVC 964 (Fig.4.3) which has previously been shown to cluster with group 2 isolates upon phylogenetic analysis of the 5′ NCR (Muerhoff *et al*, 1996; Smith *et al*, 1996). Analysis of nucleotides 1-201 in this alignment but excluding HGVC 964 produced very similar results except that the bootstrap support for group 2 isolates was increased to 75% (Fig.4.4). Sequences in groups 2 and 3 together with that of isolate GO5BD grouped separately from sequences of group 1 and the new KZN sequences (bootstrap support 90%) (Fig.4.4). None of the KZN isolates clustered with group 3 isolates described from South-East Asia.

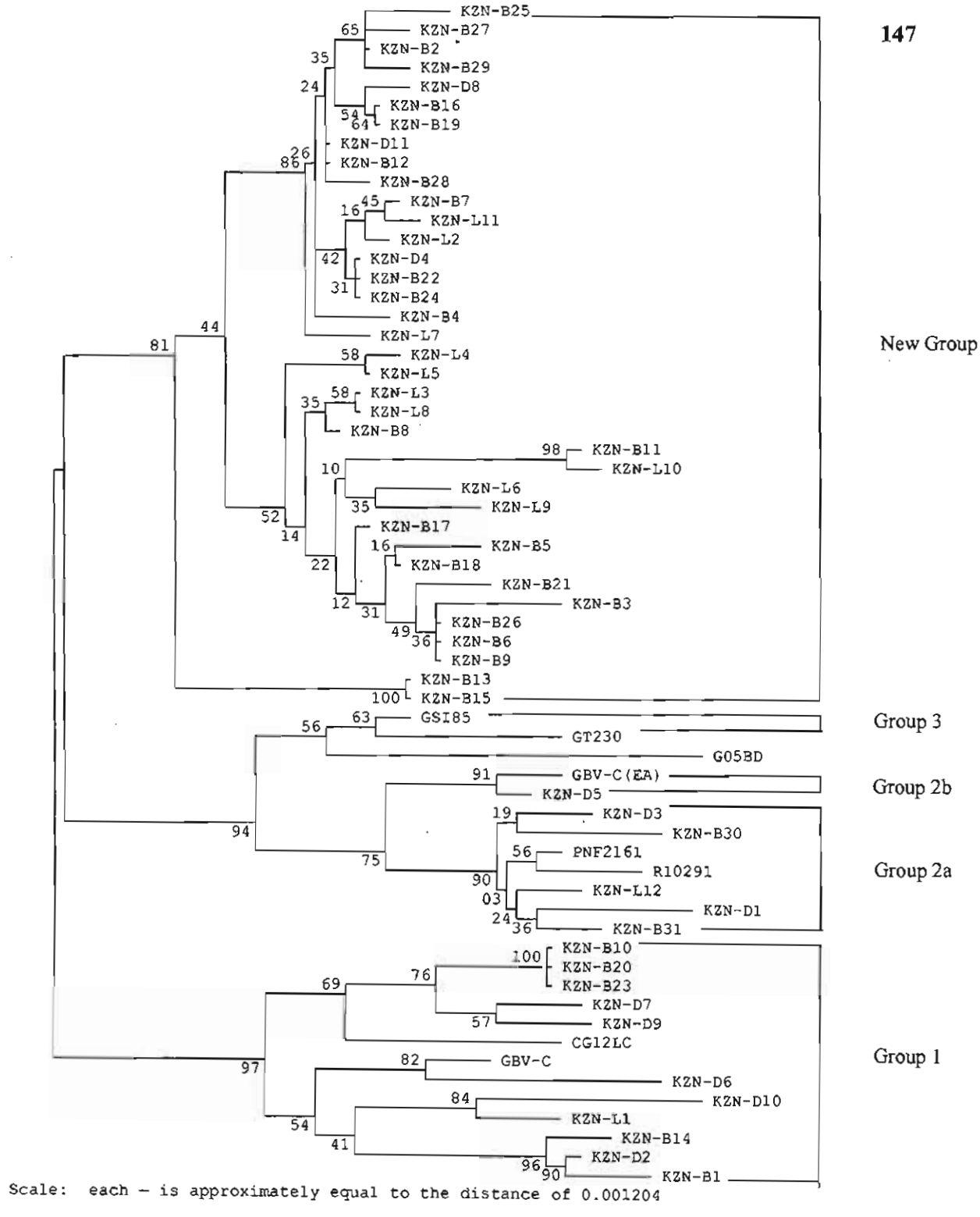


Fig. 4.4 Phylogenetic tree of GBV-C/HGV 5′ NCR sequences from blood donors and patients (shortened region [J-C 100 replicates]) excluding HGVC964. A neighbour joining tree was constructed from p-distances between representative published nucleotide sequences (GenBank/EMBL; accession numbers in parentheses) and from KZN isolates (KZN-KwaZulu Natal, South Africa; KZN D1-D11 for haemodialysis patients, KZN L1-L12 for CLD patients, KZN B1-B31 for blood donors). Bootstrap values greater than 75% are indicated .

There was a high degree of overlap in the ranges of distances between and within groups (Table 4.1). GBV-C/HGV infects all racial groups in the province of KZN, South Africa (Table 4.2). The new variant of GBV-C/HGV seems to be more prevalent amongst Africans than amongst Indians, Whites or 'Coloureds' (Table 4.2).

Table 4.1 The pairwise distances for the full length of the sequences of GBV-/HGV Groups 1-4 and the new group (mean [range])

	1	2	3	4	New Group ⊗
1	0.082 (0-0.119)				
2	0.138 (0.112-0.167)	0.059 (0.029-0.088)			
3	0.12 (0.106-0.138)	0.099 (0.073-0.122)	0.045 *		
4	0.14 (0.132-0.158)	0.114 (0.089-0.135)	0.092 •	(=G05BD) #	
5	0.116 (0.084-0.162)	0.122 (0.092-0.151)	0.109 (0.09-0.132)	0.139 (0.122-0.154)	0.059[0-0.113]

* (only 1 comparison); • only 2 comparisons);) # only 1 sequence; ⊗ KZN, South African

Table 4.2 GBV-C/HGV infection according to phylogenetic and race groups

Race	GBV-C/HGV Groups					
	1	**2a**	**2b**	**3**	**New**	**Total no.**
Africans	7	2	-	-	32	41
Indians	2	2	1	-	-	5
Whites	1	-	-	-	5	6
"Coloureds"	1	1	-	-	-	2

"Coloureds": persons of mixed origin

4.3.2.2 GBV-C/HGV isolates from the eight geographical regions of KZN

Phylogenetic analysis of the 5′ NCR sequences of 18 GBV-C/HGV isolates from the 8 health regions of KZN together with the 5'NCR sequences of 28 GBV-C/HGV isolates whose complete genomic sequences were available in Genbank database yielded evidence of five groups (Fig.4.5). Group 1 (bootstrap support 86%), Group 2 (bootstrap support 98%), Group 3 (bootstrap support 83%), Group 4 (71%) and a fifth group (Group 5) (94%) (Fig.4.5). The classification of Groups 3 and 4 as separate groups is based on the analysis of complete genomic sequences (Smith *et al*, 2000). The majority 17/18 (94%) of GBV-C/HGV sequences from the province of KZN clustered as a fifth group (Group 5) which was distinct from all other known GBV-C/HGV isolates (Fig.4.5). Only one isolate (KZN-E11) from Zone E

clustered with group 1 African variants (bootstrap support, 84%) (Fig.4.6). Excluding the Cape isolates with major deletions from the current phylogenetic analysis, improved not only the bootstrap values of the tree but clustered the majority of the GBV-C/HGV isolates from the Cape provinces with the KZN isolates as the fifth group (Fig.4.5). None of the South African isolates grouped with Group 3 and 4 variants (Fig. 4.5).

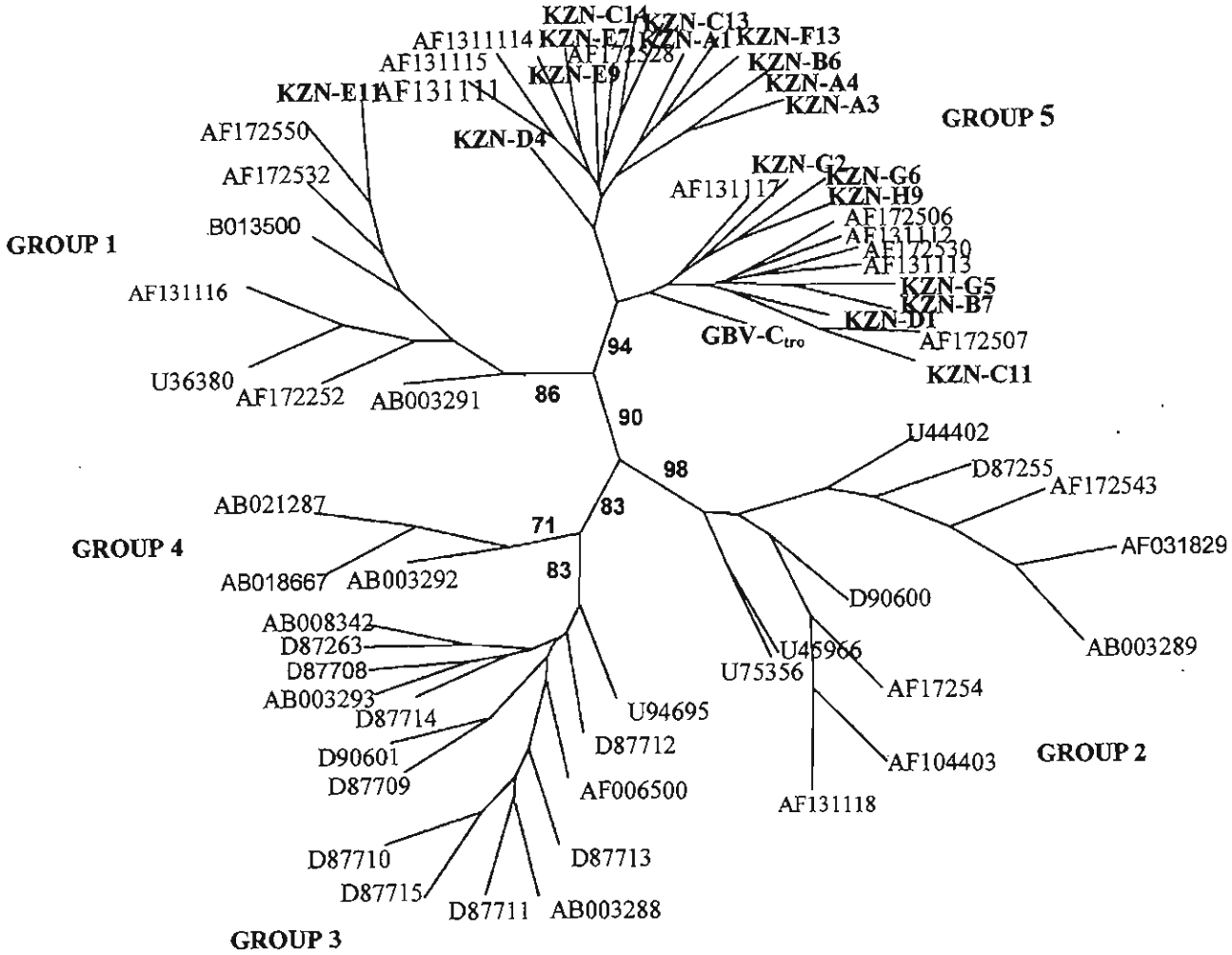


Fig. 4.5 Consensus phylogenetic tree of 5′ NCR sequences of GBV-C/HGV isolates from 8 geographical regions of KZN, South Africa and representative published sequences from Genbank/EMBL. Phylogenetic analysis was carried out on a 311bp fragment, positions 143-442 in the GBV-C prototype isolate (U36380). Maximum likelihood distances between sequences were calculated using Phylip DNADIST (ts:tv = 3, assuming no rate variation between sites), and used to produce a neighbour joining tree using Phylip NEIGHBOR. Bootstrap values (100 replicates) were obtained with the SEQBOOT and CONSENSE options of Phylip. The tree was produced using Treeview version 1.5. (KZN = KwaZulu Natal; A-H = 8 geographical health regions of KwaZulu Natal) [Sathar and York, 2001]

4.3.3 Phylogenetic analysis of the complete genome sequences of GBV-C/HGV isolates

Phylogenetic analysis of the coding region of the 33 epidemiologically unrelated complete GBV-C/HGV genome sequences yielded evidence for four distinct phylogenetic groupings each of which was supported by high levels of bootstrap support [74-100%] (Fig. 4.6). Three group 3 isolates from China (U75356, U94695 and AF006500) grouped together with high bootstrap support, while the isolates AB013501 and U63715 grouped separately from the other group 2 isolates (group 2a) and isolate AB003292 from Japan was relatively divergent from other group 4 isolates (Fig.4.6).

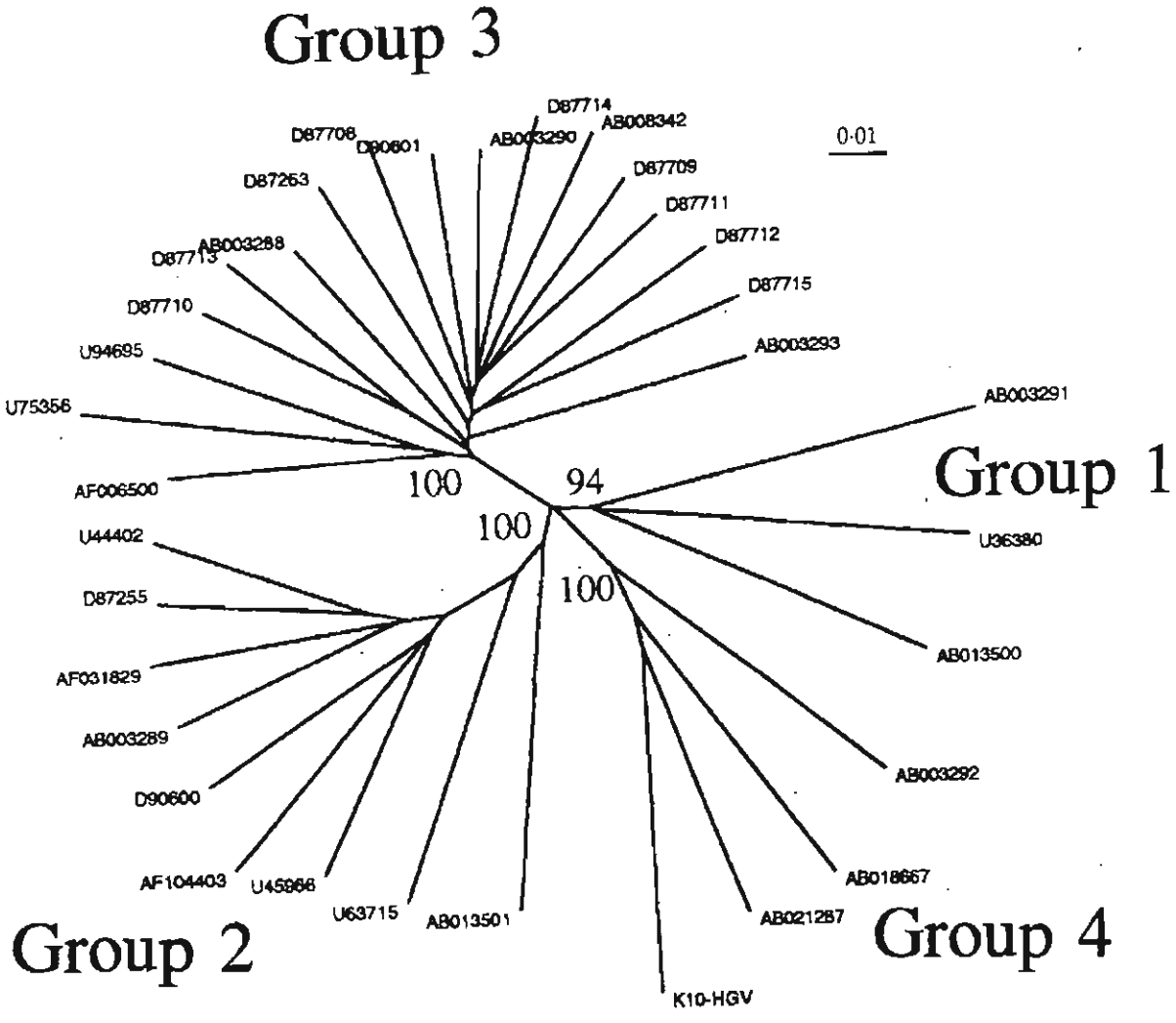


Fig. 4.6 Phylogenetic tree of GBV-C/HGV complete coding sequences. Maximum likelihood distances between sequences were calculated with Phylip DNADIST(ts:tv = 2, assuming no rate variation between sites), and used to produce a neighbour joining tree with Phylip NEIGHBOUR. Bootstrap Values (100 replicates) were obtained with the SEQBOOT and CONSENSE options of Phylip. The tree was produced using Treeview version (1.5) [Smith *et al*, 2000].

4.3.4 Phylogenetic analysis of the E2 gene of GBV-C/HGV isolates

Analysis of individual viral genes failed to produce congruent phylogenetic trees with the sole exception of the E2 gene (Fig. 4.7). Similar analysis of subgenomic fragments of 2000, 1000 or 500 nt produced congruent trees only for fragments including the COOH-terminal region of the E2 gene (Fig.4.7). Analysis of the entire 3'-terminal half of the virus genome or any of its subfragments failed to produce congruent trees (Fig.4.7).

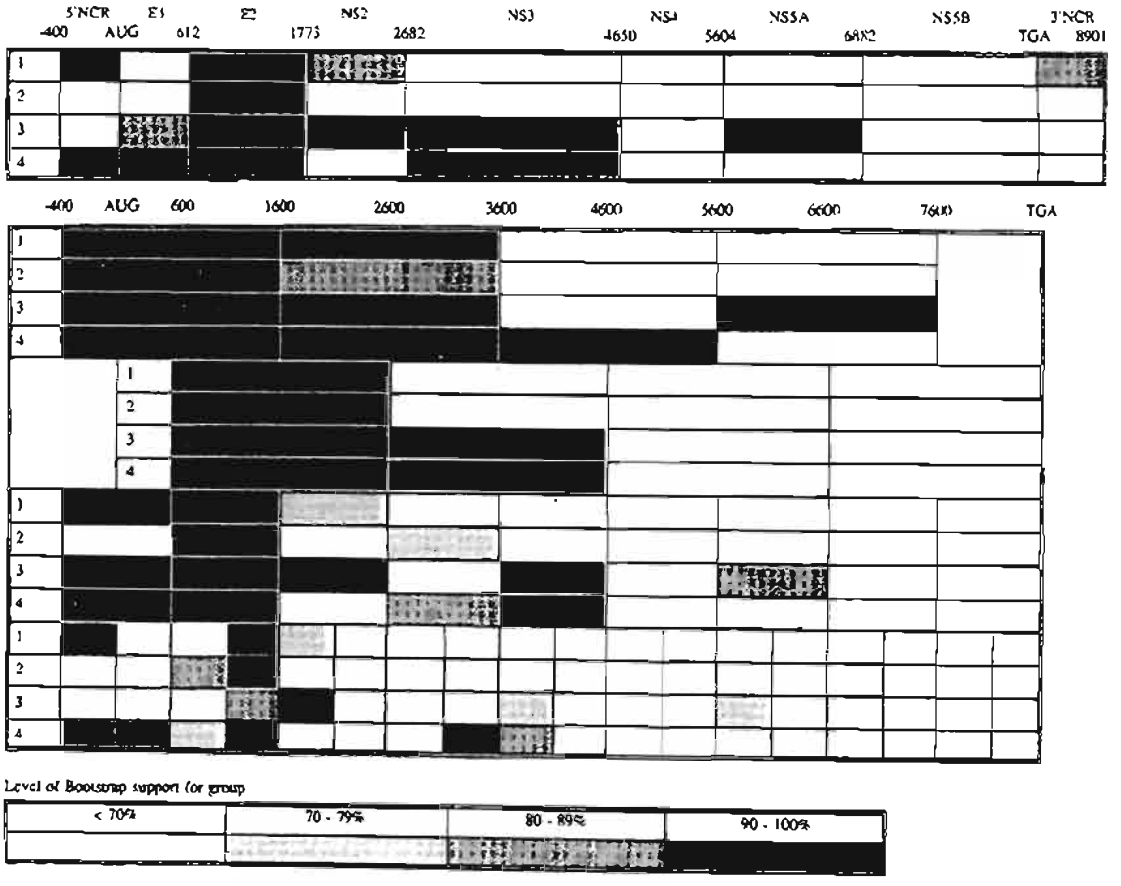


Fig. 4.7 Congruence between phylogenetic analysis of complete genomes and subgenomic fragments. The level of bootstrap support for the phylogenetic groupings 1-4 is indicated by the degree of shading for different genes and sugenomic fragments (Smith *et al*, 2000)

Congruent phylogenetic trees (boostrap support > 75%) were produced using a region as small as 200 nt (positions 1344 - 1543) [Fig.4.8] within the E2 gene. The shortest region that gave a congruent tree with more than 98% bootstrap support for each group was the 600 nt region from positions 994 – 1594 [Fig. 4.8]. This region also produced a congruent tree when analysed at synonymous sites (>85% support) but not at nonsynonymous sites

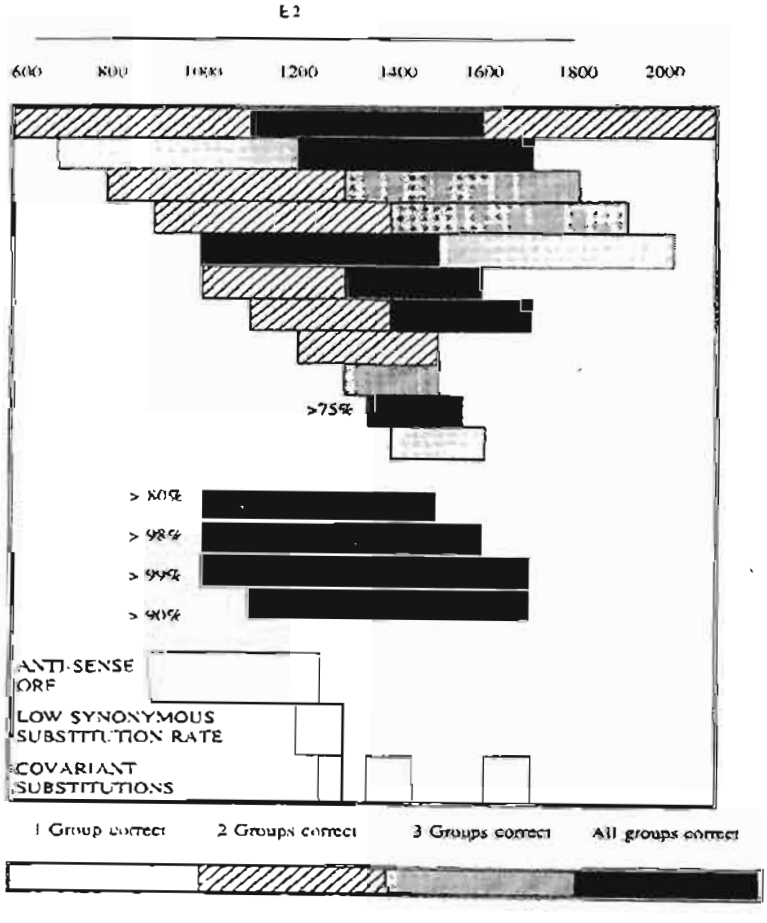


Fig.4.8 Congruence of phylogenetic relationships produced using fragments of the E2 gene. The extent to which phylogenetic groupings observed from analysis of complete genome sequences are supported by comparisons of the fragments of the E2 gene is indicated by the level of shading. The number of groups supported by more than 70% of bootstrap replicates is indicated (Smith *et al*, 2000)

E2 sequences for the 350 nt region (positions 1146 to 1495) of five GBV-C/HGV variants from KZN with unusual 5'-NCR sequences (L6 [AF172506]; L7 [AF172507]; B16 [1F172528]; B18 [AF172530]; D4 AF1722530]) were aligned and compared with published sequences of the same E2 region of 33 geographically distinct isolates whose complete genomic sequences are currently available in the Genbank database. The 4 major groups identified by analysis of the complete sequences were reproduced by the analysis of the E2 gene with good bootstrap support for each group (Fig. 4.9). Only the KZN isolates with unusual 5'NCR sequences formed an additional group (Fig.4.9).

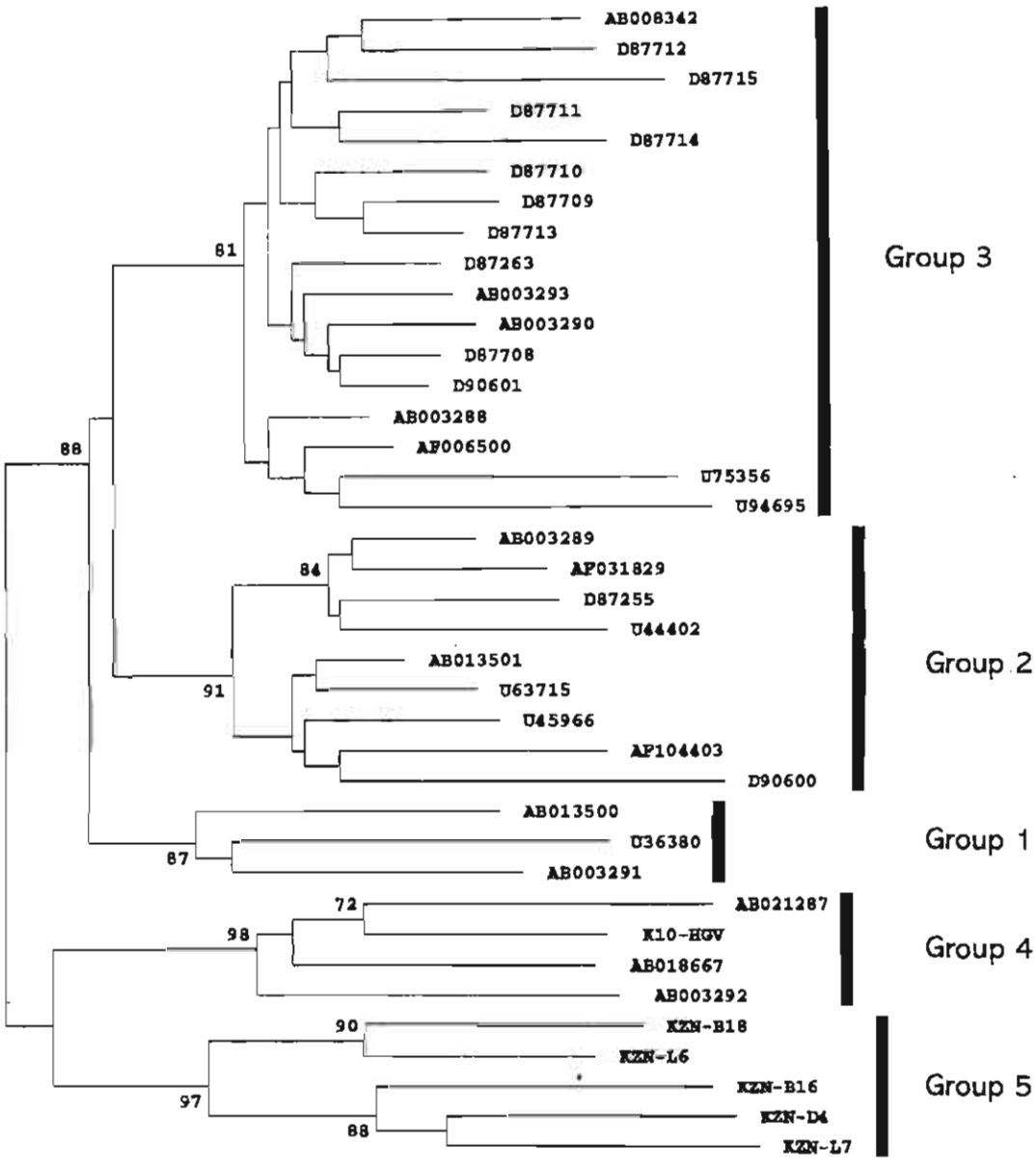


Fig.4.9 Phylogenetic tree E2 sequences of GBV-C/HGV isolates from KZN, South Africa. South African isolates form an additional E2 phylogenetic grouping (Group 5) [Smith *et al*, 2000) [KZN-KwaZulu Natal; B-blood donors; D-haemodialysis patient; L-chronic liver disease patient]

4.3.5 Genbank accession of GBV-C/HGV isolates from KZN, South Africa

Sequences of GBV-C/HGV isolates from KZN have been deposited in Genbank. The Genbank accession numbers are as follows:

AF172501 – AF172554 from Blood donors ; haemodialysis patients and patients with chronic liver disease (Fig. 4.3)

AY032956 – AY32973 from the eight geographical health regions of KZN, South Africa (Fig.4.5)

AF181977 – AF181981 are isolates used to analyse the E2 gene (Fig. 4.9)

4.4. DISCUSSION

Several groups have examined the genetic variation in GBV-C/HGV by comparing sequences from different segments of the GBV-C/HGV genome. Initial analysis of complete genome sequences suggested that GBV-C/HGV could be classified into three groups based on pairwise distances greater than 0.12 over the entire genome (Okamoto *et al*, 1997) or into four or five groups based upon the phylogenetic analysis of complete genome sequences (Takahahsi *et al*, 1997). However, studies with NS3, NS5A and envelope regions have been unsuccessful or contradictory in defining groups of GBV-C/HGV isolates (Kao *et al*, 1996; Kim *et al*, 1997; Muerhoff *et al*, 1997; Pickering et al, 1997; Viazov *et al*, 1997a). In contrast, analysis of regions within the 5´ NCR allowed the discrimination of three major groupings that correlated with the geographic origin of the isolates and with the analysis of complete genome sequences (Muerhoff *et al*, 1997; Smith *et al*, 1997). Based on this assumption, phylogenetic analysis of the 5´ NCR of isolates from blood donors and patients identified three variants of GBV-C/HGV in KZN (Fig.4.3) viz. Group 1, found predominantly in West and Central Africa; Group 2 found in Europe and North America but also including isolates from Pakistan, Japan and East Africa, and a new group of variants whose sequences are different from all other known GBV-C/HGV sequences (Fig.4.3). (Sathar *et al*, 1999b). This new grouping is supported by bootstrap analysis (88% of replicates) and although they cluster with group 1 sequences (bootstrap support of 90%) they are distinct from any previously described African group 1 isolates sequences (Muerhoff *et al*, 1997; Smith *et al*, 1997a).

Sequence analysis of the 5′ NCR of GBV-C/HGV isolates from eight health regions within the province of KZN depicts this new variant of GBV-C/HGV as a distinct group (bootstrap support of 94%) [Fig. 4.5] (Sathar and York, 2001). Seventeen of these 18 (94%) isolates clustered as an additional group while one isolate (KZN-E11) grouped with Group 1 variants (bootstrap value of 86%), confirming that this new variant of GBV-C/HGV is the predominant "genotype" in KZN. Included into the phylogenetic analysis were sequences of 5′ NCR of GBV-C/HGV isolates from the western and eastern Cape provinces of South Africa (Tucker *et al*, 1999) Based on their analysis, Tucker *et al* (1999) suggested the presence of a novel "fourth genotype." Five of their 13 isolates were characterised by major deletions in the 5′ NCR (Tucker *et al*, 1999). Unlike Tucker *et al* (1999) neither major deletions nor additional bands to the predicted 344bp PCR fragment were observed in any of the isolates from KZN (Sathar *et al*, 1999b). In their proposal that South African GBV-C/HGV isolates be classified as "Genotype 5", Tucker and Smuts (2000) did not make reference to the findings of Smith *et al* (2000) nor did they include in their analysis GBV-C/HGV isolates from the province of KZN (Sathar *et al*, 1999b). Phylogenetic analysis of 5′ NCR sequences of the "novel" South African GBV-C/HGV isolates from the provinces of the Western, and Eastern Cape (excluding the deletants) (Tucker *et al*, 1999) and from KZN (Sathar *et al*, 1999b), grouped South African isolates as an additional fifth group (Fig.4.5) (Sathar and York, 2001). In the proposal made by Tucker and Smuts (2000) that South African isolates be classified as Genotype 5, the inclusion of GBV-C/HGV isolates from KZN would have been more representative of GBV-C/HGV isolates from South Africa. South African GBV-C/HGV

isolates cluster as a phylogenetically distinct group and constitute an additional new group, i.e. Group 5 (Sathar and York, 2001).

The extreme 5′-terminal sequence is available for only a single Group 1 and none of the Group 4 isolates, so an adequate analysis could only be carried out from positions –388 to –1 (Smith *et al*, 2000). Analysis of this region and of various subfragments failed to produce congruent phylogenetic trees, with two sequences responsible for the majority of aberrant groupings; AB013500, an extreme Group 2 isolate from Bolivia, groups with Group 3 isolates for the 5′-NCR and some subgenomic fragments in the 3′ half of the genome, although the 5′-NCR is atypical (Smith *et al*, 2000). Similarly, U75356, an unpublished Group 3 isolate from China, has a 5′-NCR sequence similar to but distinct from those of Group 2 isolates (Smith *et al*, 2000). This sequence also has a frameshift within NS5A and an unusual sequence at the 3′ terminus (Smith *et al*, 2000). Hence, it is uncertain whether these isolates represent recombinants (An *et al*, 1997) or divergent variants with unique 5′-NCR sequences. When these two sequences were excluded from the data, a congruent tree is obtained when the region -388 to –1 was analysed (Smith *et al*, 2000). However, subfragments of this region, including those previously identified as reproducing the phylogenetic relationships of Group 1 and 2 isolates (Smith *et al*, 1997a) provide < 70% bootstrap support for either Groups 3 or 4 (Smith *et al*, 2000). The large number of sequences available within this region (1409 Accessions) and the inconsistent phylogenetic relationships of all but the largest fragments complicate similar analysis of 5′-NCR sequences (Smith *et al*, 2000). Visual examination for motifs

distinct from those typical of Groups 1, 2, 3 and 4 revealed a small number of unusual sequences, but of these only the variants from KZN (Sathar *et al*, 1999b) and isolates from Spain (Smith *et al*, 2000) grouped separately from the 5′ NCR sequences of complete genome sequences for the region -388 to -1) (Smith *et al*, 2000).

HGVC 964 is a group 3 isolate based on the analysis of the complete genome sequence (Takahahsi *et al*, 1997) but groups with group 2a isolates when analysis is confined to the 5′ NCR (An *et al*, 1997; Muerhoff *et al*, 1997; Smith *et al*, 1997) [Fig. 4.4]. Similarly, the isolate G05BD appears to represent a distinct phylogenetic group by analysis of the complete genome sequence (Takahahsi *et al*, 1997) but groups with Group 2 and Group 3 sequences upon analysis of the 5′ NCR sequence (Fig 4.3). In addition, Groups 3 (bootstrap support, 83%) and Group 4 (bootstrap support, 71%) isolates are not clearly differentiated by phylogenetic analysis of 5′ NCR sequences (Fig.4.5). The high degree of overlap in the ranges of sequence distances between and within groups does not permit the establishment of "cut-off" distances that define different groups or subgroups (Table 4.1). Therefore, analysis of the 5′ NCR sequences may not always provide an accurate guide to the relationship of complete genome sequences.

Phylogenetic analysis of the coding region of 33 published complete or near complete genomic sequences of GBV-C/HGV provided consistent evidence for four phylogenetic groups each supported by high bootstrap values (Fig.4.6). These groupings correspond to the

three groupings previously identified from the analysis of complete genome sequences (Okamoto *et al*, 1997) together with an additional group consisting of an unclassified Japanese sequence (Takahahsi *et al*, 1997) and three sequences one each from Thailand (K-10, Sirirurg Songsivilai, unpublished results), Vietnam (AB018667) and Myanmur (AB021287). Group 1 includes isolates from Ghana, West Africa and a single Japanese isolate; Group 2 includes isolates from Europe, North and South America and Japan; Group 3 includes isolates from Japan and China and the fourth group consists of isolates from south - east Asia (Fig.4.6). Although subgroupings of Groups 1, 2 and 3 have been distinguished by phylogenetic analysis of subgenomic regions (Muerhoff *et al*, 1996; 1997) there was only limited evidence from the analysis of complete genome sequences for subgroupings (Fig.4.6). The existence of the fourth group had been suggested from the analysis of 5′ NCR sequences of south East Asian isolates. A previous phylogenetic analysis of complete genome sequences also proposed four groups in which Groups 1 and 4 were combined and Group 3 was split into two (Charrel *et al*, 1999; Naito *et al*, 1999), but the bootstrap analysis did not support these groupings. Similarly, an analysis based upon genetic distances identified five groups with Group 1 split into two (Takahahsi *et al*, 1997).

An unexpected finding was that short COOH-terminal fragments of the E2 gene (Fig.4.8) could reproduce the phylogenetic relationships of complete GBV-C/HGV sequences (but not subgroupings). Previous studies of smaller numbers of sequences have shown that phylogenetic relationships are inconsistent when comparisons are made for individual genes (Takahahsi *et al*, 1997) or subgenomic fragments (Smith *et al*, 1997). Although three

(Cong *et al*, 1999) or four (Kim *et al*, 1997) phylogenetic groupings are observed when fragments larger than 1500 nucleotides including the E2 gene are compared, interpretation of these studies is complicated by idiosyncratic labelling, the absence of Group 4 isolates and the failure to assess the robustness of groupings. The analysis of a 200 nt fragment from the center of the E2 gene (positions 1344 - 1543) provides > 70% bootstrap support for all four phylogenetic groupings, while a 600 nt region (positions 994 - 1594) provided > 98% support (Fig. 4.8) [Smith *et al*, 2000]. This is in stark contrast to other coding regions: all subgenomic fragments of 2000 nt or less that did not contain this region of E2 failed to produce congruent trees with the sole exception of a 2,000 nt fragment encompassing the remainder of the E2 gene, NS2 and the NH_2-terminal half of NS3 (Fig. 4.7) [Smith *et al*, 2000]. Smith *et al*, (2000) provides several potential explanations for the observation that analysis of the E2 gene or specific subfragments produces phylogenetic trees congruent with those observed for complete genome sequences.

The analysis helps to clarify previous conflicting studies on the extent to which subgenomic regions can be used to identify GBV-C/HGV phylogenetic groupings. Most early studies of virus diversity concentrated on a 118 - 135 nt fragment within NS3 that was the first part of the genome to be sequenced (Simons *et al*, 1995a,b; Berg *et al*, 1996b; Heringlake *et al*, 1996a; Kao *et al*, 1996; Masuko *et al*, 1996; Schmidt *et al*, 1996; Schreier *et al*, 1996; Tsuda *et al*, 1996; Muerhoff *et al*, 1997; Pickering *et al*, 1997; Ibanez *et al*, 1998). However, the phylogenetic conclusions of these studies appear to be unreliable since analysis of even the entire NS3 gene fails to produce congruent groupings. Inconsistent phylogenetic groupings

have also been observed for the NH_2-terminus of E2 (Kim *et al*, 1997), 354 nt of NS5A / NS5B (Viazov *et al*, 1997a) or 279 nt of NS5B (Muerhoff *et al*, 1997).

Phylogenetic analysis of the 5′ NCR and E2 sequences of KZN isolates suggests that at least three groups (Groups 1, 2 and a new variant) of GBV-C/HGV are present in KZN. GBV-C/HGV in KZN infects all racial groups. In the study of blood donors and patients, Groups 1 (Central and West Africa), 2 (Europe, North and South America) and 5 (South Africa) isolates were detected in a heterogeneous population which included subjects from all four racial groups in KZN, viz. Africans, Whites, Indians and "Coloureds" (individuals of mixed origin). "Genotypes" 1, 2 and 4 were detected in a predominantly Black community in the Eastern and Western Cape provinces (Tucker *et al*, 1999). The absence of Group 2 variants in the study of GBV-C/HGV isolates from the 8 geographical health regions of KZN, is perhaps a reflection of a more homogenous study population (Black Africans). To-date both Groups 3 (China and Japan) and 4 variants (Southeast Asia) have not been detected in Southern Africa.

The diversity amongst Group 1 African isolates (Muerhoff *et al*, 1997; Smith *et al*, 1997a; Tanaka *et al*, 1998; Sathar *et al*, 1999a; Tucker *et al*, 1999) and the confirmation of a fifth group in South Africa reflects the extensive human population diversity in Southern Africa., consistent with the possibility that this virus may have emerged in Africa (Tanaka *et al*, 1998). The spread of geographically distinct GBV-C/HGV groups has been associated with human migration (Gonzales-Prez *et al*, 1997; Tanaka *et al*, 1998; Gallian *et al*, 1998; Wong *et al*, 1999) and is consistent with their co-evolution with humans during pre-historic migrations.

Group 1 and 5 isolates are African and have relatively diverse 5′ NCR sequences (Muerhoff *et al*, 1997; Smith *et al*, 1997a, Naito *et al*, 1999) while the remaining groups correspond to the three main waves of human migration from Africa to Europe (Group 2), Northern Asia (Group 3) and Southern Asia (Group 4). In addition, the presence of group 3 variants amongst native populations in South America (Pujol *et al*, 1997;Gonzales-Prez *et al*, 1997) is consistent with the first colonisation of this continent from Northern Asia via the Behring Strait. Although Japanese isolates have been found belonging to Groups 1-4 (Okamoto *et al*, 1997), most are Group 3 suggesting that the presence of other groups represents recent introductions. Since a virus related to GBV-C/HGV is present in chimpanzees (Adams *et al*, 1998; Birkenmeyer *et al*, 1998), while New World monkeys harbour more distantly related but species-specific variants (Leary *et al*, 1996a; Bukh and Apgar, 1997) it is possible that GBV-C/HGV has been continuously present in human populations since speciation.

Further sequence analysis of the E2 region from GBV-C/HGV variants isolated from different geographical regions may help clarify the origins of this unusual virus. It would certainly be interesting to obtain the E2 sequences of additional South African isolates. However, the complete sequence of one or more of the novel South African isolates would indeed settle the question of whether or not this is in fact a new phylogenetic variant. Although DNA sequencing is expensive and impractical for large scale epidemiological studies, GBV-C/HGV genotyping by phylogenetic analysis based on nucleotide sequences provides the most reliable genotyping results to-date and remains the gold standard by which other typing assays are compared. The current study confirm that the majority of the GBV-C/HGV isolates infecting

the local population in KZN are members of GBV-C/HGV Group 5. Whilst genotyping systems for GBV-C/HGV using restriction fragment length polymorphism (RFLP)[Mukaide *et al*, 1997; Fan *et al*, 2002], single strand polymorphism (SSCP)[White *et al*, 1999; Kato *et al*, 1998], heteroduplex mobility analysis (HMA)[White *et al*, 1999; Viazov *et al*, 1997a] and genotyping system based on PCR using type specific primer (Naito and Abe, 2001) methods have been reported, these methods have limitations as they are not sensitive enough in determining the degree of diversity among Groups 1-5 genomes. Group 1 and 5 GBV-C/HGV are African isolates that are closely related, however they have relatively diverse 5'NCR sequences. The complete sequence of Group 5 variants would establish whether this is a new phylogenetic variant or a recombinant. The complete sequence of Group 5 variants from KZN, the development of rapid, sensitive and inexpensive "genotyping" assays similar to that used to genotype HCV is the subject of further investigations. In view of the "potential" protective role that GBV-C/HGV genotypes may or may not play in HIV infection this could become a major focus of research in KZN where HIV is responsible for many deaths.

CHAPTER 5

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

At present there is no antibody profile that can distinguish ongoing GBV-C/HGV replication. GBV-C/HGV antibody test does not exclude viraemia. Therefore, the detection of GBV-C/HGV RNA is necessary for the diagnosis of active GBV-C/HGV infection. As with all target amplification technologies, nucleic acid contamination leading to false positive results is a continual problem and rigorous precautions and anti-contamination measures were followed. A negative control was included in each analysis. Sample handling and storage can affect the reliability of quantitative analysis especially in samples with low viral titres. In this study specimens were processed immediately, dispensed into aliquots for single use and stored frozen at – 80°C. There are numerous methods that are available to extract viral nucleic acid from biological samples, each with their own advantage. The best methods combine a quanidium-based extraction process with few steps as possible to prevent physical loss of RNA (QIAamp extraction kit used in this study). Although, the RT-PCR has its pitfalls it is the "gold standard" by which GBV-C/HGV RNA is detected. There are numerous formats using primers from the different regions of the GBV-C/HGV genome, based on single round or nested PCR, one or two tube reactions and various product detection techniques. It is perhaps not surprising that standardisation has not taken place. For this reason, results from different laboratories do not always agree. The introduction of internationally recognised

quality controls will be of major importance in the comparisons of laboratory procedures and results. For our study we created our own internal control which was used in each analysis.

The effect of GBV-C/HGV viral load on the disease process is not known. Among the techniques reported to detect GBV-C/HGV RNA, the majority are based on RT-PCR and include limiting dilution, competitive and dot plot procedures. All are extremely labour intensive, require considerable preparation and evaluation in terms of specificity and reproducibility, and are not appropriate for handling large numbers of samples. Traditional detection of amplified DNA relies upon electrophoresis of the nucleic acids in the presence of ethidium bromide and visual or densitometric analysis of the resulting bands with ultraviolet light. Southern blot detection of amplicon using hybridisation with a labeled oligo-nucleotide probe is also time consuming and requires multiple PCR product handling steps, further risking contamination. Alternatively, PCR-ELISA may be used to capture amplicon onto a solid phase using biotin or digoxigenin-labelled primers, oligonuleotide probes (oligoprobes) or directly after incorporation of the digoxigenen into the amplicon. The amplicon can be detected using an enzyme-labelled avidin or anti-digoxigenin reporter molecule similar to a standard ELISA format. The LCx assay is based on single tube, single round RT-PCR, however, the use of internal controls is lacking. The branched chain DNA assay is being developed, but is not widely used to quantitate GBV-C/HGV RNA. The technology is based on hybridisation followed by signal amplification using novel branched chain oligonucleotides and the luminescent signal compared with that obtained from external standards. Quantitative

real-time PCR is a relatively new technology that provides a broad dynamic range for detecting specific gene sequences. This technology is based on the detection of a fluorescent signal produced proportionally during amplification of a PCR product. DNA and RNA can be quantified without the laborious post-PCR processing. Whether all genotypes of GBV-C/HGV can be quantified equally using these methods are not known. For this reason selective primers and hybridisation probes is of major importance. Although these assays bring the field of quantitative virology within the technical reach of most laboratories, they are still very expensive and are not widely available. Having recently acquired a light cycler the prospect of using real-time PCR in studying the potential beneficial role of GBV-C/HGV co-infection in HIV infection has become a reality for future studies.

Recent advancements in nucleic acid sequencing have overcome many of the pitfalls that were encountered a few years ago. In the past DNA fragments had to be cloned into an M13 vector so that high concentrations of pure single strand transcripts of the insert were produced for sequencing. The finding that sequenase a T7 DNA polymerase could be used to efficiently sequence double stranded templates meant that purified plasmid DNA could be used instead of single stranded templates. The next advancement was the development of mutated Taq DNA polymerase (Taq-FS). This enzyme enabled PCR products to be sequenced directly. Taq FS is a mutant Taq polymerase that has a tyrosine substituted for a phenylalanine at the active site. Dye labeled di-deoxy chain terminators are incorporated with

far more efficiency than wild type Taq and sequencing with dye labeled primers resulted in near uniform peak heights (Appendix B and C). The technology and dyes have been further improved and it is now possible to easily sequence PCR products and with the use of capillary sequences such as the automated ABI 310 genetic analyzer (which was used for this study) many of the difficulties associated with sequencing have been overcome. The use of PCR sequencing meant that the amplicons resulting from the RT-PCR amplification of GBV-C/HGV positive samples could be sequenced directly. The PCR primers were used to sequence the PCR products and by performing two separate PCR sequencing reactions both strands of the amplicons could be sequenced.

Virus populations within individuals can be heterogenous. Virus populations are not homogenous but consist of variants that may differ at one or more positions in the viral genome. These variants are described as "quasispecies." (Smith *et al,* 1997b). The suggestion that GBV-C/HGV "quasispecies" exsist has been derived from the sequence analysis of multiple cloned copies of virus genomes derived from one or two infected individual (Viazov *et al*, 1997; Pickering *et al*, 1997; Nakao *et al*, 1997; Fan *et al*, 2002) with 4-8 years of persistent infection. Recently, Worobey and Holmes (2001) in their phylogenetic analysis of the 33 full or nearly full genome sequences of GBV-C/HGV isolates provided evidence for inter-and/or intra-genotype recombinations and suggest that GBV-C/HGV may be a recombinogenic virus. Although some subpopulations of GBV-C/HGV, may recombine freely within themselves, they may have been isolated enough from one another in the past to

accumulate characteristic differences. Although Group 5 variants from KZN cluster with Group 1 sequences they are distinct from any previously described African Group 1 isolates sequences. The sequence data (Appendices B and C) did not suggest the presence of GBV-C/HGV recombinant infections in any patient.

GBV-C also referred to as HGV are closely related Flaviviruses of human origin. Transmission by blood transfusions and from infected mothers to their infants has been documented. It is very probable that GBV-C/HGV is also transmitted by non-parenteral routes in KZN. In the absence of histochemical studies of liver biopsies to detect GBV-C/HGV, the higher prevalence of novel Group 5 GBV-C/HGV isolates in blood donors, HIV positive patients and patients with liver disease, the lack of elevation in liver enzymes or clinical hepatitis in blood donors and haemodialysis patients suggests that GBV-C/HGV is not associated with liver disease in KZN, South Africa. However, some findings suggest that GBV-C/HGV is involved with some cases of acute and chronic hepatitis, that GBV-C/HGV may be pathogenic to primates considered to be appropriate non-human hosts for viral hepatitis studies; and that GBV-C/HGV does indeed replicate in human liver. Whilst there is no consensus reached about the pathogenecity of GBV-C/HGV, it would be premature to screen blood donors for GBV-C/HGV and exclude a large proportion of blood donors from the donor pool without solid evidence that GBV-C/HGV is indeed pathogenic to humans. Thus GBV-C/HGV is a virus still searching for a disease.

There is evidence to suggest that GBV-C/HGV infection may have a beneficial influence in HIV positive patients. Longitudinal studies indicate that GBV-C/HGV infection slows down the progression to AIDS and eventually death by inhibiting HIV replication and enhancing immune competence. The definitive mechanism of how GBV-C/HGV inhibits the progression to AIDS is not known. For the first time relatively higher CD3 (due to an increase in T cell status) and an increase in γδT expression, together with a decrease in CD 30 (reflects a decrease in activation status and an increase in T helper Type 1 response) has been demonstrated in HIV positive patients co-infected with GVBV-C/HGV. These preliminary findings would tend to suggest an association with protection and/or delayed progression of HIV disease in GBV-C/HGV infected patients. Human γδ T cells possess potent cytotoxic activity and are capable of producing a large array β-chemokines and pro-inflammatory cytokines which are known to interfere with HIV replication. It is tempting to speculate that GBV-C/HGV could indirectly affect AIDS risk through the expression of γδT cells; induction of various chemokines and other soluble factors and altered expression of chemokine receptors essential for HIV co-receptors. In KZN, the epicenter of the HIV pandemic where HIV is having a devastating effect, a more detailed *in vivo* and *in vitro* longitudinal study of the observed phenomena may impact on new approaches to treat or prevent HIV infection.

AIDS is the fourth most common cause of death worldwide (Esparaza and Bhamarapravati, 2000). The impact of AIDS humbles any prior infectious disease threat in our history. In KZN the HIV epidemic and the consequential development of AIDS poses an enormous threat to the social, political and economic landscape of the province. With no effective vaccine available, the majority of HIV infected individuals living in Sub-Saharan Africa cannot afford antiretroviral drugs (70%). Despite short course antiretroviral regimens for the prevention of mother to child transmission (MTCT) of HIV, transmission at antepartum (7%) and through breastfeeding (4%) remains high. In Africa, 55% of HIV-1 positive adults are woman of child-bearing age. The cumulative risk of mother to child transmission of HIV is between 25%-45% in Africa (Dabis and Ekpini, 2002). Data from antenatal clinics show that in several parts of southern Africa, more that 30% of pregnant women are infected with HIV-1 (Dabis and Ekpini, 2002). South Africa is considered to have the fastest-growing HIV positive population in Africa if not the world. Official Department of Health figures compiled from antenatal clinics across the country show a significant increase in the infection rate among pregnant women in most provinces, the highest figures (33%) being reported for KZN (Prof. A Smith-Dept of Virology-personal communication). Therefore, effective HIV vaccines are needed to combat the pandemic.

Several candidate AIDS vaccines have been studied in human volunteers. It is currently believed that AIDS vaccines will need to induce neutralising antibody and cell-mediated immune responses to be protective (Haynes *et al*, 1996). Cell-mediated immune responses protective against intracellular pathogens typically involve $CD4^+$ T helper cells of the type 1 (IFN-γ) phenotype and $CD8^+$ cytotoxic T lymphocyte (CTL) responses (Abbas *et al*, 1996). The most promising AIDS vaccine strategy studied to-date has involved the use of live poxvirus vectors to express HIV antigens (Taylor *et al*, 1991). It has been suggested that live vaccines may be more potent than soluble protein vaccines because they induce proinflammatory cytokines, which act as adjuncts for the induction of cell-mediated immunity (Ramshaw and Ramsay, 2000).

Direct human to human microbial transmission as therapy for infectious diseases may seem anachronistic. Before the development of penicillin, syphilis was treated with the blood borne pathogen *Plasmodium vivax*. *Plasmodium vivax* has been used to treat Lyme disease (Annon, 1991) and to "stimulate" the immune response of HIV infected individuals (Heimlich *et al*, 1997). Malaria therapy has been recommended as an adjunct cancer treatment (Greentree, 1981). Enemas using stools from apparently healthy individuals were used to treat individual patients suffering from refractory *Clostridium difficile* colitis (Schwan *et al*, 1984). Recently,

2. Determine whether GBV-C/HGV could be used as an alternative vector to express HIV antigens or alternatively the insertion of GBV-C/HGV genes into viral vectors such as pox viruses (vaccinia or canarypox) or bacteria could induce more potent HIV specific immunity including a cytotoxic T cell response.
3. Determine the impact of GBV-C/HGV on HIV transmission from mother to infant and/or *vise versa*.
4. Determine whether GBV-C/HGV co-infection is an additional favourable prognostic factor in HIV positive adults (mothers) and/or their children (long term slow progressors).
5. Replication of GBV-C/HGV has been demonstrated in peripheral-blood mononuclear cells (Xiang *et al*, 2001) and CD4 T cells (Xiang et al, 2000) and the inhibitory effect of GBV-C/HGV infection on HIV replication leading to delayed disease progression was demonstrated *in vitro* (Xiang *et al*, 2001). These *In vitro* inhibition studies need to duplicated using African HIV Clade C and GBV-C/HGV Group 5 variants from KZN, South Africa in different PBMC subsets (CD4, CD8 etc.).

Current evidence suggests that other viral agents or other factors may be responsible for a large majority of post-transfusion or community-acquired non-A-E hepatitis. A new pathogen, namely, Transfusion Transmissible Virus (TTV) has become a new focus of viral hepatitis research. This DNA non-enveloped virus has numerous similarities to GBV-C/HGV. Several independent studies have cast doubts on the pathogenicity of TTV. More recently, a novel

hepatitis virus, SEN-Virus (SEN-V), has been isolated. SEN-V is now considered to be a likely candidate for previously unexplained hepatitis.

The past 5 years has seen the discovery of GBV-C, HGV and now the enlarging family of circoviruses that include, TTV, SANBAN, YONBAN and SEN-V. Each time a novel sequence is discovered, a search for disease association ensues. The sophistication and sensitivity of recombinant DNA technology for viral discovery virtually ensures that new viruses will continue to be discovered. At issue is whether these agents cause non-A to E hepatitis or some extra-hepatic manifestation and, if so, with what frequency and with what degree of clinical relevance. These viruses are so diverse that they might have very different pathogenecity and each phylogenetic grouping may have to be studied independently to assess its clinical relevance. Although some viruses recovered from blood may cause disease while others may be "innocent bystanders" or even be beneficial, the surveillance of all new and novel viral infections must be maintained.

Appendix A

Published sequences from the 5′ NCR of the GBV-C/HGV isolates PNF2161 (U44402), R10291 (U45966) and GBV-C (U36380) showing the position of the primers used in RT-PCR

```
      1                                                  50

u44402 ACGTGGGGGA GTTGATCCCC CCCCCCCGGC ACTGGGTGCA AGCCCCAGAA
u45966 .......... .......... .......... ..........
u36380 .......... .......... CCCCCCCGGC ACTGGGTGCA AGCCCCAGAA

      51                                                 100
u44402 ACCGACGCCT ATCTAAGTAG ACGCAATGAC TCGGCGCCGA CTCGGCGACC
u45966 .......... .......... CAATGAC TCGGCGCCGA CTCGGCGACC
u36380 ACCGACGCCT ACTGAAGTAG ACGTAATGGC CCCGCGCCGA ACCGGCGACC

      101                                                150
u44402 GGCCAA AAGGTGGTGGATGG GTGAT GACAGGGT TGGTAGGTCGTAAATCC
u45966 GGCCAA AAGGTGGTGGATGG GTGAT GACAGGGT TGGTAGGTCGTAAATCC
u36380 GGCCAA AAGGTGGTGGATGG GTGAT GACAGGGT TGGTAGGTCGTAAATCC

      151                                                200
u44402 CGGTCACCTT GGTAGCCACT ATAGGTGGGT CTTAAGAGAA GGTTAAGATT
u45966 CGGTCACCTT GGTAGCCACT ATAGGTGGGT CTTAAGAGAA GGTTAAGATT
u36380 CGGTCATCCT GGTAGCCACT ATAGGTGGGT CTTAAGGGGA GGCTACGGTC

      201                                                250
u44402 CCTCTTGTGC CTGCGGCGAG ACCGCGCACG GTCCACAGGT GTTGGCCCTA
u45966 CCTCTTGTGC CTGCGGCGAG ACCGCGCACG GTCCACAGGT GTTGG,CCCTA
u36380 CCTCTTGCGC ATATGGAGGA AAAGCGCACG GTCCACAGGT GTTGGTCCTA

      251                                                300
u44402 CCGGTGGGAATAAGGGCCCGACGTCAGGCTCGTCGTTAAACCGAGCCCGT
u45966 CCGGTGGGAATAAGGGCCCGACGTCAGGCTCGTCGTTAAACCGAGCCCGT
u36380 CCGGT : GTAATAAGGACCCGGCGCTAGGCACGCCGTTAAACCGAGCCCGT
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      301                                                        350
u44402 TACCCACCTGGGCAAACGACGCCCACGTACGGTCCACGTCGCCCTTCAAT
u45966 AACCCGCCTGGGCAAACGACGCCCACGTACGGTCCACGTCGCCCTTCAAT
u36380 TACTCCCCTGGGCAAACGACGCCCACGTACGGTCCACGTCGCCCTTCAAT

      351                                                        400
u44402 GTCTCTCTTGACCAATAGGCGTAGCCGGCGAGTTGACAAGGACCAGTGGG
u45966 GTCGCTCTTGACCAATAGGCTTAGCCGGCGAGTTGACAAGGACCAGTGGG
u36380 GTCTCTCTTGACCAATAGGCGTA. .CGGCGAGTTGACAAGGACCAGTGGG

      401                                                        450
u44402 GGCCGGGGGCT. TGAGAGGGACTCCAAGTCCCGCCCTTCCCGGTGGGCC
u45966 GGCCGGGGTTTATGGGGAAGGACCCCAAACCCTGCCCTTCCCGGCGGACC
u36380 GGCCGGGCGGGAGGGGGAAGGACCCCCACCGCTGCCCTTCCCGGGGAGGC

      451                                                        500
u44402 GGGAAA TGCATGGGCCCACCCAGCTCC GCGGCGGCCTGCAGCCGGGGTAG
u45966GGGAAA TGCATGGGGCCACCCAGCTCC GCGGCGGCCTGCAGCCGGGGTAG
u36380 GGGAAA TGCATGGGGCCACCCAGCTCC GCGGCGGCCTGCAGCCGGGGTAG

      501                                                        550
u44402 CCCAAGAATC C TTCGGGTGAGGGCGGGTGGCA TTTCCTTTTTCTATACCA
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u36380 CCCAAGAACC C TTCGGGTGAGGGCGGGTGGCA TTTCTTTTCCTATACCGA
```

Appendix B

The etiology of chronic liver disease at King Edward VIII Hospital, Durban.

Chronic Liver Disease	**No**
Alcohol	56
HBV	10
HCV	1
Auto-immune	5
HBV+Alcohol	10
HCV + Alcohol	4
Autoimmune + alcohol	2
HBV + HCV	1
Idiopathic	7
Bilharzia	2
Total	**98**

HBV-Hepatitis B Virus; HCV-Hepatitis C Virus

Appendix C

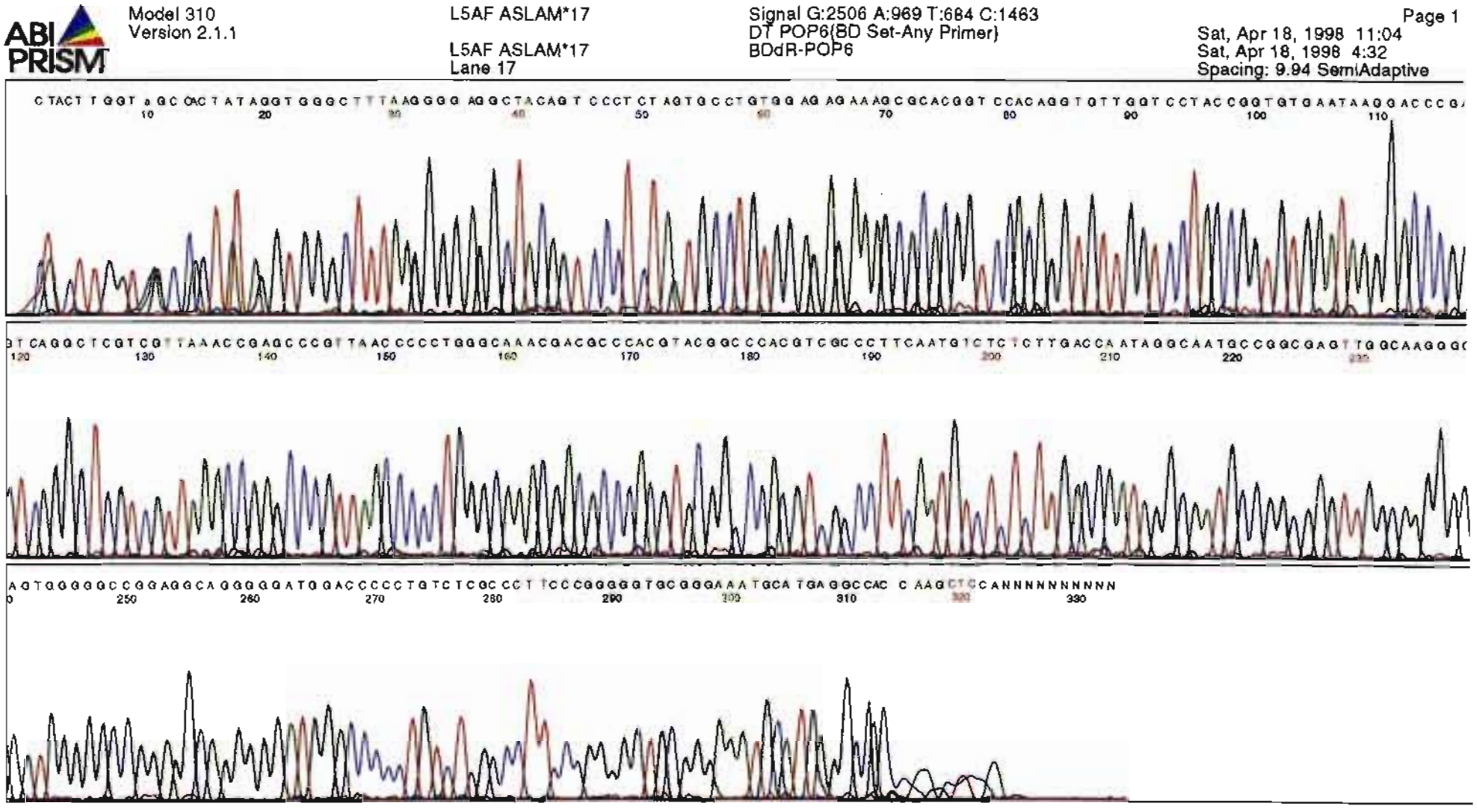
Biochemical tests in alcoholic patients with and without liver disease

Biochemical Tests	**ALD** **Mean ± SD**	**Alcoholic controls** **Mean ± SD**	**p-value**
ALB (g/L)	23.66 ± 7.19	40.74± 6.94	P<0,00001
GLOB (g/L)	53.28 ± 14.12	42.97± 8.91	P=0,0001
Total-Bil (umol/L)	61.24± 86.84	12.74± 6.03	P=0.0003
ALT (U/L)	89.78± 236.80	58.31± 34.83	ns
GGT (U/L)	227.80 ± 212.11	152.74± 212.84	ns
ALP (U/L)	233.0 1± 82.60	74.86± 44.49	P=0.0001
CDTect (U/L)	28.27± 19.31	54.91±38.13	P=0.0005

ALD, alcoholic liver disease; ALB, albumin; T-Bil, Total bilirubin; ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase ; ALP, alkaline phosphatase; CDTect, carbohydrate deficient transferrin; umol/L, micromoles per litre; U/L, units per litre; SD, standard deviation

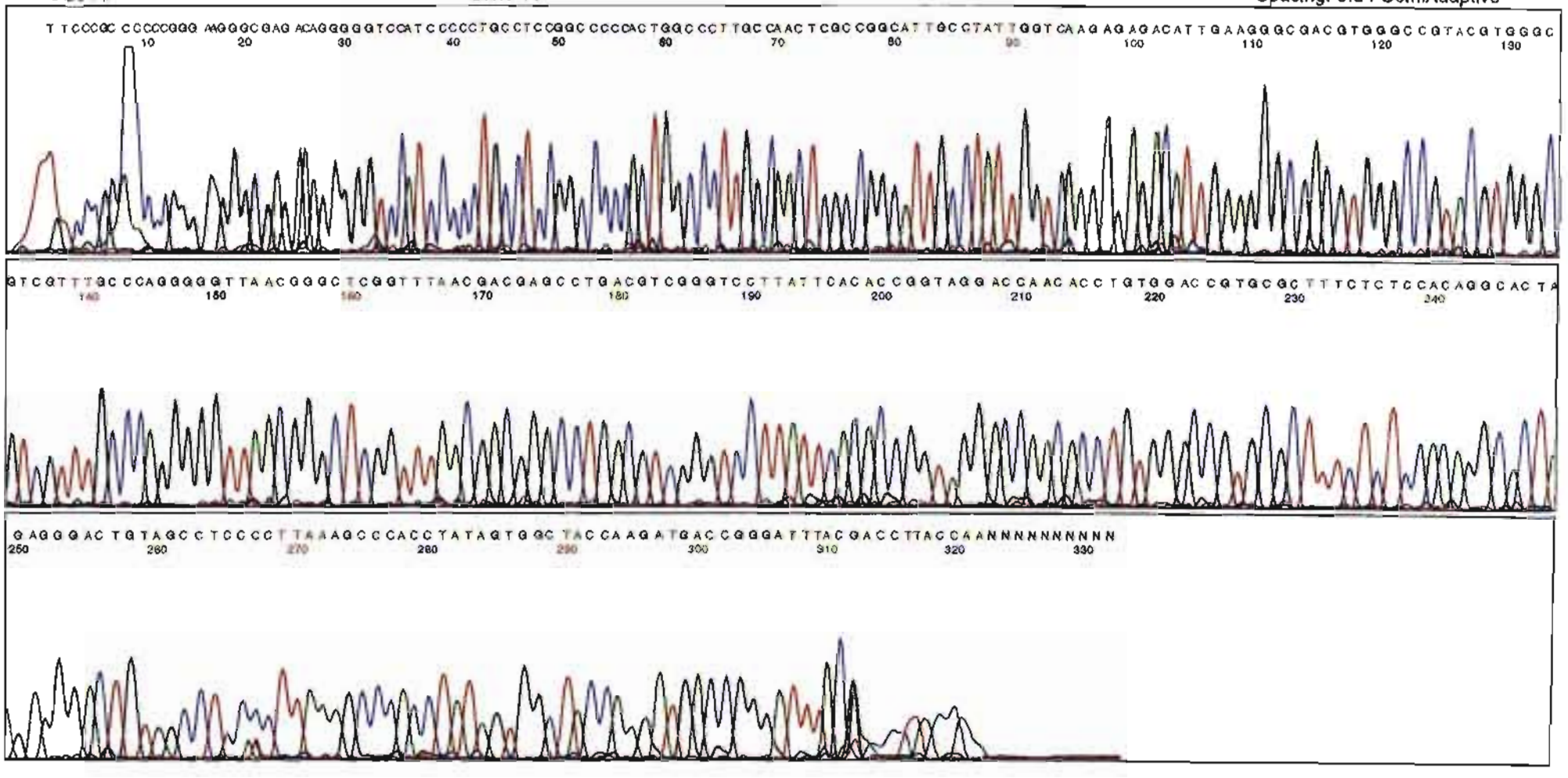
Appendix D

A typical flurograph showing the forward sequence of a GBV-C/HGB isolate



Appendix E

A typical flurograph showing the reverse sequence of a GBV-C/HGB isolate



Appendix F

Sequence data of GBV-C/HGV isolates from the eight geographical health regions of KwaZulu Natal

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D87712           TGGTAGCCAC TATAGGT-GG GT-CTTAAG- GGTTGGTCAA GGT-CCCTCT AGCGCTTGTG
D87714           TGGTAGCCAC TATAGGT-GG GT-CTTAAG- GGTTAGTCAA GGT-CCCTCT GGCGCTTGTG
D87708           TGGTAGCCAC TATAGGT-GG GT-CTTAAG- GGTTGGTCAA GGT-CCCTCT GGCGCTTGTG
AB003293         TGGTAGCCAC TATAGGT-GG GT-CTTAAG- GGTGGGTCAA GGT-CCCTCT GGCGCTTGTG
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D87710           TGGTAGCCAC TATAGGT-GG GT-CTTAAG- GGTTGGTCAA GGT-CCCTCT GGCGCTTGTG
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AY032963         ---GCGCC-C T-TAG-T-GG G--CTTACG- -GGAG---TC AGC-CCTT-- -ATGCCTG-G
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REPRINTS OF PUBLICATIONS

Journal of Medical Virology 59:38–44 (1999)

GB Virus C/Hepatitis G Virus Infection in KwaZulu Natal, South Africa

Mahomed A. Sathar,*[1] Paresh N. Soni,[1] Sarala Naicker,[1] Jan Conradie,[2] Fathima Lockhat,[1] and Eleanor Gouws[3]

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[2]*Natal Blood Transfusion Services, Durban, South Africa*
[3]*Center of Epidemiological Research, Medical Research Council, Durban, South Africa*

Sera from 70 patients on maintenance haemodialysis, 98 patients with chronic liver disease, and 232 volunteer blood donors in the province of KwaZulu Natal, South Africa, were screened for GB virus/hepatitis G virus (GBV-C/HGV) RNA and anti-E2 by reverse transcription-polymerase chain reaction (RT-PCR) and by an enzyme-linked immunosorbent assay (ELISA), respectively. GBV-C/HGV RNA was detected in 17/70 (24.3%) haemodialysis patients, 12/98(12.2%) patients with chronic liver disease, and 44/232 (18.9%) blood donors (Africans [29/76; 38.2%]; Asians [2/52; 3.8%]; Whites [11/49; 22.4%], and "Coloureds" [persons of mixed origin; 2/55; 3.6%]). Overall (anti-E2 and/or RNA) 43.9% (43/98) of patients with chronic liver disease, 47.1% (33/70) of haemodialysis patients, and 31.9% (74/232) of blood donors (Africans [44/76; 5.9%]; Asians [5/52; 9.6%]; Whites [15/49; 30.6%], and Coloureds [9/54; 16.6%]) were exposed to GBV-C/HGV infection. There was a significant difference in the prevalence of GBV-C/HGV infection (RNA and/or anti- E2) between African blood donors and the other racial groups ($P < .001$), and between blood donors and haemodialysis patients ($P = .02$) and patients with chronic liver disease ($P = .04$). Anti-E2 antibodies and GBV-C/HGV RNA were almost mutually exclusive. GBV-C/HGV-infected haemodialysis patients received more transfusions ($P = .03$) than noninfected patients. There was no significant difference in liver biochemistry between GBV-C/HGV-infected and noninfected patients and between blood donors in each of the four racial groups. The high prevalence of GBV-C/HGV infection in blood donors and chronic liver disease patients, and the lack of elevated liver enzymes and clinical hepatitis in blood donors and haemodialysis patients, suggest that GBV-C/HGV may not be associated with liver disease. ***J. Med. Virol. 58:38–44, 1999.*** © 1999 Wiley-Liss, Inc.

KEY WORDS: GBV-C/HGV; blood donors; haemodialysis; chronic liver disease; racial difference; South Africa

INTRODUCTION

Liver disease is a major cause of morbidity and mortality among patients with end-stage renal disease due primarily to hepatitis B virus (HBV), hepatitis C virus (HCV), alcohol, and hemosiderosis [Rao and Anderson, 1992; Huang, 1997]. However, the etiology of liver disease remains unknown in about 4–23% of dialysis patients [Rao and Anderson, 1992; Huang, 1997]. Approximately 10–20% of non-A-E hepatitis cases remain unidentified, suggesting the existence of additional viral agents [Linnen et al., 1996]. The recent identification of GB virus C (GBV-C) [Simmons et al., 1995] followed by the identification of hepatitis G virus (HGV) [Linnen et al., 1996], are among possible transfusion transmissible viruses currently being investigated for human hepatitis of unknown etiology.

Whilst some studies did not show a direct association between GBV-C/HGV infection and liver pathology [Alter et al., 1997a, 1997b], other studies have reported a spectrum of liver pathology [Mushahwar and Zuckerman, 1998]. The site of GBV-C/HGV infection and replication in the liver, peripheral blood mononuclear cells, or other tissues have been observed by some investigators [Madejon et al., 1997; Saito et al., 1997], whilst other studies suggest that the actual site of GBV-C/HGV replication remains to be identified [Laskus et al., 1997; Mellor et al., 1998]. There is controversy about the relevance of GBV-C/HGV infection

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in fulminant hepatic failure [Heringlake et al., 1996; Toshiba et al., 1996; Kanda et al., 1997].

Only some 12–15% of chronic non-ABC hepatitis patients are GBV-C/HGV infected [Linnen et al., 1996]. High prevalence of GBV-C/HGV has been found in subjects with frequent parenteral exposure and in groups at high risk of exposure to blood and blood products [Jarvis et al., 1996; Linnen et al., 1996], including intravenous drug abusers [Aikawa et al., 1996; Linnen et al., 1996], patients on maintenance haemodialysis [Masuko et al., 1996; Tsuda et al., 1996; Lampe et al., 1997; Shrestha et al., 1997; Wang et al., 1997; Murthy et al., 1998], multitransfused individuals [Linnen et al., 1996; Neilson et al., 1996], and haemophiliacs [Jarvis et al., 1996; Linnen et al., 1996; Castelling et al., 1998]. In non-African countries, the highest prevalence of GBV-C/HGV has been documented in patients with acute and chronic HCV (18%) infection [Linnen et al., 1996]. The risk of GBV-C/HGV infection seems to be increased in those co-infected with HBV or HCV [Linnen et al., 1996; Alter et al., 1997a]. These results indicate that GBV-C/HGV is transfusion transmissible, therefore patients receiving blood transfusions, haemodialysis, and organ transplant patients are at risk of infection. Most GBV-C/HGV infections remain subclinical and resolve after the loss of serum GBV-C/HGV RNA with the concomitant detection of antibodies to the putative envelope E2 protein (anti-GBV-C/HGV E2) [Dille et al., 1997; Tacke et al., 1997]. There is evidence to suggest that anti-E2 is a neutralising and protective antibody [Hassoba et al., 1998; Tillmann et al., 1998].

Very little is known about the epidemiology and pathogenic role of GBV-C/HGV in Southern Africa [Lightfoot et al., 1997; Mphahlele et al., 1997; Tucker et al., 1997; Castelling et al., 1998]. Because GBV-C/HGV is transmitted parenterally, we screened for the presence GBV-C/HGV RNA and anti-E2 in a high-risk population of haemodialysis and chronic liver disease patients and a representative sample of blood donors (from four racial groups) in the province of KwaZulu Natal, South Africa. The aim of the study was also to determine an association, if any, between GBV-C/HGV infection and liver disease.

METHODS

Patients

The study population included 70 patients with chronic renal failure who were undergoing maintenance haemodialysis and 98 consecutive patients with chronic liver disease. As a control group, 232 adult unpaid volunteer blood donors were studied from the four racial groups (Africans, Indians, Whites, and "Coloureds" [persons of mixed origin]). Patients gave written, informed consent, and the study was approved by the Ethics Committees of the University and the Natal and Blood Transfusion Services.

Serological Assays

Sera were tested for serological markers for HBV (HBsAg, HBeAg) by commercial radioimmunoassay (RIA; Abbott Laboratories, North Chicago, IL). Antibodies to the putative envelope protein (E2) of GBV-C/HGV were determined by enzyme-linked immunosorbent assay (ELISA) "μ Plate Anti-HG_{env}" (Boehringer Mannheim, Germany) according to the manufacturer's instructions. Liver enzymes and liver function tests were carried out by routine multianalysis systems in the central laboratory.

RNA Extraction and Reverse Transcription

HCV and GBV-C/HGV viral RNA were extracted and purified from serum with a QIAmp Viral RNA Kit (Qiagen, Gmbh, Germany). Complementary DNA (cDNA) was synthesized from 10 μl of extracted RNA melted at 70°C for 10 min and added to 15 μl of a mix containing 1.5 μM $pd(N)_6$ random hexamers (Pharmacia Biotech, Uppsala, Sweden), 5× first strand buffer (50 mM Tris-HCl [pH 8.3]; 40 mM KCl; 6 mM $MgCl_2$; 1 mM DTT) (GibcoBRL, Technologies, Paisley, UK); 250 U M-MLV reverse transcriptase (GibcoBRL), 0.5 mM dNTPs (Promega, Madison, WI), and diethylpyrocarbonate treated-H_2O and incubated at 37°C for 1 hr. The reaction was terminated by incubation at 95°C for 10 min.

HCV RNA Detection

The presence of HCV RNA in serum was determined by reverse-transcription nested polymerase chain reaction (RT-PCR) using primers from the 5′ NCR region, as described previously [Brown et al., 1992] .

GBV-C/HGV RNA Detection

Highly conserved nested primers using published sequences from the 5′ NCR of GBV-C/HGV genomes were used to carry out the nested PCR [Jarvis et al., 1996]. Briefly, 5 μl of the cDNA were added to 95 μl of a first round PCR mix containing 0.2 mM dNTPs (Promega); 10× PCR buffer (1.5 mM $MgCl_2$, 20 mM $[NH_4]_2SO_4$; 75 mM Tris-HCl [pH 9.0]; 0.01% [w/v] Tween) (Advanced Biotechnologies, Surrey, UK); diethylpyrocarbonate (DEPC)-H_2O, 2.5 U Thermoprine ^{plus} DNA Polymerase (Advanced Biotechnologies) and 50 pmol of each primer located at positions 108 (5′-AGGTGGTGGATGGGTGAT-3′; sense, outer) and 531 (5′-TGCCACCCGCCCTCACCCGAA-3′; antisense, outer) (Oswald DNA Services, Southampton, UK). Two microliters of the first-round PCR product were added to 48 μl of PCR mix containing 0.2 mM dNTPs (Promega); 10× PCR buffer; DEPC-H_2O, 2.5 U Thermoprine ^{plus} DNA Polymerase, and 50 pmol/μl of each primer located at positions 134 (5′-TGGTAGGTCGTAAATCCCGGT-3′; sense, inner) and 476 (5′-TGRGCTGGGTGGCCYCATGCWT-3′; antisense, inner). Amplification was over 25 cycles for both first and second rounds for PCR, using the following temperatures: 96°C 1 min, 50°C 1 min, 72°C 2.5 min, with a final extension at 72°C 10 min followed by

TABLE I. Demographic and Biochemical Features in Blood Donors (n = 232) With and Without GBV-C/HGV Infection

Blood donors	GBV-C/HGV$^+$ Mean ± SD (Range)	GBV-C/HGV$^-$ Mean ± SD (Range)	P
African (n = 76)	(n = 29)	(n = 47)	
ALT (U/L)	17.24 ± 11.40 (9–69)	15.68 ± 6.6 (5–45)	NS
Age (years)	32.10 ± 10.06 (19–48)	28.38 ± 10.53 (18–59)	NS
Sex (M:F)	8.6:1	4.2:1	NS
Asian (n = 52)	(n = 2)	(n = 50)	
ALT (U/L)	16.5 ± 2.12 (15–18)	23.48 ± 16.82 (7–92)	NS
Age (years)	47.0 ± 7.07 (42–52)	30.68 ± 12.09 (17–67)	NS
Sex (M:F)	1:1	11.5:1	NS
White (n = 49)	(n = 11)	(n = 38)	
ALT (U/L)	24.45 ± 11.64 (11–54)	23.76 ± 13.83 (8–63)	NS
Age (years)	40.27 ± 9.71 (19–53)	39.32 ± 15.19 (17–67)	NS
Sex (M:F)	2:0	2.25:1	NS
Coloured (n = 55)	(n = 2)	(n = 53)	
ALT (U/L)	12.5 ± 2.12 (11–14)	19.06 ± 12.51 (5–73)	NS
Age (years)	19.0 ± 1.41 (18–20)	31.23 ± 12.46 (17–68)	NS
Sex (M:F)	2.67:1	1.9:1	NS

GBV-C, GB virus C; HGV, hepatitis G virus; ALT, alanine aminotransferase; NS, not significant. Coloured, persons of mixed origin.

a 4°C soak. Known positive and negative GBV-C/HGV sera together with DEPC-H_2O were included in each run. The 344-bp PCR product was detected in 2% agarose gel prepared in 1× TBE buffer stained with ethidium bromide (10 mg/ml).

Statistical Analysis

Blood donor, haemodialysis, and chronic liver disease patients' data were analysed separately. The chi-square test was used to compare groups (GBV-C/HGV positive vs. GBV-C/HGV negative) with respect to categorical data, and Student's t-test was applied to continuous data. Analysis of covariance was used to compare GBV-C/HGV-positive and GBV-C/HGV-negative patients with respect to liver function test, adjusting for age, sex, and disease group. Multiple logistic regression was used to determine any confounding influence of age, sex, and disease group on GBV-C/HGV. Blood donors were compared with patients with chronic liver disease and haemodialysis patients with regard to demographic parameters using Student's t-test or the chi-square test.

RESULTS

GBV-C/HGV RNA

Of the 232 unpaid volunteer blood donors 76 (32.8%) were African, 49 (21.1%) Whites, 52 (22.4%) Asians, and 55 (23.7%) Coloureds. All the blood donors were negative for HCV, HBV, and human immunodeficiency virus (HIV). None of the donors bad a history or clinical evidence of liver disease. Overall, GBV-C/HGV RNA was detected in 44 of 232 (18.9%) blood donors (29/76 [38.2%] African, 2/52 [3.8%] Asian, 2/55 [3.6%] Coloured, and 11/49 [22.4%] White) (Table I). There was no significant difference in the prevalence of GBV-C/HGV RNA between Asian and Coloured blood donors (P = .81) and between African and White blood donors (P = .07). However, there was a significant difference in the prevalence of GBV-C/HGV RNA between the other racial groups (Asians vs. Whites [P < .005]; Asians vs. Africans [P < .00001; Coloureds vs. Whites [P < .005) and Coloureds vs. Africans [P < .00001]).

Among the 70 haemodialysis patients, there were 30 (42.9%) Africans, 29 (42.4%) Asians, 3 (4.3%) Coloureds, and 8 (11.4%) Whites. Seventeen (17) of 70 (24.3%) haemodialysis patients (5/30 [16.7%] Africans; 8/29 [27.6%] Asians; 1/3 [33%] Coloureds; and 3/8 [11.43%] Whites) were infected with GBV-C/HGV (95% confidence interval [CI] = 5.7%; 18.7%). GBV-C/HGV-positive patients tended to have a longer duration of dialysis (not significant) and have had more transfusions (P = .03) than noninfected patients (Table II). Four of 70 patients (5.7%) were infected with HCV but only 2/17 (11.76%) GBV-C/HGV-positive patients were co-infected with HCV.

Most of the patients with chronic liver disease were Black [88 Africans (90%), 7 Asians (7%), 2 Coloureds (2%), and 1 White (1%)]. The diagnosis of chronic liver disease was confirmed by histology in 81 patients and by peritoneoscopy alone in 17 patients. The etiology of chronic liver disease in most of the patients was due to alcohol abuse, viral infection, or a combination of the two. In 5 patients, chronic liver disease was due to autoimmune liver disease and in 7 it was cryptogenic. None of the patients gave a history of blood transfusions or intravenous drug abuse. The prevalence of GBV-C/HGV RNA in patients with chronic liver disease was 12.2% (12/98) (95% CI = 5.7%; 18.7%) (Table II). Only 2 of 10 patients with HBV were co-infected with GBV-C/HGV. GBV-C/HGV RNA was detected more frequently in patients with an alcoholic etiology alone (6/56 [11%]) or in combination with alcohol and HBV (1/10 [10%]) or alcohol and HCV (1/4 [25%]). Two of seven (29%) patients with cryptogenic liver disease were GBV-C/HGV RNA positive.

There was no significant difference in the prevalence

TABLE II. Demographic and Biochemical Features of Chronic Liver Disease and Haemodialysis Patients With and Without GBV-C/HGV Infection

	GBV-C/HGV$^+$ Mean ± SD (Range)	GBV-C/HGV$^-$ Mean ± SD (Range)	*P*
Chronic liver disease			
(n = 98)	(n = 12)	(n = 86)	
Age (years)	45.7 ± 15.9 (18–75)	44.8 ± 16.8 (2–81)	NS
Sex (M:F)	1.4:1	1.2:1	NS
T-Bil (umol/L)	68.6 ± 73.4 (7–235)	63.3 ± 83.3 (7–506)	NS
ALP (U/L)	197.0 ± 163.29 (18–576)	173.8 ± 143.41 (3–838)	NS
AST (U/L)	92.2 ± 48.4 (23–173)	154.6 ± 350.6 (14–2454)	NS
Haemodialysis (n = 70)	(n = 17)	(n = 53)	
Age (years)	38.17 ± 12.06 (17–65)	39.57 ± 12.76 (15–67)	NS
Sex (M:F)	1:0.8	1:0.8	NS
Dialysis (months)	76.23 ± 50.90 (12–180)	59.60 ± 54.82 (3–216)	NS
TRANSF (no.)	12.58 ± 7.79 (5–31)	8.07 ± 7.17 (2–30)	.03
AST (U/L)	14.88 ± 5.33 (6–28)	18.60 ± 13.22 (6–90)	NS
ALT (U/L)	12.58 ± 6.97 (5–32)	17.92 ± 18.24 (4–101)	NS
ALP (U/L)	132.58 ± 146.39 (36–667)	107.11 ± 131.50 (5–920)	NS
T-BIL (umol/L)	11.94 ± 2.22 (8–17)	11.32 ± 2.25 (5–17)	NS

GBV-C, GB virus C; HGV, hepatitis G virus; TRANSF, transfusion; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; T-BIL, total bilirubin; NS, not significant.

of GBV-C/HGV RNA between blood donors and patients. There was a significant difference in the prevalence of GBV-C/HGV RNA between with chronic liver disease (12/98; 12.2%) and haemodialysis patients (17/70; 24.2%) (P = .04).

Anti-E2

There was a higher prevalence of anti-E2 in African blood donors (21/76; 27.6%) than White (14/49; 8.2%), Coloured (7/55; 12.7%), or Asian (3/52; 5.7%) blood donors (Table III), and this difference was significant (P < .05). There was a significant difference (P < .05) in the prevalence of anti-E2 in chronic liver disease (32/98 [32.7%]), and haemodialysis patients (18/70 [25.7%]) compared with blood donors (35/232 [15.1%]) (Table III). There was no significant difference in anti-E2 seroprevalence between chronic liver disease and haemodialysis patients (P = .33).

African blood donors (45/76; 59.2%) had a greater exposure to GBV-C/HGV infection (RNA and/or anti-E2 positive) compared with White (15/49; 30.6%; P = .002), Coloured (9/55; 16.4%; P < .00001), and Asian (5/52; 9.6%; P < .00001) blood donors. There was a significant difference (P < .05) in the overall exposure to GBV-C/HGV (RNA and/or anti-E2 positive) between blood donors (31.9% [74/232]) and patients (43.9% [49/98] vs. 47.1% [33/70]), respectively (Table III). GBV-C/HGV RNA and anti-E2 were mutually exclusive in almost all (88–100%) patients and blood donors. GBV-C/HGV RNA and anti-E2 was positive simultaneously in only 1/98 (1.1%) patients with chronic liver disease, 2/70 (2.9%) haemodialysis patients, and in 5/232 (2.2%) blood donors.

Liver Biochemistry

Elevated alanine aminotransferase (ALT) levels were observed in only 2/70 (2.85%) dialysis patients. Mildly elevated ALT levels were observed in 6/232 (2.6%) blood donors. Only 1/44(2.3%) GBV-C/HGV-positive donors had a mild elevated ALT level (69 U/L). Using multivariate analysis and controlling for the influence of confounding factors (age, sex, and disease groups), no significant differences were observed between GBV-C/HGV infected and noninfected blood donors in each of the four racial groups (Table I), nor were such differences observed in patients with chronic liver disease and haemodialysis patients (Table II).

DISCUSSION

Although the study population may not be representative of the overall prevalence of GBV-C/HGV in KwaZulu Natal, the results indicate that there is a high prevalence of GBV-C/HGV infection in KwaZulu Natal, South Africa and that there are racial differences in the prevalence of GBV-C/HGV infection. The overall prevalence of GBV-C/HGV RNA in blood donors in the province of KwaZulu Natal (18.9%) is higher than that of blood donors in Gauteng (11.1%) [Castelling et al., 1998], with the prevalence being higher among African (38%) and White (22%) blood donors in KwaZulu Natal compared with African (29%) and White (8,5%) blood donors in Gauteng [Castelling et al., 1998]. Tucker et al. [1997] reported a GBV-C/HGV RNA prevalence of 6.3% in a predominantly Coloured rural community in the Eastern Cape that was higher than the 4% of Coloured blood donors infected with GBV-C/HGV in KwaZulu Natal. However, volunteer blood donors represent a healthier population than rural communities.

The prevalence of anti-E2 in healthy blood donors ranges from 2.7% to 20.3% in different parts of the world [Dille et al., 1997; Tacke et al., 1997; Ross et al., 1998]. The overall anti-E2 prevalence in the blood donor population (14.9%) was lower than the 20.3% reported by Ross et al. [1998] for South African blood donors. However, Ross et al. [1998] did not define the racial breakdown of the blood donor population, and our results indicate a racial difference in anti-E2 prevalence. The lower prevalence of viraemia (3.8%)

TABLE III. GBV-C/HGV RNA and Anti-E2 Seroprevalence Among Blood Donors and Patients on Maintenance Haemodialysis or With Chronic Liver Disease

Study population	No.	GBV-C/HGV RNA+ (%)	GBV-C/HGV anti-E2+ (%)	GBV-C/HGV RNA and anti-E2+ (%)	GBV-C/HGV exposure RNA and/or anti-E2+ (%)
Blood donors					
African	76	29 (38.2)	21 (27.6)	5 (6.5)	45 (59.2)
White	49	11 (22.4)	4 (8.2)	0 (0)	15 (30.6)
Asian	52	2 (3.8)	3 (5.7)	0 (0)	5 (9.6)
Coloured	55	2 (3.6)	7 (12.7)	0 (0)	9 (16.4)
Total	232	44 (18.9)	35 (15.1)	5 (2.1)	74 (31.9)
Chronic liver disease	98	12 (12.2)	32 (32.7)	1 (1.0%)	43 (43.9)
Haemodialysis	70	17 (24.3)	18 (25.7)	1 (2.9)	33 (47.1)

GBV-C, GB virus C; HGV, hepatitis G virus; Coloured, persons of mixed origin.

and anti-E2 response (5.7%) in the Asian blood donors compared with other racial groups is in agreement with the low prevalence of anti-E2 (2.7–6.3%) in Asian countries [Ross et al., 1998] and reflects the infrequent exposure to GBV-C/HGV in this community. Anti-E2 prevalence in potential risk groups was higher (25.7% vs. 32.7%) than in blood donors (15.1%) ($P < .05$). Concurrent detection of viraemia and seropositivity was seen in only 2–3% of our study population. This low rate probably indicates an overlap between E2 seroconversion and presence of viraemia [Dille et al., 1997; Tacke et al., 1997]. It may be possible that at the time of testing these patients were still viraemic and in the process of seroconversion. Viraemia and seroreactivity was almost mutually exclusive. The combined overall exposure of GBV-C/HGV infection in African blood donors (59.2%) was higher compared with 46.7% (PCR + anti-E2) reported in a West African population [Dille et al., 1997]. GBV-C/HGV infection appears to be a common infection in our community.

There is a higher prevalence of GBV-C/HGV infection in apparently healthy individuals in African countries (10–40%) [Dawson et al., 1996; Dille et al., 1997; Mphahlele et al., 1997; Tucker et al., 1997; Castelling et al., 1998], with infection occurring more commonly during childhood [Mphahlele et al., 1997], compared with blood donor groups in non-African countries (1–4%) [Linnen et al., 1996; Masuko et al., 1996; Moaven et al., 1996; Alter et al., 1997b; Roth et al., 1997; Wang et al., 1997]. The reason for the high prevalence of GBV-C/HGV in blood donors worldwide and basis for the racial differences in GBV-C/HGV infection in the South African blood donor populations is not known. Racial differences in the prevalence of HAV [Sathar et al., 1994], HBV [Dusheiko et al., 1989], and HCV [Soni et al., 1993] infections due to differences in socioeconomic factors in South Africa is well documented. Whether this difference holds true for GBV-C/HGV is not known, although a relationship was noted between GBV-C/HGV infection and the lack of waterborne sewage [Tucker et al., 1997]. The differences in the prevalence of detecting GBV-C/HGV infection may be due to the differences in the sensitivity of the various PCR protocols and primers (derived from various regions of the genome) used by various investigators. The nested PCR used in this study is likely to contribute to an increase in the sensitivity of the assay as compared with the one step PCR procedures. In addition the testing of anti-E2 greatly extends the ability of RT-PCR to define the epidemiology of GBV-C/HGV.

In this study, most haemodialysis patients and blood donors, including those with GBV-C/HGV infection, had normal liver enzymes. The high prevalence of GBV-C/HGV in blood donors and haemodialysis patients without biochemical evidence of liver damage suggests that many patients are viraemic in the absence of liver disease. However, normal ALT levels do not prove definitely normal histology, as shown for HCV infection [Alberti et al., 1991]. No significant difference was observed between GBV-C/HGV RNA-positive and -negative patients. More recently, no association was reported between GBV-C/HGV and hepatocellular carcinoma in Black South Africans [Lightfoot et al., 1997]. The prevalence of HCV in haemodialysis patients (4/70; 5.7%) and in patients with chronic liver disease (6/98; 6.1%) in KwaZulu Natal is similar to that reported previously (4.8%) and confirms the low prevalence of HCV [Soni et al., 1993].

In non-African countries, the mode of transmission of GBV-C/HGV occurs parenterally and is considered to be similar to HCV [Linnen et al., 1996]. In this study, GBV-C/HGV-infected dialysis patients tended to have had more transfusions and a longer duration of dialysis than noninfected patients. Therefore, most patients on maintenance haemodialysis acquired their GBV-C/HGV infection through the transfusions they received [De Lamballeric et al., 1996; Masuko et al., 1996; Tsuda et al., 1996; Lampe et al., 1997; Shrestha et al., 1997; Wang et al., 1997; Castelling et al., 1998; Murthy et al., 1998]. The absence of a history of blood transfusions and intravenous drug abuse in patients with chronic liver disease, in addition to the high prevalence of GBV-C/HGV in blood donors and in rural communities, especially in African children, tends to suggest that GBV-C/HGV is being transmitted by as yet undefined nonparenteral routes in South Africa. Both vertical [Feucht et al., 1996] and sexual [Ibanez et al., 1998; Scallan et al., 1998] transmission of GBV-C/HGV has been suggested. HBV infection is endemic amongst the African population of South Africa and the risk of horizontal transmission of HBV is well recognised [Dusheiko et al., 1989]. Whether the association of

GBV-C/HGV with HBV implies that GBV-C/HGV might spread by sexual or prenatal routes cannot be determined by this study and would require a more comprehensive epidemiological study to elucidate the high prevalence of GBV-C/HGV in our population.

Although histochemical and hybridisation studies of liver biopsies to detect GBV-C/HGV were not undertaken in this study, the higher prevalence of GBV-C/HGV in blood donors and patients with chronic liver disease and the lack of elevation in liver enzymes as well as clinical hepatitis in blood donors and haemodialysis patients would suggest that GBV-C/HGV may not be associated with liver disease.

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A new variant of GB virus C/hepatitis G virus (GBV-C/HGV) from South Africa☆

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Abstract

Phylogenetic analysis of the 5′ non-coding region (5′NCR) sequences has demonstrated that GB virus C/hepatitis G virus (GBV-C/HGV) can be separated into three major groups that correlate with the geographic origin of the isolate. Sequence analysis of the 5′NCR of 54 GBV-C/HGV isolates from 31 blood donors, 11 haemodialysis patients and 12 patients with chronic liver disease suggests the presence of a new variant of GBV-C/HGV in the province of KwaZulu Natal, South Africa. Eleven isolates grouped as group 1 variants (bootstrap support, 90%) found predominantly in West and Central Africa, a further six isolates grouped as group 2 variants (bootstrap support, 58%) found in Europe and North America; five of which grouped as 2a (bootstrap support, 91%) and one as 2b (bootstrap support, 87%), the latter also includes isolates from Japan, East Africa and Pakistan. Although the remaining 37 GBV-C/HGV isolates were more closely related to group 1 variants (bootstrap support, 90%), they formed a cluster, which was distinct from all other known GBV-C/HGV sequences. None of the South African isolates grouped with group 3 variants described from Southeast Asia. Three variants of GBV-C/HGV exist in KwaZulu Natal: groups 1, 2 and a new variant, which is distinct from other African isolates.

Keywords: Hepatitis GBV-C/HGV; Sequence and phylogenetic analysis; 5′ Non-coding region (5′NCR); South Africa

1. Introduction

The clinical significance of human infection with the recently identified flavivirus, GB virus-C (GBV-C) (Simons et al., 1995) also called hepatitis

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G virus (HGV) (Linnen et al., 1996) is currently unclear (Mushahwar and Zukerman, 1998). Although originally proposed as an agent of hepatitis a direct association with liver pathology is still lacking (Alter et al., 1997a,b), and it is not yet clear as to whether GBV-C/HGV is indeed a hepatotropic virus (Laskus et al., 1997; Miyakawa and Mayumi, 1997; Mellor et al., 1998). Only some 12–15% of chronic non-A, B, C hepatitis cases are GBV-C/HGV infected (Alter and Bradley, 1995). High prevalences of GBV-C/HGV have been found in subjects with frequent parenteral exposure and in groups at high risk of exposure to blood and blood products (Jarvis et al., 1996; Linnen et al., 1996), including intravenous drug abusers (IVDA) (Linnen et al., 1996), patients on maintenance haemodialysis (Masuko et al., 1996), multitransfused individuals (Linnen et al., 1996) and haemophiliacs (Jarvis et al., 1996). There is some controversy about the relevance of GBV-C/HGV infection in fulminant hepatic failure (Heringlake et al., 1996; Toshiba et al., 1996; Kanda et al., 1997).

Attempts to genotype GBV-C/HGV based on sequence analysis of the NS3 (Kao et al., 1996; Tsuda et al., 1996; Pickering et al., 1997) and NS5A (Viazov et al., 1997) genomic regions have been unsuccessful. However, sequence analysis of 5′ non-coding region (5′NCR) has provided evidence that GBV-C/HGV can be separated into three major groups that correlate with the geographic origin of the isolates (Muerhoff et al., 1996, 1997; Smith et al., 1997). Group 1 is prevalent in West and Central Africa, group 2 in Europe and North America, but also includes isolates from Japan, Pakistan and East Africa and group 3 in Southeast Asia. In addition, group 2 can be subdivided into groups 2a and 2b; the sub-division of group 1 is less certain (Muerhoff et al., 1997).

It has been suggested that GBV-C/HGV may have originated in Africa (Tanaka et al., 1998). Very little is known about the molecular epidemiology and pathogenic role of GBV-C/HGV in southern Africa (Castelling et al., 1998; Lightfoot et al., 1997; Tucker et al., 1997). Since analysis of the 5′NCR is considered to be predictive of the classification of GBV-C/HGV into the three or more major groups displayed by sequence analysis of the complete genome (Muerhoff et al., 1996, 1997; Smith et al., 1997), we determined the nucleotide sequences of the 5′NCR region of GBV-C/HGV isolates from 31 blood donors (KZN B1-B31), 11 haemodialysis patients (KZN D1-D11) and 12 patients with chronic liver disease (KZN L1-L12) from the province of KwaZulu Natal (KZN) in South Africa. The sequences were then compared by phylogenetic analysis with published sequences to establish their phylogenetic relationship with GBV-C/HGV isolates from other geographic regions of the world.

2. Materials and methods

2.1. Patients

Sera from individuals previously shown to be infected with GBV-C/HGV were used as a source of viral RNA (Sathar et al., 1999). They included 17 of 70 (23%) patients with chronic renal failure who were undergoing maintenance haemodialysis,12 of 98 (12.2%) consecutive patients with chronic liver disease and 44 of 232 (18.9%) adult volunteer blood donors from the four racial groups (Africans, Indians, Whites and 'Coloureds' [mixed origin]) in the province of KwaZulu Natal, South Africa. Patients gave written, informed consent, and the study was approved by the Ethics Committee of the University of Natal.

2.2. Detection of GBV-C/HGV RNA

GBV-C/HGV viral RNA was extracted from sera with a QIAmp Viral RNA Kit (Qiagen, Gmbh, Germany) with modifications (Marshall et al., 1998). Complementary DNA (cDNA) was synthesised from 10 μl of extracted RNA at 37°C for 1 h using Moloney murine leukaemia virus reverse transcriptase (M-MLV-RT) (Gibco-RL, Paisley, UK) and 1.5 μM pd (N) 6 random hexamers (Pharmacia Biotech, Uppsala, Sweden). Highly conserved nested primers using published sequences (Jarves et al., 1996) from the 5′NCR of GBV-C/HGV genomes were used to perform the nested PCR. Briefly, 5 μl of the cDNA was added

to 95 μl of a first round PCR mix containing: 0.2 mM dNTPs (Promega, Madison, USA); 10 × PCR buffer (1.5 mM $MgCl_2$, 20 mM $[NH_4]_2SO_4$, 75 mM Tris–HCl [pH 9.0]; 0.01% (w/v) Tween 20) (Advanced Biotechnologies, Surrey, UK); diethylpyrocarbonate treated water (DEPC-H_2O), 2.5 U Thermoprine^{plus} DNA polymerase (Advanced Biotechnologies, Surrey, UK) and 50 pmol of each primer located at positions 108 (5′-AGGTGGTGGATGGGTGAT-3′; sense, outer); and 531 (5′-TGCCACCCGCCCTCACCCGAA-3′; antisense, outer) (Oswald DNA Services, Southampton, UK). Two microlitres of the first round PCR product was added to 48 μl of a PCR mix containing: 0.2 mM dNTPs; 10 × PCR buffer; DEPC-H_2O; 2.5 U Thermoprine^{plus} DNA polymerase and 50 pmol/μl of each primer located at positions 134 (5′-TGGTAGGTCGTAAATCCCGGT-3′; sense, inner) and 476 (5′-TGRGCTGGGTGGCCYCATGCWT-3′; antisense, inner). Amplification was over 25 cycles for both first and second rounds for PCR, using the following temperatures: 96°C for 1 min, 50°C for 1 min, 72°C for 2.5 min with a final extension at 72°C for 10 min followed by a 4°C soak. Standard precautions for avoiding contamination for PCR were observed. Known positive and negative GBV-C/HGV sera together with DEPC-H_2O were included in each run to ensure specificity. The 344 bp PCR product was detected in 2% agarose gel prepared in 1 × TBE buffer stained with ethidium bromide (10 mg/ml).

2.3. Sequence analysis

Purification of the amplified products from the PCR reactions were performed using the High Pure PCR Product Purification Kit (Boehringer Mannheim, Germany) according to the manufacturer's instructions. Both strands of the 344 bp PCR product were sequenced by direct cycle sequencing using the Perkin Elmer dRhodamine Terminator Cycle Sequencing Kit (Perkin Elmer, Biosystems, CA, USA) on the Perkin Elmer Geneamp PCR system 2400 thermal cycler. The sequences were determined using an Applied Biosynthesis Prism 310 Automated Genetic Analyser (Perkin Elmer, Biosystems, CA, USA). The sequences were analysed using the Sequence Navigator Software (V.1.0.1). (Perkin-Elmer, Biosystems, CA, USA). Nucleotide sequences were aligned manually using the Simmonic 2000-sequence editor package (P. Simmonds) (Fig. 1).

2.4. Phylogenetic analysis

Phylogenetic analysis of GBV-C/HGV sequences was carried out on a 311 base pair fragment in the 5′NCR region (positions 160–470 in the HGV type 2a clone, PNF2161 (Muerhoff et al., 1996)). Sequences were compared using a distance based method (p-distance at all sites followed by neighbour joining) as implemented on the MEGA package (Kumar et al., 1993). Robustness of grouping was assessed by bootstrap re-sampling; numbers on branches indicate the percentage of 500 bootstrap replicates that supported the observed phylogeny (restricted to values of 75% or greater). p-Distances are indicated on scale bar (Fig. 2). Sequences compared included those of designated genotype for which complete genomic sequences are available (GenBank/EMBL accession numbers in parentheses). These include the group 1 sequences, GBV-C (U36380) and CG12LC (AB003291) which corresponds to variants recently found in Central African Pygmy populations, which are characterised by an insertion in the NS5 region (Tanaka et al., 1998); group 2a sequences, PNF2161 (U44402) and R10291 (U45966); group 2b sequence GBV-C (EA) (U63715); group 3 sequences GT230 (D90601) and GSI85 (D87262). The following sequences of unclassified genotypes were also included: HGVC-964 (U75356) from China and G05BD (AB003292) from Japan.

3. Results

Fifty-four of 73 (74%) GBV-C/HGV isolates were sequenced, the remainder giving rise to faint or poor nucleotide sequences. The 5′NCR sequences from 54 GBV-C/HGV isolates were aligned for phylogenetic analysis with representative isolates from each group and are presented in Fig. 1. The phylogenetic tree comparing sequences

from the 5′NCR of our 54 isolates (31 blood donors [KZN B1–31]; 11 haemodialysis patients [KZN D1–11] and 12 patients with chronic liver disease KZN 1–12]) with isolates from other geographic regions is shown in Fig. 2. Using either the full sequence, or only the first 200 nucleotides in order to exclude regions that are variable but do not correlate with the geographic origin of the isolates (Smith et al., 1997), our isolates segregated into three distinct phylogenetic groups (groups 1, 2 and a new group) (Fig. 2).

The majority (37/54) of GBV-C/HGV sequences formed a cluster, which was distinct from all other known GBV-C/HGV sequences, sup-

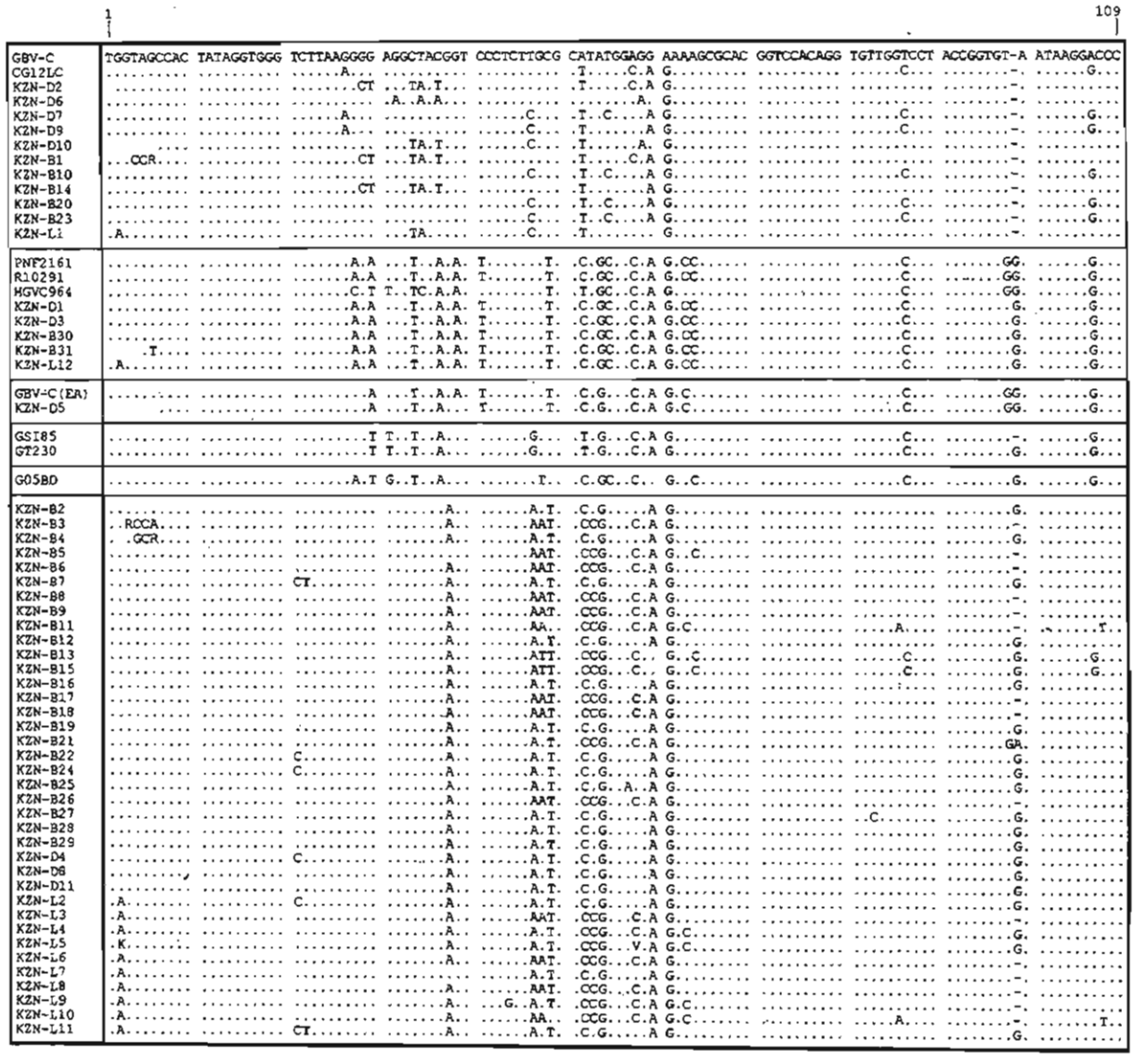


Fig. 1. Alignment of nucleotide sequences from the 5′NCR of GBV-C/HGV isolates from blood donors (B), haemodialysis (D) and patients with chronic liver disease (L) from the province of KwaZulu Natal (KZN), South Africa. Sequences were compared with GBV-C/HGV or representative variants of GBV-C/HGV (sources listed in Section 2). Symbols: '.', similar nucleotide to GBV-C; '', nucleotide not determined; and '–', gap introduced to preserve alignment.

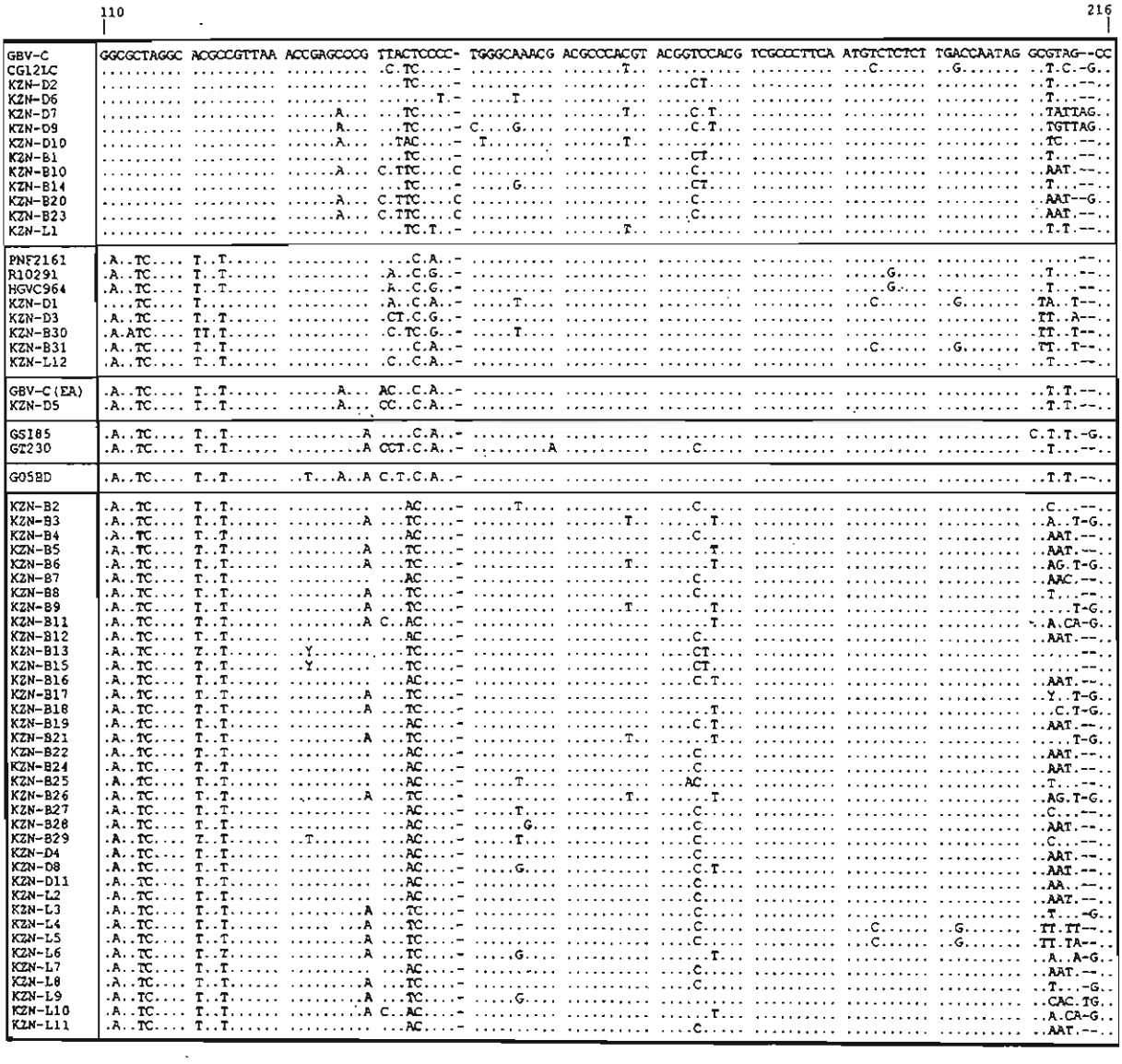


Fig. 1. (*Continued*)

ported by 88% of bootstrap replicates (Fig. 2). Eleven isolates clustered as group 1, with a bootstrap support of 90%. Six additional isolates clustered as group 2 variants, five of which grouped with 2a (bootstrap support of 91%) and one (KZN D5) with 2b (bootstrap support of 87%). Although this analysis did not strongly support the groupings of groups 2a and 2b sequences (58% of replicates), this analysis included the group 3 isolate HGVC 964 which has previously been shown to cluster with group 2 isolates upon phylogenetic analysis of the 5′NCR (An et al., 1997; Muerhoff et al., 1997; Smith et al., 1997). Analysis of nucleotide 1–201 in this alignment but excluding HGVC 964 produced very similar results except that the bootstrap support for group 2 isolates was increased to 75% (data not shown). Sequences in groups 2 and 3 together

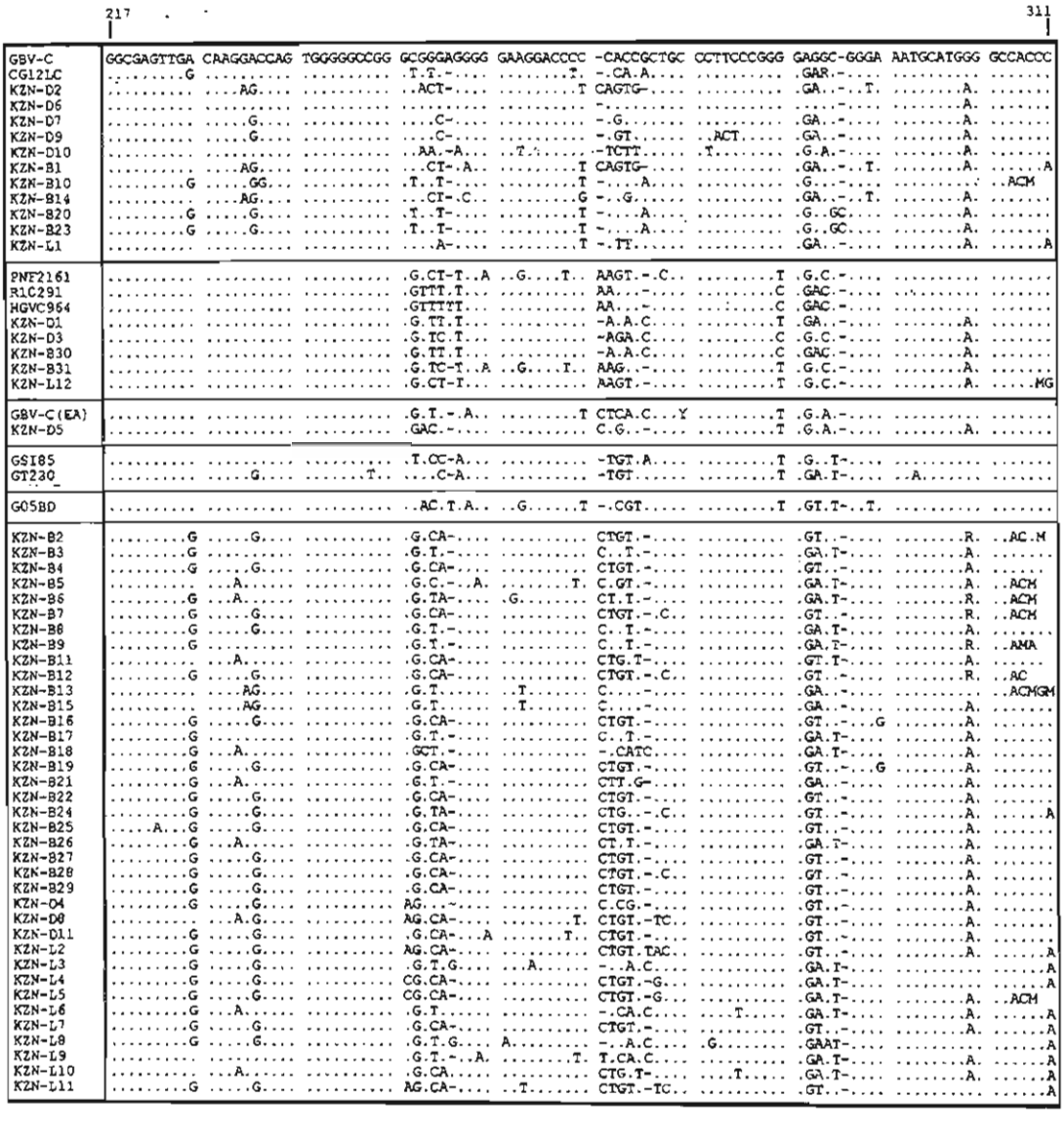
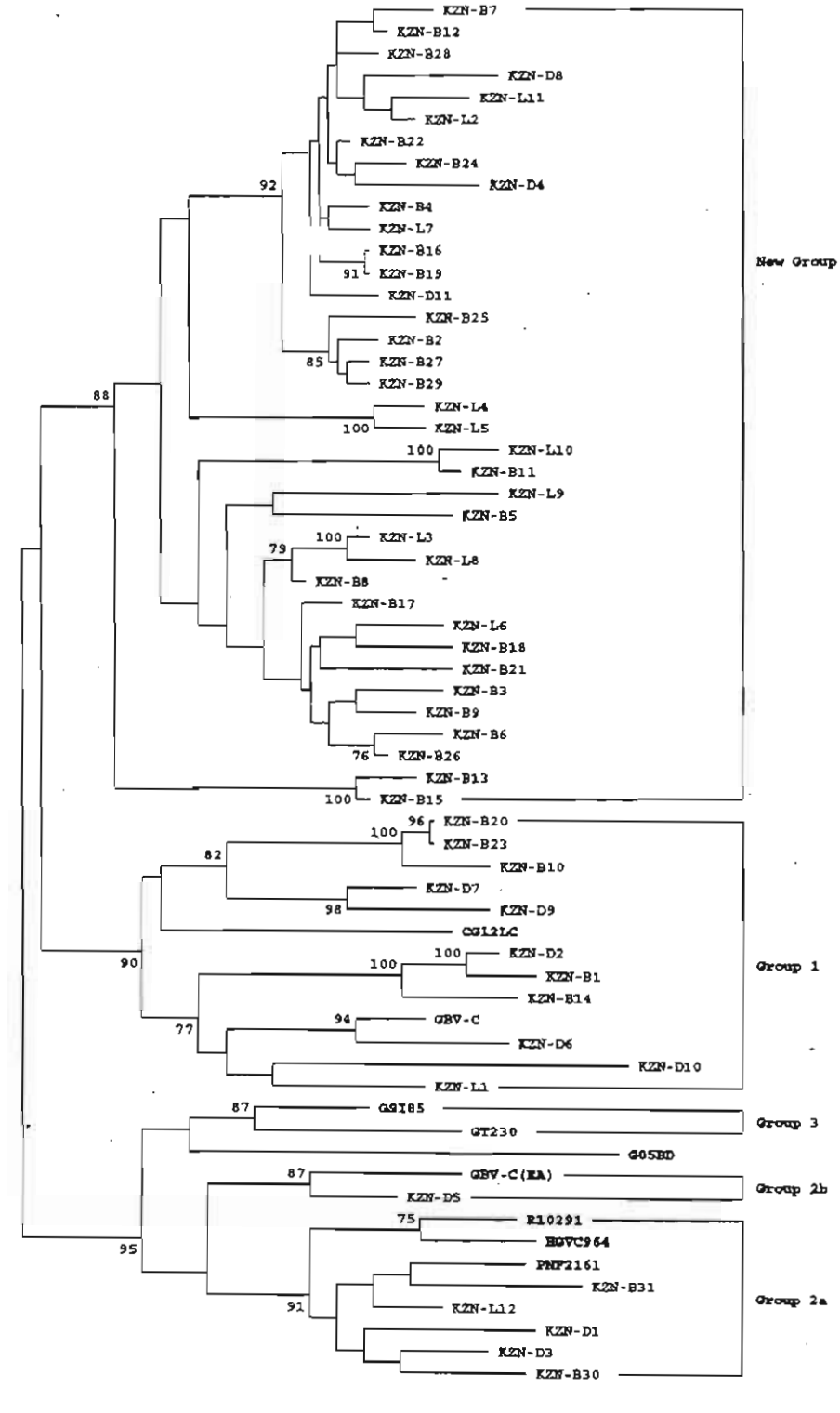


Fig. 1. (*Continued*)

with that of isolate G05BD grouped separately from sequences of group 1 and the new South African sequences (bootstrap support 90%). None of our isolates clustered with group 3 isolates described from Southeast Asia. There was a high degree of overlap in the ranges of distances between and within groups (data not shown). GBV-C/HGV infects all racial groups in the province of KwaZulu Natal, South Africa. The new variant of GBV-C/HGV' seems to be more prevalent amongst Africans than amongst Indians, Whites or 'Coloureds' (Table 1).

4. Discussion

Several groups have examined the genetic variation in GBV-C/HGV by comparing sequences


KZN-B7
KZN-B12
KZN-B28
KZN-D8
KZN-L11
KZN-L2
KZN-B22
KZN-B24
KZN-D4
KZN-B4
KZN-L7
KZN-B16
KZN-B19
KZN-D11
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KZN-B20
KZN-B23
KZN-B10
KZN-D7
KZN-D9
CG12LC
KZN-D2
KZN-B1
KZN-B14
GBV-C
KZN-D6
KZN-D10
KZN-L1
G9I85
GT230
G05BD
GBV-C(HA)
KZN-D5
R10291
BGVC964
PNP2161
KZN-B31
KZN-L12
KZN-D1
KZN-D3
KZN-B30
92
91
85
88
100
100
79
100
98
82
96
100
90
100
77
94
87
87
75
95
91
New Group
Group 1
Group 3
Group 2b
Group 2a
Scale: equals a distance of 0.0131

Fig. 2.

from different segments of the GBV-C/HGV genome. Analysis of complete genome sequences suggests that GBV-C/HGV can be classified into three groups based on pairwise distances greater than 0.12 over the entire genome (Okamoto et al., 1997) or into four or five groups based upon the phylogenetic analysis of complete genome sequences (Takahashi et al., 1997). However, studies with NS3, NS5A and envelope regions have been unsuccessful or contradictory in defining groups of GBV-C/HGV isolates (Kao et al., 1996; Muerhoff et al., 1997; Pickering et al., 1997; Viazov et al., 1997; Lim et al., 1998). In contrast, analysis of regions within the 5′NCR allows the discrimination of three major groupings that correlate with the geographic origin of the isolates and with the analysis of complete genome sequences (Muerhoff et al., 1996, 1997; Smith et al., 1997). Based on this assumption, phylogenetic analysis of the 5′NCR of our isolates identified three variants of GBV-C/HGV in KwaZulu Natal, South Africa with group 1 which is found predominantly in West and Central Africa; group 2 which is found in Europe and North America but also including isolates from Pakistan, Japan and East Africa, and a new group of variants whose sequences are different from all other known HGV/GBV-C sequences published to date. This new group is supported by bootstrap analysis (88% of replicates) and although they cluster with group 1 sequences (bootstrap value of 90%) they are distinct from any previously described African group 1 isolates (Muerhoff et al., 1996, 1997; Smith et al., 1997).

These new South African variants were also distinct from other GBV-C/HGV groups when phylogenetic analysis was based on a shorter region of the 5′NCR previously shown to reproduce the groupings observed for the complete genome sequences (Smith et al., 1997) (data not shown). However, recent evidence suggests that phylogenetic analysis of the 5′NCR may not always provide an accurate guide to the relationship of complete genome sequences. HGVC 964 is a group 3 isolate based on the analysis of the complete genome sequence (Takahashi et al., 1997) but groups with group 2a isolates when analysis is confined to the 5′NCR (An et al., 1997; Muerhoff et al., 1997; Smith et al., 1997). Similarly, the isolate G05BD appears to represent a distinct phylogenetic group by analysis of the complete genome sequence (Takahashi et al., 1997) but groups with groups 2 and 3 sequences upon analysis of the 5′NCR sequence (Simmonds and Smith, unpublished results). In addition, the analysis of complete genome sequences do not provide any evidence for the groupings as suggested by our analysis of the 5′NCR sequences (Smith and Simmonds, unpublished data). Finally, the high degree of overlap in the ranges of sequence distances between and within groups (data not shown) does not permit the establishment of 'cut-off' distances that define different groups or subgroups. Confirmation that the novel South African variants described here comprise a new grouping of GBV-C/HGV will require sequence analysis of complete virus genomes.

Current information suggests that diversity amongst African isolates of GBV-C/HGV is greater than amongst the other major groupings, consistent with the possibility that this virus may have emerged in Africa (Tanaka et al., 1998). Further evidence for this possibility comes from

Table 1
GBV-C/HGV groups according to race

Race	GBV-C/HGV groups					
	1	2a	2b	3	New	Total no.
Africans	7	2	–	–	32	41
Indians	2	2	1	–	–	5
Whites	1	–	–	–	5	6
'Coloureds'[a]	1	1	–	–	–	2

[a] 'Coloureds': persons of mixed origin.

Fig. 2. Phylogenetic tree of GBV-C/HGV 5′NCR sequences. A neighbour joining tree was constructed from p-distances between representative published nucleotide sequences (GenBank/EMBL; accession numbers in parentheses) and from South African isolates (KZN-KwaZulu Natal, South Africa; KZN D1-D11 for haemodialysis patients, KZN L1-L12 for CLD patients, KZN B1-B31 for blood donors). Bootstrap values greater than 75% are indicated (500 replicates).

the recent discovery of a virus closely related to GBV-C/HGV infecting wild-caught chimpanzees (Adams et al., 1998; Birkenmeyer et al., 1998). It is possible that sequences detected in the current study are a further reflection of the extensive human population diversity in the southern area of Africa.

Phylogenetic analysis of the 5'NCR sequences of our isolates suggests that at least three groups (groups 1, 2 and a new variant) of GBV-C/HGV are present in the province of KwaZulu Natal, South Africa.

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Phylogenetic analysis of GBV-C/hepatitis G virus

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Comparison of 33 epidemiologically distinct GBV-C/hepatitis G virus complete genome sequences suggests the existence of four major phylogenetic groupings that are equally divergent from the chimpanzee isolate GBV-C_{tro} and have distinct geographical distributions. These four groupings are not consistently reproduced by analysis of the virus 5′-noncoding region (5′-NCR), or of individual genes or subgenomic fragments with the exception of the E2 gene as a whole or of 200–600 nucleotide fragments from its 3′ half. This region is upstream of a proposed anti-sense reading frame and contains conserved potential RNA secondary structures that may be capable of directing the internal initiation of translation. Phylogenetic analysis of this region from certain South African isolates is consistent with previous analysis of the 5′-NCR suggesting that these belong to a fifth group. The geographical distribution of virus variants is consistent with a long evolutionary history that may parallel that of pre-historic human migrations, implying that the long-term evolution of this RNA virus is extremely slow.

Introduction

The similarity in genome organization between GB virus-C/hepatitis G virus (GBV-C/HGV) and hepatitis C virus (HCV) has led to the naïve expectation that variation of these closely related and persistent flaviviruses might also be similar. However, our limited understanding of the causal reasons for virus variability is underscored by the increasing evidence that these viruses vary in quite different ways. Although both viruses have a similar rate of nucleotide substitution during persistent infection [$0{\cdot}4–1{\cdot}9 \times 10^{-3}$ for HCV (Major *et al.*, 1999; Smith *et al.*, 1997*a*; Okamoto *et al.*, 1992; Ogata *et al.*, 1991), $0{\cdot}4–2{\cdot}4 \times 10^{-3}$ for HGV (Khudyakov *et al.*, 1997; Nakao *et al.*, 1997)], GBV-C/HGV lacks a hypervariable region comparable to that present at the NH_2 terminus of the HCV E2 protein (Takahashi *et al.*, 1997*a*; Nakao *et al.*, 1997) and observed ratios of synonymous to nonsynonymous substitution are higher for GBV-C/HGV (30:1) than within HCV subtypes (9:1) although the latter are less divergent (Muerhoff *et al.*, 1997; Smith *et al.*, 1997*b*). In addition, while different genotypes of HCV differ by more than 30%, the most extreme GBV-C/HGV variants differ by only 14%. Previous studies have identified three (Suzuki *et al.*, 1999; Okamoto *et al.*, 1997), four (Charrel *et al.*, 1999) or five (Takahashi *et al.*, 1997*b*) phylogenetic groupings of GBV-C/HGV, although some of these groupings are weak and inconsistent between different studies. However, whereas HCV genotypes can be distinguished by phylogenetic analysis of a variety of subgenomic regions as small as 222 nt, variants of GBV-C/HGV cannot be reliably identified in this way. Systematic analysis of six complete GBV-C/HGV genome sequences revealed that congruent phylogenetic relationships were obtained for only a minority of 300, 600 and 1200 nt fragments, and that the optimal region was all or part of the 5′-noncoding region (5′-NCR) (Muerhoff *et al.*, 1997; Smith *et al.*, 1997*b*).

At present 33 epidemiologically unrelated GBV-C HGV

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complete virus genome sequences are available, including three of group 1 and several isolates of uncertain relationship to previously defined groupings. In addition, the recent discovery of closely related chimpanzee viruses (Adams *et al.*, 1998) and the availability of a complete genome sequence (GBV-C_{tro}) (Birkenmeyer *et al.*, 1998) allows phylogenetic trees to be constructed using a more appropriate outgroup. We have therefore undertaken a re-analysis of the phylogenetic relationships of GBV-C/HGV complete genome sequences, the extent to which these can be reproduced by analysis of subgenomic regions and the implications of virus geographical variation for theories about its evolutionary history.

Methods

■ **Nucleotide sequences.** Nucleotide sequences were obtained from GenBank and manipulated and aligned using Simmonic 2000 software (P. Simmonds, unpublished). Nucleotide positions of aligned sequences are numbered relative to the AUG codon at the beginning of the E1 gene of the prototype isolate GBV-C (U36380; Leary *et al.*, 1996*b*). The 32 other epidemiologically unrelated complete genome sequences were AB003288–AB003293 (Takahashi *et al.*, 1997*b*), AB013500 (Saito *et al.*, 1999), AF104403 (Charrel *et al.*, 1999), AB013501 (Konomi *et al.*, 1999), AF031829 (Bukh *et al.*, 1998), D87255 (Shao *et al.*, 1996), D90600, D90601 (Okamoto *et al.*, 1997), U44402, U45966 (Linnen *et al.*, 1996), U63715 (Erker *et al.*, 1996), AB008342 (Kaneko *et al.*, 1998), AF006500 (L. Lu *et al.*, unpublished). D87263 (Nakao *et al.*, 1997), D87708–D87715 (Katayama *et al.*, 1998), U75356 (Y. S. Zhou and H. T. Wang unpublished), U94695 (Wang *et al.*, 1997), AB018667 and AB021287 (H. Naito & K. Abe, unpublished), together with the outgroup AF070476 (GBV-C_{tro}; Birkenmeyer *et al.*, 1998). The partial sequence (positions −396 to 6118) of an additional isolate from Thailand (K-10) was made available by Sirirrug Songsivilai (personal communication). The boundaries between genes were as given in Charrel *et al.* (1999).

■ **Serum samples.** Sera were collected from individuals from Papua New Guinea, Sudan and The Gambia as part of population-based hookworm or malaria surveys, from pregnant women in the Democratic Republic of Congo (Mokili *et al.*, 1999) and from blood donors from Saudi Arabia. Sera from South Africa were as described previously (Sathar *et al.*, 1999).

■ **PCR amplification of GBV-C/HGV genomes.** RNA was extracted from plasma using a proteinase K–SDS lysis buffer (Jarvis *et al.*, 1994). The 5′-NCR was detected by nested RT–PCR amplification using the outer primers 5′ TGCCACCCGCCCTCACCCGAA 3′ (positions −21 to −41) and 5′ AGGTGGTGGATGGGTGAT 3′ (−443 to −426), and the inner primers 5′ GGRGCTGGGTGGCCYCATGCWT 3′ (−76 to −97) and 5′ TGGTAGGTCGTAAATCCCGGT 3′ (−415 to −397). Amplification reactions were 30 cycles for each round and consisted of 94 °C for 36 s, 55 °C for 42 s and 72 °C for 90 s.

For samples that were PCR positive for the 5′-NCR [7/74 (9·4%) Papua New Guinea, 2/66 (3%) Sudan, 3/74 (4%) The Gambia and 1/48 (2%) Saudi Arabia], the E2 region was reverse transcribed and amplified from RNA purified in a combined reaction using a standard buffer system (Access PCR, Promega) according to the manufacturer's instructions. Two sets of primers were used to amplify adjoining regions of the E2 gene. Set 1 consisted of outer primers 5′ GCCTCHGCCAGCTTCATCAGRTA 3′ (1682–1660) and 5′ GGYAAYCCGGTGCGGTCVCCCYTGC 3′ (1255–1279), and inner primers 5′ AAAYACAAARTCCARVAGCARCCA 3′ (1650–1627) and 5′ TCCTACRCCATGACCAARATCCG 3′ (1288–1310). Amplification conditions were 30 cycles of 94 °C for 18 s, 55 °C for 21 s and 72 °C for 90 s. Set 2 consisted of outer primers 5′ ARCTYYGAACACCRSCGVACCAG 3′ (1499–1477) and 5′ GCCASYTGYACCATAGCYGC 3′ (979–998), and inner primers 5′ ACCCRAACGTYCCRGTBGGAGGC 3′ (1375–1353) and 5′ GTNGYBGAGCTSTYCGAGTGGGG 3′ (1027–1046).

The region of NS5A where duplications are observed in some isolates (Tanaka *et al.*, 1998) was amplified for 5′-NCR-PCR positive samples from the Democratic Republic of Congo ($n = 7$), The Gambia ($n = 4$), Sudan ($n = 2$) and Papua New Guinea ($n = 8$) using outer primers 5′ CACAATAGGCTGTATGGTTCTGG 3′ (positions 6736–6714) and 5′ CCATCGCCWGCACTWATCTCGG 3′ (positions 6409–6430), and inner primers 5′ TACRGARAGRGCCACRTTGAAGAC 3′ (positions 6573–6550) and 5′ ACNGAGAGCAGCTCAGATGAG 3′ (positions 6433–6453). Amplifications were started at 80 °C followed by 30 cycles of 94 °C 18 s, 52 °C 21 s and 72 °C 90 s. The size of amplified DNA fragments was assessed by electrophoresis through 4% Metaphor agarose gels, with expected sizes of 140 or 180 bp for fragments with and without a duplication.

■ **Nucleotide sequencing and phylogenetic analysis.** Nucleotide sequences of amplified fragments were obtained by direct sequencing of amplified genome regions using Thermosequenase (Amersham) in reactions containing ^{33}P-labelled dideoxynucleotides. Potential RNA secondary structures were investigated using RNADraw version 1.0 (Matzura, 1995).

Complete coding region sequences were analysed by three different methods. (1) Distances were estimated using an F84 model of substitution (Felsenstein, 1984), which allows for unequal transition/transversion rates and unequal base frequencies. 100 bootstrapped datasets, distance matrices, neighbour joining trees and a consensus tree were produced using SEQBOOT, DNADIST, NEIGHBOR and CONSENSE, all part of the PHYLIP package (Felsenstein, 1993). (2) Synonymous and non-synonymous distances were estimated using Method 1 of Nei & Gojobori (1986) for 200 datasets produced by bootstrapping codons (D. Haydon, unpublished). Neighbour joining trees and a consensus tree were produced as above. (3) Distances were estimated using a Tamura & Nei (1993) model of substitution, a more general form of the F84 model that also allows for unequal transition rates between purines and pyrimidines, together with rate heterogeneity modelled as a proportion of invariable sites plus eight rates taken from a discrete gamma distribution using parameters estimated from the data. 100 bootstrapped datasets were produced using SEQBOOT, and distance matrices calculated with the programs PUZZLE version 4.0.2 (Strimmer & von Haeseler, 1996) and PUZZLEBOOT version 1.01 (Holder & Roger, 1999) using parameters estimated from the dataset. Trees with the lowest least-squares deviation were produced using the program FITCH applying global search, and a consensus tree produced using CONSENSE. Phylogenetic trees of subgenomic regions were produced with MEGA (Kumar *et al.*, 1993) using the Kimura 2-parameter model of substitution on datasets of 100 bootstrap replicates.

In order to determine which GBV-C/HGV group was closest to the outgroup GBV-C_{tro} (AF070476), we used a reduced dataset consisting of three sequences from each group (Group 1, HGU36380, AB003291, AB013500; Group 2, AB013501, U44402, U63715; Group 3, AB003288, D90601, U94695; Group 4, AB003292, AB018667, AB021287). We estimated the likelihood of the outgroup clustering with each of the four groups under four different models of nucleotide substitution: (i) an F84

model; (ii) an F84 model allowing for rate variation modelled using a discrete gamma distribution with eight categories; (iii) as for (ii), but allowing for different transition/transversion ratios, different base frequencies, different rates and different levels of rate heterogeneity for each gene; and (iv), as for (ii) but allowing parameters to differ for each codon position. These models were fitted and the bootstrap support for each cluster compared using the fast approximate bootstrap procedure of Kishino & Hasegawa (1989) as implemented in the PAML version 2.0 package (Yang, 1999). Similar analyses of amino acid sequences were carried out using Phylip 3.57c (Kimura substitution matrix), or RELL [Jones *et al.* (1992) substitution matrix)] assuming gamma rate heterogeneity.

Results

Complete genome sequences

Phylogenetic analysis of the coding region of 33 epidemiologically unrelated complete GBV-C/HGV genome sequences yielded evidence for four distinct phylogenetic groupings each of which was supported by high levels of bootstrap support (Fig. 1). These groupings correspond to the three groupings previously identified from the analysis of complete genome sequences (Okamoto *et al.*, 1997) together with an additional group consisting of an unclassified Japanese sequence (Takahashi *et al.*, 1997*b*) and three sequences from Thailand (K-10; Sirirrug Songsivilai, unpublished results), Vietnam (AB018667) and Myanmur (AB021287). Similar groupings and levels of bootstrap support were obtained when the analysis was confined to either synonymous or non-synonymous sites, or to amino acid substitutions, if comparisons were extended to include the entire virus genome, or if the chimpanzee isolate GBV-C_{tro} was used as an outgroup (data not shown).

Although subgroupings of groups 1, 2 and 3 have been distinguished by phylogenetic analysis of subgenomic regions (Muerhoff *et al.*, 1996; Takahashi *et al.*, 1997*a*; Muerhoff *et al.*, 1997) there was only limited evidence from the analysis of complete genome sequences for subgroupings. Three group 3 isolates from China (U75356, U94695 and AF006500) grouped together with high bootstrap support, while the isolates AB013501 and U63715 grouped separately from the other

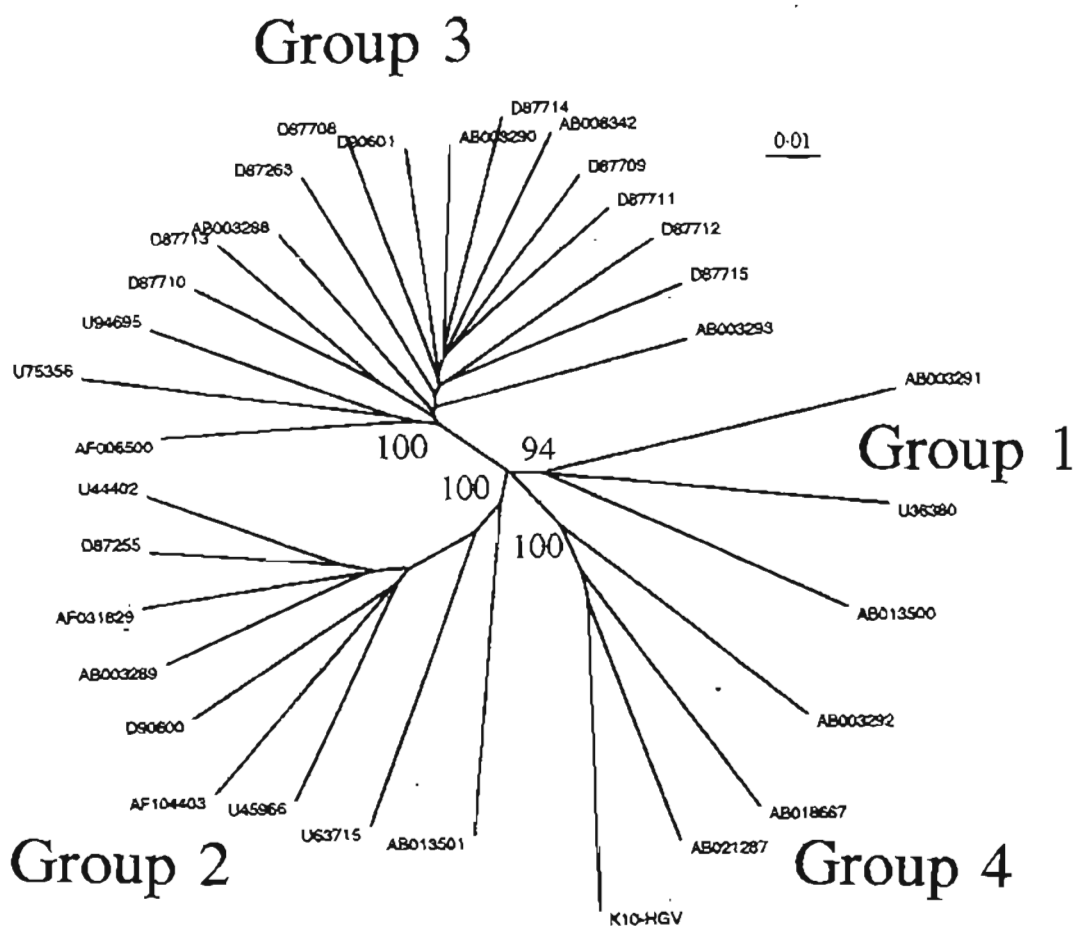

Fig. 1. Phylogenetic tree of GBV-C/HGV complete coding region sequences. Maximum likelihood distances between sequences were calculated with Phylip DNADIST (ts:tv = 2, assuming no rate variation between sites), and used to produce a neighbour joining tree with Phylip NEIGHBOR. Bootstrap values (100 replicates) were obtained with the SEQBOOT and CONSENSE options of Phylip. The tree was produced using Treeview version 1.5.

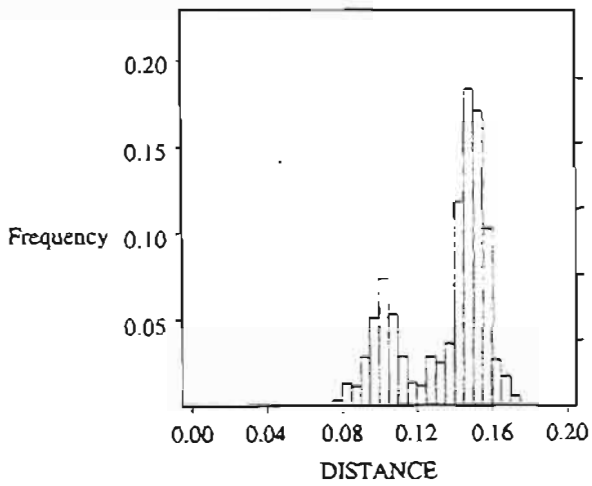

Fig. 2. Frequency distribution of pairwise evolutionary distances between GBV-C/HGV complete coding region sequences. Distance were calculated as in Fig. 1.

group 2 isolates (group 2a) and isolate AB003292 from Japan was relatively divergent from other group 4 isolates. However, analysis of pairwise distances between GBV-C/HGV sequences revealed a distribution with only two overlapping peaks (Fig. 2) corresponding to comparisons between sequences belonging to different groups (distances of 0·131–0·171) or within one of the four phylogenetic groups (0·076–0·137). The overlap between these two distributions comprised 18/528 pairwise comparisons (3·5%), more than half of which involved a divergent group 2 isolate from South America (AB013501).

A previous study suggested that certain group 1 isolates containing a duplicated region within NS5A (e.g. isolate AB003291) were ancestral to other GBV-C/HGV isolates relative to GBV-A (Tanaka *et al.*, 1998). However, this was not confirmed when the analysis was extended to complete genome sequences (Suzuki *et al.*, 1999), or when we investigated the relative likelihood with which the more closely related GBV-C_{uro} isolate clustered with three representatives from each group (Table 1). Under four different models of substitution, clustering with group 1 could be rejected at the 5% level. Although clustering with group 2 gave the best fit to the data for the models which included rate heterogeneity, differences between genes in transition/transversion ratios, rate heterogeneity and base frequencies were relatively small (results not shown), and the bootstrap support for this clustering was not high enough to be conclusive. The best fit to the data were obtained with the model in which parameters could vary according to position within codons, reflecting the strong bias against non-synonymous substitution. Bootstrap resampling analysis using simpler models of substitution at synonymous or non-synonymous sites supported group 4 as being ancestral, while analysis of amino acid sequences supported either groups 1 or 3 as being ancestral depending on the substitution matrix used. Hence, our analysis does not consistently place any one of the four human GBV-C/HGV groups as being ancestral.

Furthermore, the duplicated region may be derivative rather than ancestral since this requires fewer evolutionary steps (Suzuki *et al.*, 1999). Most isolates that lack the duplication have instead an 8 nt direct repeat (ACCCCGTC, positions 6457–6464 and 6487–6494) flanking a sequence with the potential to form a structure with an 11 nt stem and a 3 nt loop. The formation of this stem–loop during RNA synthesis could lead to slippage and mispairing between the direct repeats resulting in the duplication of the hairpin loop and the direct repeat. We did not detect the duplication in NS5A amongst other isolates from Africa (n = 13) or Papua New Guinea (n = 8) or other NS5A sequences available in GenBank (n = 94).

Subgenomic regions

Next, we investigated the extent to which subgenomic regions of GBV-C/HGV bear the same phylogenetic relationships as do entire virus genomes. Analysis of individual virus genes failed to produce congruent phylogenetic trees with the sole exception of the E2 gene (Fig. 3). Similar analysis of subgenomic fragments of 2000, 1000 or 500 nt produced congruent trees only for fragments including the COOH-

Table 1. Log likelihood and approximate bootstrap support for the clustering of the outgroup GBV-C_{uro} with groups 1–4 under different models of substitution

Model of substitution	Log likelihood for clustering (bootstrap support)			
	Group 1	Group 2	Group 3	Group 4
F84	−54051·387 (0·44%)	−54035·720 (10·60%)	−54032·371 (28·72%)	−54022·015 (60·24%)
F84 with rate variation	−49041·927 (1·58%)	−49038·439 (59·52%)	−49041·905 (2·46%)	−49039·305 (36·44%)
F84 with rate variation gene specific	−49018·110 (1·52%)	−49014·258 (67·18%)	−49018·031 (3·24%)	−49015·900 (28·06%)
F84 with rate variation codon specific	−47139·032 (0·5%)	−47133·093 (78·82%)	−47137·713 (9·06%)	−47137·046 (11·62%)

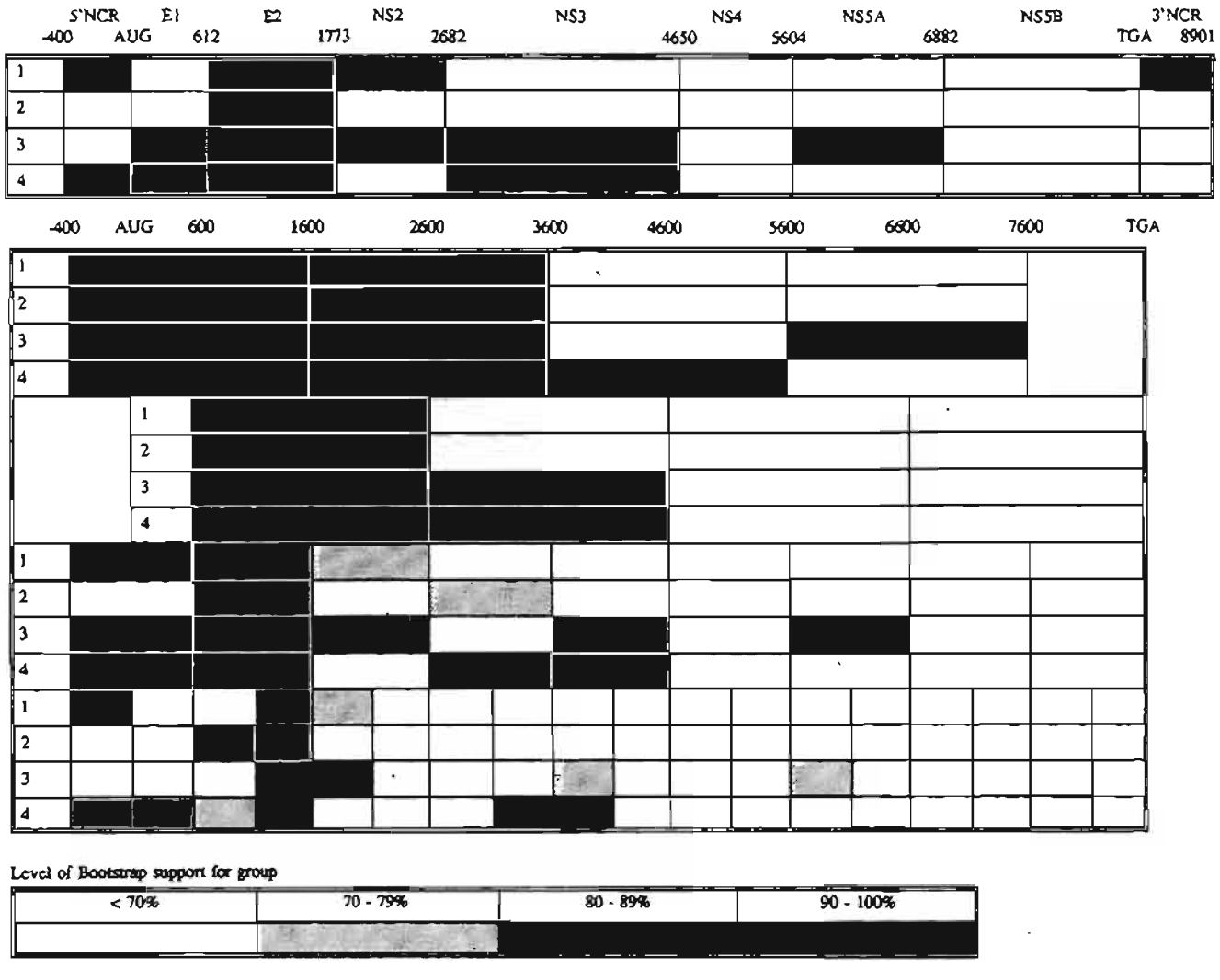

Fig. 3. Congruence between phylogenetic analysis of complete genomes and subgenomic fragments. The level of bootstrap support for phylogenetic groupings 1–4 is indicated by the degree of shading for different genes and subgenomic fragments.

terminal region of the E2 gene. Analysis of the entire 3′-terminal half of the virus genome or any of its subfragments failed to produce congruent trees.

In most cases, sequences that grouped aberrantly were not consistently associated with a particular group. The only exception was the group 4 sequence AB0021287 that grouped with group 2 sequences for subgenomic fragments encompassing the NH_2 terminus of the NS2 gene or the COOH-terminal half of the NS5B gene. Even if the assumption is made that this isolate is a recombinant between isolates of group 4 and 2 [the major GBV-C/HGV groups found in this geographical region (Naito *et al.*, 1999)], the only additional subgenomic region that then produces a congruent phylogenetic tree is a 1000 nt fragment encompassing the COOH terminus of the E2 gene and most of the NS2 gene.

Since a 500 nt fragment of the E2 gene could reproduce the phylogenetic relationships of the complete genome sequences, we next analysed this region in more detail (Figs 4 and 5). Congruent phylogenetic trees were produced using a region as small as 200 nt (positions 1344–1543). The shortest region that gave a congruent tree with more than 98% bootstrap support for each group was the 600 nt region from positions 994–1594. This region also produced a congruent tree when analysed at synonymous sites (> 85% support) but not at nonsynonymous sites.

Previous studies have suggested that the phylogenetic relationships of GBV-C/HGV complete genome sequences can be reproduced by analysis of the whole or part of the 5′-NCR (Muerhoff *et al.*, 1996; Smith *et al.*, 1997*b*; Muerhoff *et al.*, 1997). The extreme 5′-terminal sequence is available for only a single group 1 and none of the group 4 isolates, so an adequate analysis can only be carried out from positions −388 to −1. Analysis of this region and of various subfragments failed to produce congruent phylogenetic trees (Fig. 6), with

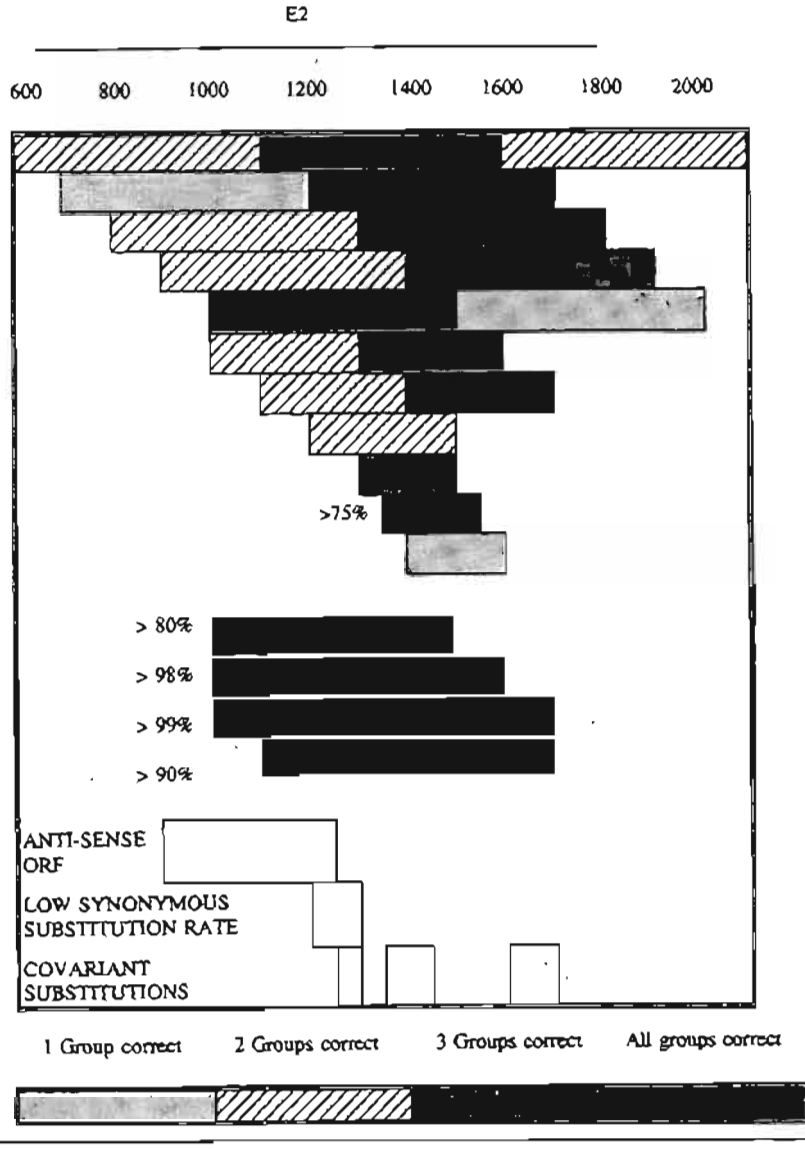

Fig. 4. Congruence of phylogenetic relationships produced using fragments of the E2 gene. The extent to which phylogenetic groupings observed from analysis of complete genome sequences are supported by comparison of fragments of the E2 gene is indicated by the level of shading. The number of groups supported by more than 70% of bootstrap replicates is indicated. The minimum degree of bootstrap support is given for selected examples. Also indicated are the position of an anti-genome reading frame (Kondo *et al.*, 1998), a region where synonymous substitutions are suppressed (Simmonds & Smith, 1999) and regions where the presence of covariant nucleotide substitutions suggests the presence of RNA secondary structures (Simmonds & Smith, 1999).

two sequences responsible for the majority of aberrant groupings AB013500, an extreme group 2 isolate from Bolivia, groups with group 3 isolates for the 5′-NCR and some subgenomic fragments in the 3′ half of the genome, although the 5′-NCR is atypical. Similarly, U75356, an unpublished group 3 isolate from China, has a 5′-NCR sequence similar to but distinct from those of group 2 isolates. This sequence also has a frameshift within NS5A and an unusual sequence at the 3′ terminus (Smith *et al.*, 1997*b*). Hence, it is uncertain whether these isolates represent recombinants (An *et al.*, 1997) or divergent variants with unique 5′-NCR sequences. If these two sequences are excluded from the dataset, a congruent tree is obtained when the region −388 to −1 is analysed. However, subfragments of this region, including those previously identified as reproducing the phylogenetic relationships of group 1 and 2 isolates (Smith *et al.*, 1997*b*), provide < 70% bootstrap support for either groups 3 or 4.

Evidence for additional groupings from the analysis of subgenomic regions

Having identified a region within the E2 gene that consistently reproduces the phylogenetic relationships of complete genome sequences, we compared published sequences of this region for evidence of additional GBV-C/HGV groups. Of 16 additional sequences for the region 994–1594 (accession nos U87653–U87664, AF063827, AF063828, AF063830, AF017533), 14 (from USA, Jamaica, Greece, UK and Egypt) grouped with group 2, and one each with groups 1 (USA) and 3 (Hong Kong) (> 99% support for all groups; data not shown). Similarly, nine additional sequences of the region 1414–1693 from Italian patients (AF015842–AF015862) clustered with group 2, although as expected from the analysis of complete genome sequences (Fig. 4), the bootstrap support was low (44%). In contrast, E2

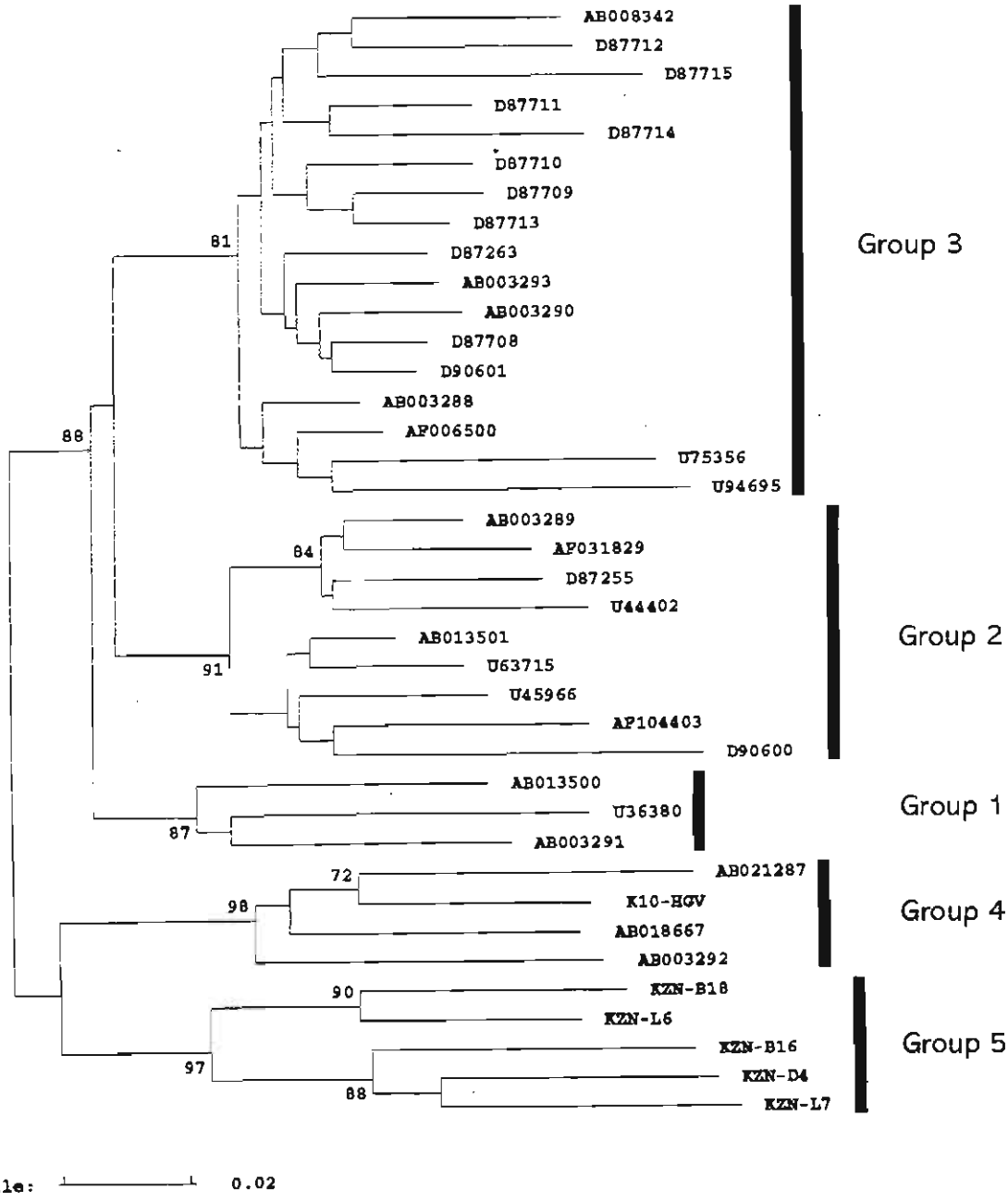

Fig. 5. South African isolates form an additional E2 phylogenetic grouping. A phylogenetic tree was constructed by neighbour joining using Jukes–Cantor distances for the region 1146–1496 for selected South African isolates and for the corresponding region from complete genome sequences. Bootstrap values (500 replicates) for branches are indicated where they were greater than 70%.

sequences for the 350 nt region 1146–1495 of five GBV-C variants from South Africa with unusual 5′-NCR sequences (Sathar *et al.*, 1999) formed an additional group (Fig. 5), while other South African isolates with 5′-NCR sequences that clustered with groups 1 ($n = 3$) or 2 ($n = 2$) clustered with groups 1 or 2 respectively. Isolates from Papua New Guinea had E2 sequences that clustered with group 2 ($n = 2$) or group 4 ($n = 2$), while an isolate from Sudan ($n = 1$) clustered with group 2.

Similar analysis of 5′-NCR sequences is complicated by the large number of sequences available within this region (1409 accessions) and the inconsistent phylogenetic relationships of all but the largest fragments (Fig. 6). Visual examination for motifs distinct from those typical of groups 1, 2, 3 and 4 revealed a small number of unusual sequences, but of these only the variants from South Africa (Sathar *et al.*, 1999) described above, and isolates from Spain (Lopez-Alcorocho *et al.*, 1999) grouped separately from the 5′-NCR sequences of

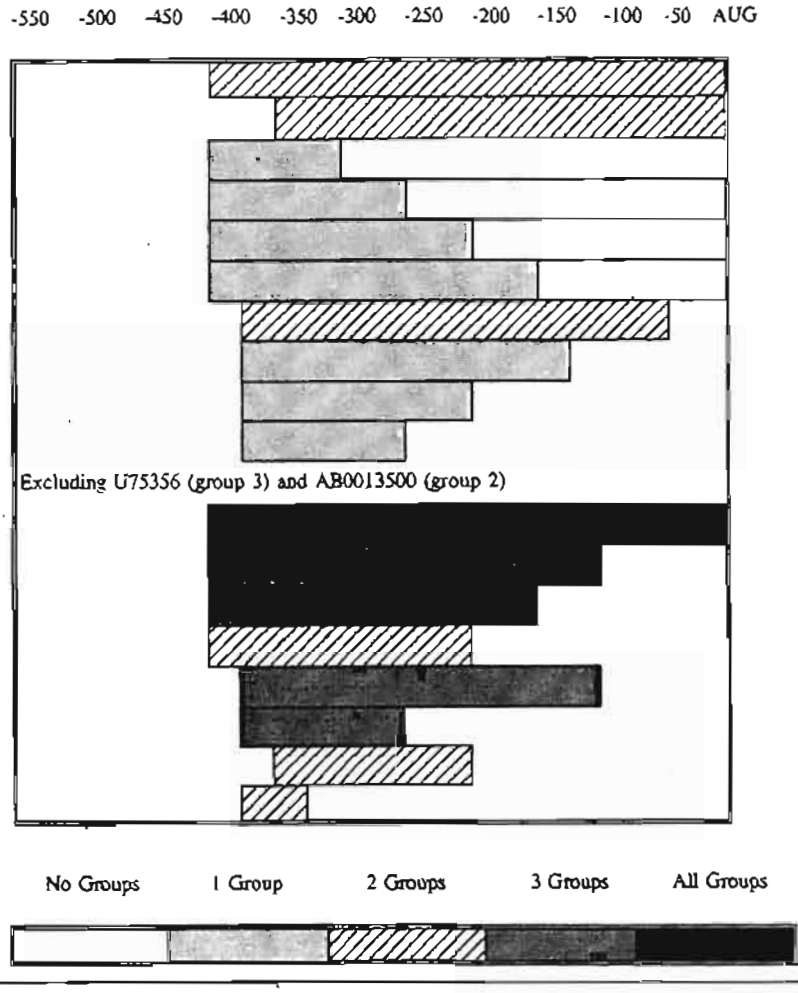

Fig. 6. Congruence of phylogenetic relationships produced using fragments of the 5′-NCR. The extent to which phylogenetic groupings observed from analysis of complete genome sequences are supported by comparison of fragments of the 5′-NCR is indicated by the level of shading. Groupings were considered to be supported if they were observed in > 70% of bootstrap replicates.

complete genome sequences for the region −388 to −1. These later isolates could represent recombinants since they have motifs typical of group 3 sequences between positions −489 and −459 but the remainder of the 5′-NCR is similar to, although distinct from, that of group 2 isolates.

Discussion

Identification of phylogenetic groups

This analysis of complete genome sequences of GBV-C/HGV identifies four main phylogenetic groupings (Fig. 1). The existence of the fourth group had been suggested from the analysis of 5′-NCR sequences of Southeast Asian isolates (Naito *et al.*, 1999). A previous phylogenetic analysis of complete genome sequences also proposed four groups in which groups 1 and 4 were combined and group 3 was split into two (Charrel *et al.*, 1999), but the published bootstrap analysis did not support these groupings. Similarly, an analysis based upon genetic distances identified five groups with group 1 split into two (Takahashi *et al.*, 1997*b*), but our analysis suggests that phylogenetic groups cannot be defined using pairwise distances (Fig. 2).

An unexpected finding of this study is that the phylogenetic relationships of complete GBV-C/HGV sequences (but not subgroupings) can be reproduced by short COOH-terminal fragments of the E2 gene (Fig. 4). Previous studies of smaller numbers of sequences have shown that phylogenetic relationships are inconsistent when comparisons are made for individual genes (Takahashi *et al.*, 1997*b*) or subgenomic fragments (Smith *et al.*, 1997*b*). Although three (Cong *et al.*, 1999) or four (Lim *et al.*, 1997) phylogenetic groupings are observed when fragments larger than 1500 nucleotides including the E2 gene are compared, interpretation of these studies is complicated by idiosyncratic labelling, the absence of group 4 isolates and the failure to assess the robustness of groupings. We show here that analysis of a 200 nt fragment from the centre of the E2 gene (positions 1344–1543) provides > 75% bootstrap support for all four phylogenetic groupings, while a 600 nt region (positions 994–1594) provided > 98% support. This is in stark contrast to other coding regions: all subgenomic fragments of 2000 nt or less that did not contain this region of E2 failed to produce congruent trees with the sole exception of a 2000 nt fragment encompassing the remainder of the E2 gene, NS2 and the NH_2-terminal half of

NS3 (Fig. 3). An important unresolved question is the extent to which some complete genome sequences represent recombinants between different groups. Inconsistent relationships were observed for some sequences between the 5′-NCR and coding regions or between different coding regions. However, the aberrant groupings were typically weak and inconsistent suggesting that these do not represent simple recombinants.

Our analysis helps to clarify previous conflicting studies on the extent to which subgenomic regions can be used to identify GBV-C/HGV phylogenetic groupings. Most early studies of virus diversity concentrated on a 118–135 nt fragment within NS3 that was the first part of the genome to be sequenced (Simons *et al.*, 1995; Masuko *et al.*, 1996; Heringlake *et al.*, 1996; Kao *et al.*, 1996; Berg *et al.*, 1996; Schreier *et al.*, 1996; Tsuda *et al.*, 1996; Schmidt *et al.*, 1996; Muerhoff *et al.*, 1997; Pickering *et al.*, 1997; Ibanez *et al.*, 1998). However, the phylogenetic conclusions of these studies appear to be unreliable since analysis of even the entire NS3 gene fails to produce congruent groupings. Inconsistent phylogenetic groupings have also been observed for the NH_2 terminus of E2 (Lim *et al.*, 1997; Muerhoff *et al.*, 1997), 354 nt of NS5A/NS5B (Viazov *et al.*, 1997) or 279 nt of NS5B (Muerhoff *et al.*, 1997). Finally, although analysis of a 2600 nt fragment containing both NS5A and NS5B produced a congruent tree for 12 isolates from groups 1, 2 and 3 (Khudyakov *et al.*, 1997), comparison of this region from the 33 complete genome sequences gave aberrant groupings of some isolates with only marginal support for groups 1 and 4 (data not shown).

The inconsistent phylogenetic relationships of GBV-C/HGV subgenomic coding regions contrasts with that observed for HCV where analysis of a variety of subgenomic regions reproduces the phylogenetic relationships of complete genomes (Simmonds *et al.*, 1994; Tokita *et al.*, 1998). This difference could arise if different regions of the GBV-C/HGV genome were subject to different evolutionary processes in which case combining these regions could produce a less reliable reconstruction than the analysis of individual regions (Bull *et al.*, 1993). However, the validity of the analysis based on complete genome sequences is supported by the correlation between their phylogenetic relationships and their geographical origin. An alternative explanation is that the amino acid sequence of the GBV-C/HGV polyprotein is well conserved (d_N:d_S 0·033, divergence < 11%) relative to HCV subtypes (d_N:d_S 0·094, divergence < 10%). Phylogenetic groupings of GBV-C/HGV therefore rely more on variation at synonymous sites, many of which are invariant or saturated, possibly because of the presence of extensive RNA secondary structures within the GBV-C/HGV genome (Simmonds & Smith, 1999). Consequently, GBV-C/HGV isolates with a common evolutionary origin may share only a small number of polymorphisms and analysis of subgenomic regions could often fail to produce congruent phylogenetic trees.

There are several potential explanations for our observation that analysis of the E2 gene or specific subfragments produces

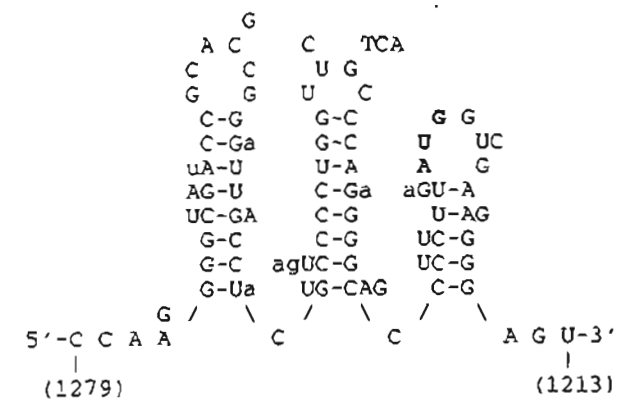

Fig. 7. Potential RNA secondary structures upstream of a potential anti-sense reading frame. Potential hairpin loops formed by the nucleotide sequence of the antisense strand corresponding to positions 1279–1213 of the sense strand are indicated. Covariant nucleotide substitutions observed amongst sequences from phylogenetic groups 1, 2 and 3 are indicated in upper case, while substitutions that disrupt the proposed structures are indicated in lower case. The conserved AUG codon at the beginning of the anti-sense reading frame is indicated by bold lettering.

phylogenetic trees congruent with those observed for complete genome sequences. First, since E2 is the most variable part of the GBV-C/HGV polyprotein (Katayama *et al.*, 1998; Erker *et al.*, 1996), the phylogenetic relationships of complete genome sequences could depend entirely on substitutions in E2. However, identical groupings are observed if E2 is excluded and most amino acid substitutions occur at the NH_2 terminus of E2 (Lim *et al.*, 1997; Katayama *et al.*, 1998; Cong *et al.*, 1999), whereas it is only the central and 3′ regions that produce congruent phylogenetic trees, and then only for synonymous rather than nonsynonymous substitutions. A second potential explanation is that this region of the genome encodes an open reading frame on the anti-sense strand (nucleotides 870–1226, Fig. 4), possibly encoding a nucleocapsid protein (Kondo *et al.*, 1998). This would constrain the accumulation of substitutions and so retain evidence of phylogenetic relationships. However, although synonymous substitutions are suppressed in this part of the genome (Muerhoff *et al.*, 1997; Simmonds & Smith, 1999), phylogenetic analysis of the anti-sense reading frame fails to produce a congruent tree (Fig. 4). Another potential source of constraint in this region of the genome would be if translation of the anti-sense reading frame was dependent on an upstream internal ribosome entry site. Indeed, substitutions in this region of the genome are frequently covariant and associated with potential RNA secondary structures (Simmonds & Smith, 1999) (Fig. 7), while RNA folding predictions for representatives of groups 1–3 identify structures with free energies one standard deviation below those of random sequences of the same base composition. However, these RNA structures were not observed in sequences from group 4 isolates, and the anti-sense reading frame is 10 residues longer in some group 3 isolates and lacks the initial AUG codon in GBV-C_{tro}.

Geographical distribution and origin of GBV-C HGV isolates

The distinct geographical distribution of GBV-C/HGV variants is consistent with their co-evolution with humans during pre-historic migrations. Group 1 and 5 isolates are African and have relatively diverse 5′-NCR sequences (Muerhoff *et al.*, 1997; Smith *et al.*, 1997*b*; Naito *et al.*, 1999; Sathar *et al.*, 1999), while the remaining groups correspond to the three main waves of human migration from Africa to Europe (group 2), northern Asia (group 3) and southern Asia (group 4). In addition, the presence of group 3 variants amongst native populations in South America (Pujol *et al.*, 1998; Gonzalez-Perez *et al.*, 1997) is consistent with the first colonization of this continent from northern Asia via the Bering Strait. Although Japanese isolates have been found belonging to groups 1–4 (Takahashi *et al.*, 1997*b*; Okamoto *et al.*, 1997), most are group 3 suggesting that the presence of other groups represents recent introductions.

Finally, since a virus related to GBV-C/HGV is present in chimpanzees (Adams *et al.*, 1998; Birkenmeyer *et al.*, 1998), while New World monkeys harbour more distantly related but species-specific variants (Bukh & Apgar, 1997; Leary *et al.*, 1996*a*) it is possible that GBV-C/HGV has been continuously present in human populations since speciation. In this case the virus appears to have evolved at an overall rate of less than 10^{-5} per site per year (Suzuki *et al.*, 1999; Simmonds & Smith, 1999), although its rate of sequence evolution measured in longitudinal studies is similar to that of other RNA viruses. Further sequence analysis of the E2 region from GBV-C/HGV variants isolated from different geographical regions may help to clarify the origins of this unusual virus.

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GB Virus C/Hepatitis G Virus (GBV-C/HGV): still looking for a disease

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Summary. GB Virus C and Hepatitis G Virus (GBV-C/HGV) are positive, single-stranded flaviviruses. GBV-C and HGV are independent isolates of the same virus. Transmission via the blood-borne route is the commonest mode, although vertical and sexual transmission is well documented. GBV-C/HGV is distributed globally; its prevalence in the general population is 10 fold higher in African countries than in non-African countries. High prevalences of GBV-C/HGV have been found in subjects with frequent parenteral exposure and in groups at high risk of exposure to blood and blood products. The clinical significance of human infection with GBV-C/HGV is currently unclear. The virus can establish both acute and chronic infection and appears to be sensitive to interferon. Only some 12–15% of chronic Non-A, B, C hepatitis cases are infected with GBV-C/HGV. A direct association with liver pathology is still lacking and it is not yet clear as to whether GBV-C/HGV is indeed a hepatotropic virus. Current evidence suggests that the spectrum of association of GBV-C/HGV infection with extrahepatic diseases ranges from haematalogical diseases, aplastic anaemia, human immunodeficiency virus (HIV)-positive idiopathic thrombocytopenia and thalassemia, through to common variable immune deficiency and cryoglobunemia.

Keywords: Flavivirus, PCR, Anti-E2, hepatitis, liver disease, clinical features

Introduction

Discovery of GBV-C and HGV

The first successful transmission of viral hepatitis from humans to nonhuman primates was achieved by Deinhardt *et al.* (Deinhardt *et al.* 1967), when serum from a 34-year-old surgeon (whose initials were GB) with acute hepatitis was inoculated into tamarins (*Saguinus* spp.). Animals inoculated with GB serum developed hepatitis, as did animals inoculated with sera of tamarins with GB serum-induced hepatitis. GB Virus C (GBV-C) was identified in the serum of a human in West Africa that contained recombinant nonstructural proteins of two other novel flaviviruses, designated GBV-A and GBV-B (Simons *et al.* 1995a). These viruses were cloned from the serum of a tamarin inoculated with the GB agent (Deinhardt *et al.* 1967) and are now known to be of animal origin. Using degenerate primers derived from the homologous sequences shared by GBV-A,

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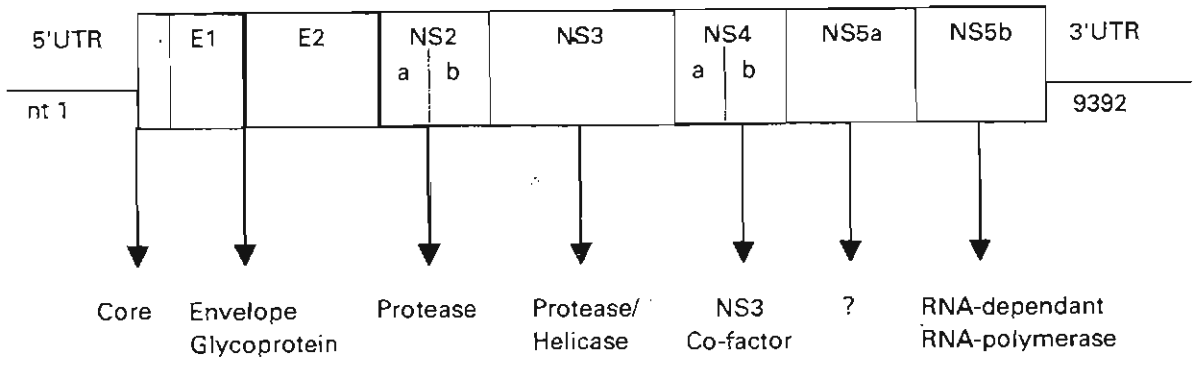

Figure 1. HGV (PNF2161) genome. Proteolytic processing and functions of the structural and non-structural proteins (nt = nucleotide; UTR = untranslated region; E = envelope; NS = nonstructural).

GBV-B, and HCV from the NS3/helicase region of these viruses, amplification products were obtained from immunoreactive sera whose sequence was different from the other viruses. The new virus was named GBV-C (Simons *et al.* 1995b; Leary *et al.* 1996). Independently, in an effort to identify additional agents for post-transfusion non-A-non-B hepatitis (NANB), Linnen *et al.* (Linnen *et al.* 1996) performed molecular cloning with plasma from a patient with presumed NANB hepatitis, and a virus like RNA sequence was identified and designated Hepatitis G Virus (HGV).

Genomic organization of GBV-C/HGV

The genomic organization of GBV-C/HGV and HCV is similar and both viruses belong to the family of *Flaviviridae*. They are both linear, positive single-stranded RNA molecules composed of about 9500 nucleotides (nt)(Figure 1).

The viral polyprotein is preceded by a 5′ untranslated region (UTR), followed by a long open reading frame (ORF) terminating with 3′ UTR (Figure 1)(Linnen *et al.* 1996). The polyprotein is cleaved into smaller fragments with different functions by host-encoded signal peptidases and viral proteases. These fragments include the envelope proteins (E1 and E2) at the amino or N-terminal end followed by nonstructural (NS) proteins (NS2, NS3, NS4, and NS5) at the carboxy or C-terminal end (Figure 1). The GBV-C/HGV genome is unusual in that the region between the 5′ UTR and the envelope proteins is absent or truncated (Simons *et al.* 1995a; Leary *et al.* 1996; Linnen *et al.* 1996). This region normally encodes the nucleocapsid/core protein that encases the viral genome. The 5′ UTR contains an internal ribosome entry site (IRES) that is capable of directing CAP-independent translation of the polyprotein (Simons *et al.* 1996). Sequence comparisons of the two prototype isolates, HGV and GBV-C, show that they have 86% and 95% homology at the nucleotide and amino-acid levels, respectively. They are therefore considered to be independent isolates of the same virus (Leary *et al.* 1996; Linnen *et al.* 1996).

Phylogenetic analysis of GBV-C/HGV

Comparisons of 33 epidemiologically distinct complete or near complete genomic sequences of GBV-C/HGV suggest the existence of four major phylogenetic groups that are equally divergent from the chimpanzee isolate, GBV-C_{trop} (Birkenmeyer *et al.* 1998), and have distinct geographical distribution (Figure 2) (Smith *et al.* 2000). Group 1 includes isolates from Ghana, West Africa and a single Japanese isolate; Group 2 includes isolates from Europe, North and South America and Japan; Group 3 includes isolates from Japan and China and the fourth group consists of isolates from South-east Asia (Figure 2) (Smith *et al.* 2000). With the sole exception of E2 gene segments, phylogenetic analysis of individual genes and subgenomic regions have failed to consistently produce congruent phylogenetic trees of the four major groups that correlate with the geographical origin of the isolates (Smith *et al.* 2000). Recently, a new variant of GBV-C/HGV whose sequences of the 5′ NCR were different from all other known GBV-C/HGV sequences was identified in the province of KwaZulu Natal (KZN), South Africa (Sathar *et al.* 1999a). Phylogenetic analysis of the E2 gene segment from certain KZN isolates is consistent with previous analysis of the 5′ NCR(Sathar *et al.* 1999a; Smith *et al.* 2000), suggesting that these belong to a fifth group (Smith *et al.* 2000)(Figure 3).The spread of geographically distinct GBV-C/HGV groups has been associated with human migration (Smith *et al.* 2000).The greater diversity amongst Group 1 African isolates compared to the

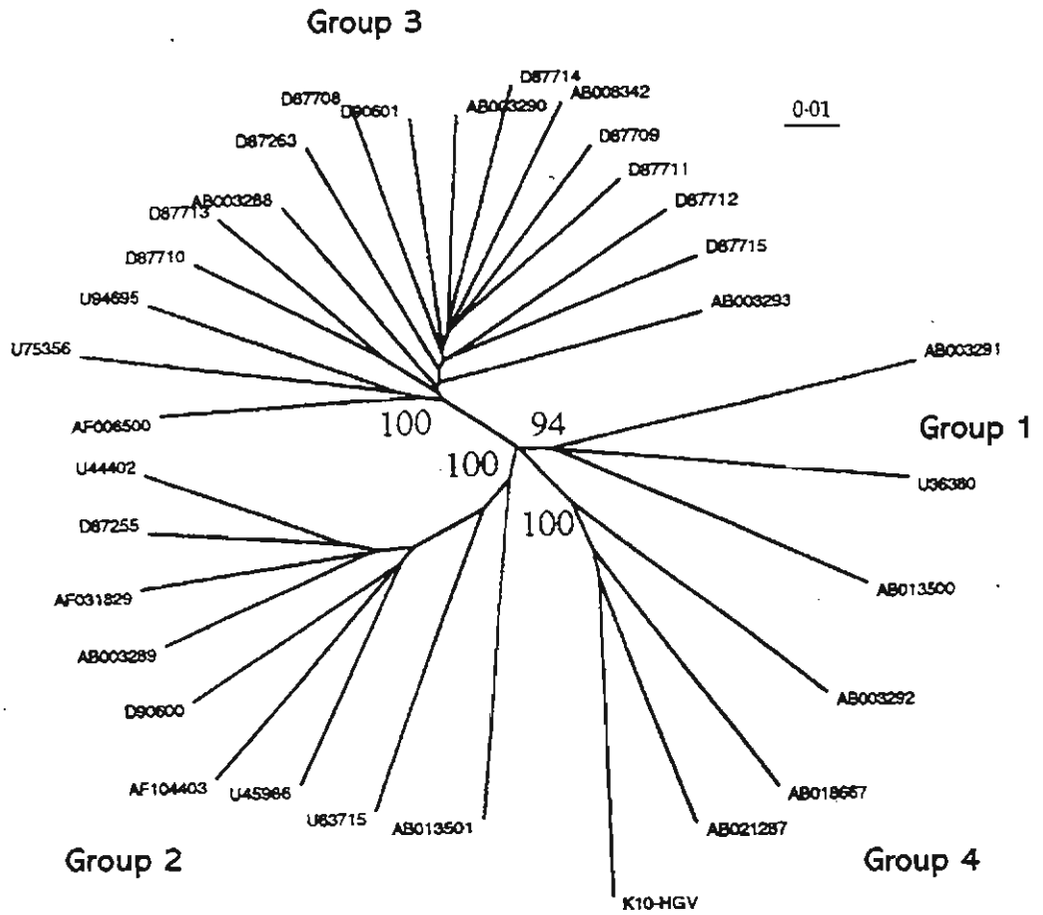

Figure 2. Phylogenetic tree of GBV-C/HGV complete coding region sequences (with permission, Smith *et al.* 2000).

other major groups, and the confirmation of a fifth group in South Africa (Smith *et al.* 2000), is consistent with the possibility that GBV-C/HGV may have emerged in Africa (Tanaka *et al.* 1998) and evolved together with its human host during their prehistoric migration.

Detection of GBV-C/HGV

GBV-C/HGV NA

The reverse-transcription polymerase chain reaction (RT-PCR) is the only diagnostic tool available to detect current GBV-C/HGV infection. Initially, primers from the NS3 region of the genome were used to detect viral RNA (Simons *et al.* 1995b; Linnen *et al.* 1996). Degenerate primers to the NS region have also been shown to be sensitive in detecting GBV-C/HGV RNA (Yoshiba *et al.* 1995). Primers from the NS5A (Linnen *et al.* 1996) or 5′ UTR (Schlueter *et al.* 1996) have been shown to be more reliable; the latter region appears to be more sensitive and more widely used in this respect (Kao *et al.* 1997a). Detection of PCR products can be accomplished by the relatively simple procedure of agarose gel electrophoresis and staining the gel with ethidium bromide, or alternatively, by a single tube assay based on RT-PCR amplification of the 5′ UTR, followed by oligomer hybridization. Detection employs a microparticle immunoassay in the automated LCx system (Marshall *et al.* 1998). While RT-PCR remains the 'gold standard' for detecting GBV-C/HGV, its sensitivity is not fully resolved.

GBV-C/HGV Antibodies

An antibody response to GBV-C/HGV directed against the envelope glycoprotein, E2, has been detected following its expression as a recombinant protein in Chinese hamster ovary cells (Pilot-Matias *et al.* 1996; Tacke *et al.* 1997a). The secreted E2 protein has been purified and used in a solid phase enzyme-linked immunosorbent assay (ELISA) for the detection of anti-E2 (Dille *et al.* 1997). Interestingly, almost all sera

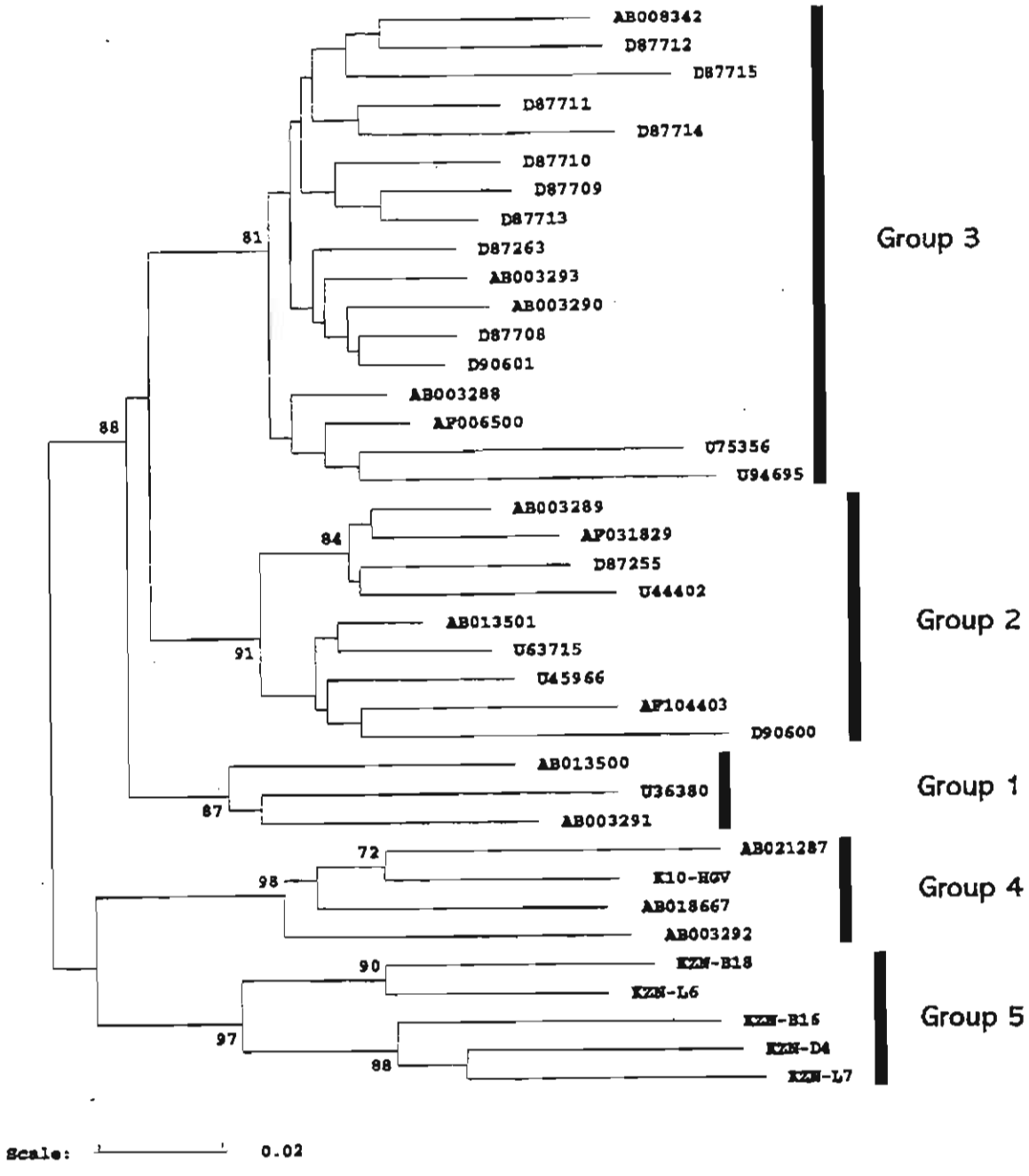

Figure 3. South African isolates (KZN) form an additional E2 phylogenetic grouping (Group 5) (with permission, Smith *et al.* 2000).

positive for anti-E2 are negative for viral RNA, and vice versa, implying that anti-E2 is associated with virus clearance and is perhaps, protective or neutralizing (Pilot-Matias *et al.* 1996; Tacke *et al.* 1997a). Testing for anti-E2 greatly extends the ability of RT-PCR to define the epidemiology of GBV-C/HGV (Sathar *et al.* 1999b). However, the specificity for anti-E2 has not been established.

Routes of GBV-C/HGV transmission

Since the discovery of GBV-C/HGV, attempts have been made to clarify its principle mode of transmission. In non-African countries the predominant route of transmission of GBV-C/HGV is parenteral. High prevalences of GBV-C/HGV have been found in subjects with frequent parenteral exposure and in groups at high risk

of exposure to blood and blood products, including intravenous drug abusers (IVDA), patients on maintenance haemodialysis, multitransfused individuals and haemophiliacs. The high prevalence in blood donors worldwide suggests that the principle route of transmission is via contaminated blood and blood products. However, maintenance of the virus at high levels in blood donors and the general population requires an effective nonparenteral route of transmission. Blood donors, however, are not representative of the general population since they are highly selective.

Various studies have pointed to the important role of sexual exposure as a likely route of transmission of GBV-C/HGV, in both non-HIV infected subjects without the risk for parenteral transmission (IVDU and multitransfused individuals including haemophiliacs)(Kao *et al.* 1997b; Scallan *et al.* 1998; Sawayama *et al.* 1999) and HIV-infected individuals with the risk for parenteral and sexual transmission (homosexuals, heterosexuals and prostitutes)(Bourlet *et al.* 1999; Nubling *et al.* 1997; Ibanez *et al.* 1998; Nerurkar *et al.* 1998). Infection with GBV-C/HGV appears to be more frequent in patients with a sexual risk than those with parenteral exposure (Bourlet *et al.* 1999; Ibanez *et al.* 1998). In a study of 600 antenatal patients there was an overall prevalence (GBV-C/HGV RNA and/or Anti-E2 positive) of 11.8%. Since this group represents a young, sexually active population, the authors concluded that sexual or close contact might play a role in the transmission of GBV-C/HGV (Skidmore & Collingham 1999). Rubio *et al.* (Rubio *et al.* 1997) reported a GBV-C/HGV prevalence of 21.7% among heterosexual partners of 150 index cases. Stark *et al.* (Stark *et al.* 1996) found GBV-C/HGV prevalence of 10.9% among non-drug injecting homosexual and bisexual men. Scallan *et al.* (Scallan *et al.* 1998) found a high prevalence of markers for GBV-C/HGV in non-intravenous drug using prostitutes (40%) and male homosexuals (47%). A positive correlation was demonstrated between GBV-C/HGV infection in prostitutes and the number of years of prostitution (Kao *et al.* 1997b; Sawayama *et al.* 1999) and the high frequency of paid sex (Wu *et al.* 1997). The near absence of GBV-C/HGV infection among heterosexual men (4%) and the comparatively higher prevalence among heterosexual women (15%) suggests that, as in HIV infection, the receptive partner is at high risk for acquiring GBV-C/HGV (Nerurkar *et al.* 1998). Interspousal transmissions of GBV-C/HGV have been reported (Kao *et al.* 1997c; Sarrazin *et al.* 1997). Although the role of semen in the transmission of GBV-C/HGV is controversial (Semprini *et al.* 1997; Hollingsworth *et al.* 1998), recent reports have suggested that human saliva may contribute to the spread of GBV-C/HGV RNA (Seemayer *et al.* 1998; Chen *et al.* 1997). Tucker *et al.* (Tucker *et al.* 2000) did not detect GBV-C/HGV replicative intermediaries in the cadaver biopsies of salivary glands and the gonads of GBV-C/HGV positive patients, implying that the virus may be present in the saliva and semen of infected individuals, but not transmitted by these routes. Despite the evidence for increased frequencies of GBV-C/HGV infection in association with sexual exposure, the mechanism of transmission remains unclear.

There is a higher risk of mother to infant transmission in high risk groups (Feucht *et al.* 1996; Fischler *et al.* 1997; Viazov *et al.* 1997; Zanetti *et al.* 1997; Wejstal *et al.* 1999). However, it is not clear whether co-infection with HCV, HIV-1 (or both), or IVDU are the underlying cause for transmission of GBV-C/HGV from mother to infant. Nor is it clear as to whether transmission of GBV-C/HGV is influenced by breastfeeding or by the mode of delivery (Viazov *et al.* 1997; Zanetti *et al.* 1997; Wejstal *et al.* 1999). Although the rate of perinatal transmission of GBV-C/HGV exceeds that of HCV, in most studies GBV-C/HGV did not induce liver disease in the infants studied (Wejstal *et al.* 1999; Zanetti *et al.* 1997).

Among 220 cases of needle-stick injuries, GBV-C/HGV RNA was detected in 21 (9.5%) donors (Shibuya *et al.* 1998). At the time of injury none of the 21 recipients were positive for GBV-C/HGV RNA or anti-E2; only 1/14 (7.1%) recipients was positive for GBV-C/HGV RNA which persisted for approximately 3 years without any evidence of liver disease (Shibuya *et al.* 1998). It has been suggested that iatrogenic infection with GBV-C/HGV could possibly occur through insufficient sterilization of needles and syringes (Ohshima *et al.* 2000). Confirmation that GBV-C/HGV is indeed an occupational hazard in hospital employees (Gartner *et al.* 1999; Schaade *et al.* 2000) will require more comprehensive longitudinal studies.

Prevalence of GBV-C/HGV infection

The prevalences of GBV-C/HGV infection in selected groups of subjects from some published studies are listed in Tables 1–4. The frequency of positivity for RNA or anti-E2 varies among groups, depending on the subjects' origins and the methods used to detect GBV-C/HGV markers. Generally, infection with GBV-C/HGV is significantly associated with a history of IVDA, exposure to blood transfusions, dialysis and with HCV infection. There is a higher prevalence of GBV-C/HGV RNA in blood donors and the general population of African countries (10–19%) compared to non-African

Table 1. Reported prevalences of GBV-C/HGV RNA in blood donors in some published studies

Continent	Country	*n*	RNA + (%)	References
N America	USA	769	13(1.7)	(Linnen *et al. 1996*)
S America	Brazil	11	2(1.8)	(Lampe *et al. 1997*)
Africa	Egypt	82	16(12.2)	(El-Zayadi *et al. 1999*)
	South Africa	248	32(12.9)	(Mphahlele *et al.* 1998)
	South Africa	249	26(10.4)	(Tucker *et al.* 1997)
	South Africa	167	21(12.6)	(Lightfoot *et al.* 1997)
	South Africa	532	59(11.1)	(Castelling *et al.* 1998)
	South Africa	232	44(18.9)	(Sathar *et al.* 1999b)
Caribbean	Martinique	221	9 (4.1)	(Cesaire *et al.* 1999)
Asia	Japan	448	4(0.9)	(Masuko *et al.* 1996)
	China	205	2(1)	(Wang *et al.* 1997b)
	Thailand	69	3(4.3)	(Raengsakulrach *et al.* 1997)
	Vietnam	890	11(1.2)	(Kakumu *et al.* 1998)
	Nepal	181	4(2)	(Shrestha *et al.* 1997)
	Mongolia	121	8(6.6)	(Kondo *et al.* 1997)
Australia		120	5(4)	(Moaven *et al.* 1996)
Europe	Austria	92	3(3)	(Schlueter *et al.* 1996)
	Germany	1048	14(1.34)	(Roth *et al.* 1997)
	Germany	106	59(4.7)	(Heringlake *et al.* 1996)
	UK	125	4(3.2)	(Jarvis *et al.* 1996)
	Italy	100	1(1)	(Fiordalisi *et al.* 1996)
	Spain	200	6(3)	(Saiz *et al.* 1997)

countries (1–6%) (Table 1). The high prevalence in commercial blood donors (5–26%) (Table 2) is probably due to the increased risk of parenteral acquisition in this group. The prevalence of GBV-C/HGV anti-E2 antibodies in healthy individuals ranges from 3–15.1% (Table 4).

The simultaneous detection of Anti-E2 greatly extends the ability of RT-PCR to define the epidemiology of GBV-C/HGV (Table 4). For example, in non-African countries, 1–2.5% of blood donors is GBV-C/HGV RNA positive. Using Anti-E2 assays, the same population of blood donors showed 3–9% seroprevalence. The overall prevalence of GBV-C/HGV in non-African blood donors was 4–16%, compared to 20–30% in Africa (Table 4). In the high risk group of patients the overall prevalence of GBV-C/HGV infection ranged from 20 to 89% (Table 4). The combined overall prevalence of GBV-C/HGV infection is higher in African countries than in non-African countries (Table 4). The simultaneous detection of GBV-C/HGV RNA and Anti-E2 may represent the seroconversion state.

Thus, the total exposure to GBV-C/HGV should take into account both the number of PCR-positive samples (i.e. viraemic/RNA positive) and anti-E2 positive samples (i.e. previously infected but cleared) in a given population. GBV-C/HGV infection appears to be a common infection globally. The reason for the high prevalence of GBV-C/HGV in blood donors worldwide and the basis for the racial differences in GBV-C/HGV infection in blood donor populations are not known. Whether socio-economic factors are associated with prevalence of GBV-C/HGV is not known for certain, although a relationship was noted between GBV-C/HGV infection and the lack of water-borne sewage (Tucker *et al.* 1997). The differences in the prevalence of detecting GBV-C/HGV infection (Tables 1–4) may be due to the differences in the sensitivity of the various PCR protocols and primers (derived from various regions of the genome) used by various investigators, and preselection of patients in terms of status for other viral markers as well as different patient histories. Further investigations are required to determine whether genetically distinct isolates from different geographical regions of the world escape detection by current PCR methods and anti-E2 assays.

GBV-C/HGV Anti-E2: A protective/neutralizing antibody and a marker for recovery

Analysis of serial samples for both RNA and anti-E2 suggests that GBV-C/HGV infection follows one of two paths: acute infection followed by recovery (appearance of GBV-C/HGV E2 antibody), or acute infection progressing to chronicity (persistence of GBV-C/HGV RNA). Follow-up of 16 post-transfusion patients for up to 16 years revealed that individuals who develop an anti-E2 response become GBV-C/HGV-RNA negative, while

Table 2. Reported prevalences of GBV-C/HGV RNA in high risk groups in some published studies

Clinical group	Country	RNA⁺No. (%)	References
Haemodialysis	Egypt	79 (30)	(El-Zayadi *et al.* 1999)
	South Africa	70(24.3)	(Sathar *et al.* 1999b)
	Brazil	65(15.4)	(Lampe *et al.* 1997)
	China	79(54)	(Wang *et al.* 1997)
	France	61(57.5)	(Lamballerie *et al.* 1996)
	Japan	519(3.1)	(Masuko *et al.* 1996)
	Indonesia	58(55)	(Tsuda *et al.* 1996)
Haemophiliacs	Scotland	95(14)	(Jarvis *et al.* 1996)
	Europe	49(9)	(Linnen *et al.* 1996)
	France	92(17.4)	(Gerolami *et al.* 1997)
	Japan	63(24)	(Kinoshita *et al.* 1997)
	Nicaragua	45(38)	(Gonzales-Prez *et al.* 1997)
	South Africa	102(23.5)	(Castelling *et al.* 1998)
IVDUs	Greece	106(32.1)	(Anastassopoulou *et al.* 1998)
	US	27(4)	(Dille *et al.* 1997)
	US	102 (14.7)	(Gutierrez *et al.* 1997)
	Sweden	19(16)	(Shev *et al.* 1998)
	Europe	60(33.3)	(Linnen *et al.* 1996)
	Germany	99(38)	(Tacke *et al.* 1997a)
	Japan	49(12)	(Aikawa 1996)
Commercial blood donors	China	205(8)	(Roth *et al.* 1997)
	US	50(26)	(Dille *et al.* 1997)
	US	42(5)	(Pilot-Matias *et al.* 1996)
	US	711(13.1)	(Gutierrez *et al.* 1997)
Healthcare workers	Egypt	30(6,6)	(El-Zayadi *et al.* 1999)
Drug addicts	Nepal	72(44)	(Shrestha *et al.* 1997)
Prostitutes	UK	50(18)	(Scallan *et al.* 1998)
	China	140(21)	(Wu *et al.* 1997)
Homosexuals	UK	52(17)	(Scallan *et al.* 1998)
Homosexual & bisexual men	Germany	101(11)	(Schlueter *et al.* 1996)

those who do not develop anti-E2 are persistently infected (Tacke *et al.* 1997b). The presence of anti-E2 and the subsequent loss of viraemia have been confirmed by other investigators (Dille *et al.* 1997; Gutierrez *et al.* 1997; Hassoba *et al.* 1997). Anti-E2 appears to be long-lasting, circulating antibodies and once acquired generally tends to persist (Masuko *et al.* 1996; Lefrere *et al.* 1997)

In chronic HCV infection the co-existence of E2/NS1 antibody and viraemia suggests that anti-E2 is not a neutralizing/protective antibody, but serves as a marker of active HCV replication (Yuki *et al.* 1996). GBV-C/HGV Anti-E2 on the other hand, has been described as a marker of viral clearance (recovery/past) and is considered to be protective against GBV-C/HGV reinfection. In 54 recipients who underwent orthotopic liver transplantation (OLT), the presence of anti-E2 pre-transplant was associated with a relatively low rate (15%) of post-transplantation GBV-C/HGV infection compared to 46% in anti-E2 negative (pre-transplant) patients (Hassoba *et al.* 1998). Post-transplantation immune suppression apparently had only a minor effect on the prevalence of anti-E2 in patients who were anti-E2 positive prior to transplantation (Hassoba *et al.* 1998). A negative association between the presence of GBV-C/HGV RNA and the presence of anti-E2 was found in all patients tested pre- and post-transplantation, suggesting viral clearance (Hassoba *et al.* 1998). Anti-E2 appears to be a neutralizing antibody whose presence at the time of liver transplantation protects against acquisition of GBV-C/HGV infection post-OLT (Bizollon *et al.* 1998; Hassoba *et al.* 1998; Silini *et al.* 1998; Tillmann *et al.* 1998). No new GBV-C/HGV infections were noted among subjects with anti-E2, despite ongoing drug use (Thomas *et al.* 1998).

Site(s) of replication

The site of GBV-C/HGV replication has been an area of intense interest and remains uncertain. A true hepatotropic virus replicates in the liver. GBV-C/HGV is a positive-stranded flavivirus whose genomic organization is similar to HCV, as such replication should proceed via a negative-strand RNA intermediate, the detection of which should be possible in the liver. GBV-C/HGV RNA was detected by RT-PCR in washed hepatocytes of 9/58 (15%) children with chronic viral hepatitis (Lopez-Alcorocho *et al.* 1997). Madejon *et al.* (Madejon *et al.*

Table 3. Reported prevalences of GBV-C/HGV RNA in liver diseases in some published studies

Clinical Group	Country	RNA$^+$ No. (%)	References
Acute/Chronic HBV	Europe	72(9.7)	(Linnen *et al.* 1996)
	Egypt	63(11.1)	(El-Zayadi *et al.* 1999)
	Japan	83(4)	(Sugai *et al.* 1997)
	US	100(32)	(Alter *et al.* 1997a)
	South Africa	106(26.4)	(Mphahlele *et al.* 1998)
Acute/Chronic HCV	Egypt	100(14)	(El-Zayadi *et al.* 1999)
	Germany	100(9)	(Schleicher *et al.* 1996)
	Italy	83(26.5)	(Francesconi 1997)
	Japan	88(8)	(Sugai *et al.* 1997)
	Russia	22(41)	(Yashina *et al.* 1997)
	Spain	143(5.6)	(Saiz *et al.* 1997)
	Taiwan	52(10)	(Hwang *et al.* 1997)
	US	116(20)	(Alter *et al.* 1997a)
	South Africa	82(30.5)	(Mphahlele *et al.* 1998)
Acute/Chronic HAV	Japan	21(0)	(Nakatsuji *et al.* 1996)
	US	100(25)	(Alter *et al.* 1997a)
Non A-E hepatitis	China	108(16.7)	(Wang & Jin 1997)
	Japan	43(0)	(Nakatsuji *et al.* 1996)
	Russia	28(3.6)	(Yashina *et al.* 1997)
	US	149(8.7)	(Dawson *et al.* 1996)
Chronic Liver Disease	Indonesia	149(5)	(Tsuda *et al.* 1996)
	Nepal	145(3)	(Shrestha *et al.* 1997)
	South Africa	92(12)	(Sathar *et al.* 1999b)
	Japan	226(7.5)	(Nakatsuji *et al.* 1996)
	US	326(12.2)	(Linnen *et al.* 1996)
	Italy	36(39)	(Fiordalisi *et al.* 1996)
Hepatocellular Carcinoma	Japan	111(10)	(Kanda *et al.* 1997)
	Japan	109(10)	(Nishiyama *et al.* 1999)
	Thailand	101(6)	(Tangkijvanich *et al.* 1999)
	China	114(14,9)	(Cao *et al.* 1998)
	Europe	57(7)	(Brechot *et al.* 1998)
	South Africa	135(14)	(Lightfoot *et al.* 1997)
Fulminant hepatitis	Japan	6(50)	(Yoshiba *et al.* 1995)
	Japan	10(0)	(Kanda *et al.* 1997)
	Germany	22(50)	(Heringlake *et al.* 1996)
	UK	23(21.7)	(Haydon *et al.* 1997)
	UK	20(0)	(Sallie *et al.* 1996)
	United States	36(38.8)	(Munoz *et al.* 1999)
	Taiwan	32(9)	(Liu *et al.* 1999)

Table 4. Reported prevalences of GBV-C/HGV RNA and Anti-E2 antibodies in some published studies

Clinical Group	Country	*n*	RNA+ (%)	Anti-E2+ (%)	Exposure (%)	References
Blood Donors	Japan	200	2(1)	10(5)	12(6)	(Tanaka *et al.* 1998)
	Germany	200	5(2.5)	7(9)	33(16.5)	(Tacke *et al.* 1997a)
	US	199	3(1.5)	9(4.5)	11(5.5)	(Gutierrez *et al.* 1997)
	US	100	1(1)	3(3)	4(4)	(Dille *et al.* 1997)
	Spain	200	5(2.5)	28(14)	32(16)	(Tacke *et al.* 1997b)
	South Africa	248	32(12.9)	30(12.1)	52(21.1)	(Mphahlele *et al.* 1999)
	South Africa	232	44(18.9)	35(15.1)	74(31.9)	(Sathar *et al.* 1999b)
Commercial Donors	US	711	93(13.1)	195(27.4)	288(40.5)	(Gutierrez *et al.* 1997)
Plasmapheresis Donors	US	50	13(26)	17(34)	30(60)	(Dille *et al.* 1997)
	West Africa	30	10(33.3)	4(13.3)	14(46.7)	(Dille *et al.* 1997)
IVDU	Germany	99	38(38)	41(41)	75(75)	(Tacke *et al.* 1997a)
	US	27	1(3.7)	23(85.2)	24(88.9)	(Dille *et al.* 1997)
	US	102	15(14.7)	76 (74.5)	91(89.2)	(Gutierrez *et al.* 1997)
Haemophiliacs	Spain	62	22(34%)	20(32)	33(53)	(Tacke *et al.* 1997b)
	France	92	16(17.4)	33(35)	47(51)	(Gerolami *et al.* 1997)
Haemodialysis	South Africa	70	17(24.3)	18(25.7)	33(47.1)	(Sathar *et al.* 1999b)
Renal Transplant	Germany	221	31(14)	89(40)	118(53)	(Stark *et al.* 1997)
Chronic liver disease	South Africa	98	12(12.2)	32(32.7)	33(47.1)	(Sathar *et al.* 1999b)

1997) and Saito *et al.* (Saito *et al.* 1997) detected GBV-C/HGV antigenomic RNA in 12/13 livers and peripheral blood mononuclear cells (PBMCs) of one of the same 13 patients examined. Because hepatocytes and PBMCs are bathed in blood, it is possible that the PCR signal noted may be due to cell-bound virus rather than active replication occurring in these cells (Laras *et al.* 1999). Using RT-PCR with tagged primers and southern blot analysis, antigenomic GBV-C/HGV RNA was detected in 4/6 liver specimens; using *in situ* hybridization in two such specimens GBV-C/HGV infection was restricted to hepatocytes (Seipp *et al.* 1999). Hepatotropism of GBV-C/HGV has been demonstrated by *in vitro* infection of PBMC and cells of human hepatoma cell lines (Ikeda *et al.* 1997; Fogeda *et al.* 1999; Seipp *et al.* 1999). Hepatocytes may not be the only site of viral replication; antigenomic GBV-C/HGV RNA was also detected in the mononuclear cell infiltrates in the portal areas of the liver (Kobayashi *et al.* 1999). These results suggest that GBV-C/HGV replicates in the liver.

Using RT-PCR with tagged primers and *in vitro* derived templates, Mellor *et al.* (Mellor *et al.* 1998) were unable to detect antigenomic GBV-C/HGV RNA in either liver biopsies or in the PBMCs of 20 GBV-C/HGV infected individuals. Radkowski *et al.* suggested that PBMCs may not be the replication site of GBV-C/HGV (Radkowski *et al.* 1998). In 5/17 patients undergoing liver transplantation, GBV-C/HGV RNA was detected in sera and not in the liver on repeated testing for viral RNA from different portions of the liver (Fan *et al.* 1999). In patients co-infected with GBV-C/HGV and HCV, the hepatotropism of HCV and not GBV-C/HGV was consistently proven (Kudo *et al.* 1997; Laskus *et al.* 1997; Pessoa *et al.* 1998). These findings suggest that GBV-C/HGV is not a hepatotropic virus and that neither the liver nor PBMCs may be the actual site of GBV-C/HGV replication.

In their study of six cadaver biopsies from one GBV-C/HGV positive patient co-infected with HIV, Mushahwar *et al.* (Mushahwar *et al.* 1998) detected glyceraldehyde-3-phosphate dehydrogenase (GAP-DH) mRNA and GBV-C/HGV RNA only in the liver, which was localized to individual hepatocytes. In multiple cadaver autopsies of 12 patients (four with AIDS, six HIV positive and two with end-stage liver disease) (Laskus *et al.* 1998; Radkowski *et al.* 1999), GBV-C/HGV RNA intermediaries were consistently demonstrated in the bone marrow and spleen. However, these results are difficult to interpret in immunocompromised patients. In a preliminary study of 23 cadaver biopsies from four GBV-C/HGV positive patients who were HIV negative, the spleen and bone marrow biopsies were uniformly positive for both negative- and positive-strand GBV-C/HGV RNA (Tucker *et al.* 2000). The authors (Tucker *et al.* 2000) concluded that GBV-C/HGV is a lymphotropic virus that replicates primarily in the spleen and bone marrow. These findings require confirmation using *in situ* hybridization and immunohistochemical staining.

Strand-specific detection of RNA is fraught with problems such as false priming of the incorrect strands or self-priming related to RNA secondary structure. All of the strand-specific studies used methods to reduce false-priming and self-priming events viz. chemical modification of the 3′ ends (Madejon *et al.* 1997; Saito *et al.* 1997); conducting cDNA synthesis at high temperature with the thermostable enzyme (Tth) (Laskus *et al.* 1997; Tucker *et al.* 2000); using *in vitro* derived templates (Laskus *et al.* 1997; Mellor *et al.* 1998; Tucker *et al.* 2000); using 'tagged' primers (Mellor *et al.* 1998; Seipp *et al.* 1999); *in situ* hybridization of liver biopsies (Kobayashi *et al.* 1999; Seipp *et al.* 1999) and *in vitro* infection of human hepatoma cells with GBV-C/HGV monoinfected serum (Seipp *et al.* 1999). Only Laskus *et al.* (Laskus *et al.* 1997) and Mellor *et al.* (Mellor *et al.* 1998) qualified their reactions using *in vitro* derived templates and provided end point titration data.

GBV-C/HGV infection and liver disease

Most GBV-C/HGV infections appear to be asymptomatic, transient, and self-limiting, with slight or no elevation of alanine aminotransferase (ALT) (Alter *et al.* 1997a; Alter *et al.* 1997b). Co-infection with GBV-C/HGV does not alter the clinical course of community-acquired hepatitis A, B or C (Alter *et al.* 1997a; Alter *et al.* 1997b). Most of these subclinical cases resolve after loss of serum GBV-C/HGV RNA with a concomitant appearance of anti-E2 (Dille *et al.* 1997; Gutierrez *et al.* 1997). Figure 4 depicts a typical clinical picture of a patient with acute post-transfusion GBV-C/HGV infection. To evaluate the clinical course of GBV-C/HGV infection, patients who were infected with GBV-C/HGV only were studied by Wang *et al.* (Wang *et al.* 1996). Among 25 such patients who acquired GBV-C/HGV infection by transfusion, 20 patients who were followed up at 2–4 week intervals over six months maintained normal ALT activities. The other five patients showed only moderate elevations in ALT (< 124 IU/l) over the first six months, with no further elevations in the subsequent follow-up period of two years. In these five patients, there were no other clinical signs of liver disease. Jaundice was absent in the 25 patients, whereas it was present in two out of the seven patients with HCV co-infection (Wang *et al.* 1996). GBV-C/HGV

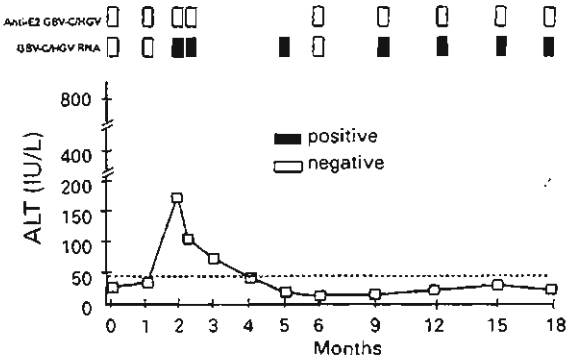

Figure 4. Clinical picture of a patient with acute post-transfusion GBV-C/HGV infection (with permission, Hwang *et al.* 1999).

is capable of inducing persistent infection in about 5–10% of infected individuals. Masuko *et al.* (Masuko *et al.* 1996) retrospectively followed eight haemodialysis patients with GBV-C/HGV infection for 7–16 years. In two patients, the virus was present at the start of haemodialysis. One had a history of transfusion, and GBV-C/HGV RNA persisted over a period of 16 years, the other cleared GBV-C/HGV RNA after 10 years. In five patients, GBV-C/HGV RNA was first detected 3–20 weeks after blood transfusion and persisted for up to 13 years. No elevations in serum ALT or signs of active liver disease were found in these patients. It would appear that in many patients infected with GBV-C/HGV, virus replication could occur without detectable damage to the liver. GBV-C/HGV transmission to chimpanzees and tamarins resulted in infection without elevation in ALT (Bukh *et al.* 1998). On the contrary, GBV-C/HGV infection in macaques (Cheng *et al.* 2000) produced mildly elevated ALT levels with mild hepatitis and positive antigenic expression in hepatocytes, suggesting that GBV-C/HGV may be pathogenic to primates.

A strong association between GBV-C/HGV and fulminant hepatitis has been suggested (Yoshiba *et al.* 1995; Heringlake *et al.* 1996) which may be associated with a specific strain of GBV-C/HGV (Heringlake *et al.* 1996). However, these studies did not clearly define whether GBV-C/HGV was transmitted by the transfusions they received prior to the onset of fulminant hepatitis. Additional studies by Yoshiba *et al.* (Yoshiba *et al.* 1996) showed that only a few of the fulminant hepatitis patients studied had received a blood transfusion prior to the onset of fulminant hepatitis. In a similar study, GBV-C/HGV RNA was detected in 3/15 (20%) patients with HBV infection and in 3/25 (12%) patients without markers of hepatitis A–E infection (Tameda *et al.* 1996). Of the six patients with GBV-C/HGV RNA, only three had a history of transfusion and all of these patients were co-infected with HBV. According to Tameda *et al.* (Tameda *et al.* 1996) these results indicate a role of GBV-C/HGV in inducing fulminant hepatitis either by itself or in concert with other hepatitis viruses. Using real time detection polymerase chain reaction (RTD-PCR), GBV-C/HGV RNA was measured serially in the sera of three Japanese patients with non A–E fulminant hepatitis, none of whom received any therapeutic transfusions before admission (Inoue *et al.* 1999). Serum ALT levels paralleled GBV-C/HG RNA in all three cases and sequence analysis revealed that the same GBV-C/HGV strain infected the patients during their entire clinical course, despite plasma exchange therapy. In one patient, hepatocyte destruction continued with persistent viraemia, although ALT levels decreased. The authors (Inoue *et al.* 1999) concluded that their assumption of an association of GBV-C/HGV with fulminant hepatitis in these three patients was further strengthened by the disappearance or persistence of GBV-C/HGV RNA in serum which appeared to be linked to the prognosis. However, several other studies have provided no evidence of an association of GBV-C/HGV with fulminant hepatitis (Hadziyannis 1997). The discrepancies in the association of GBV-C/HGV with fulminant hepatitis may be influenced by the sensitivity of the detection system and the differences in the GBV-C/HGV infection rates in the different populations studied. The role of GBV-C/HGV in the aetiology of fulminant hepatitis remains controversial.

Some investigators have reported histological features in liver biopsies of GBV-C/HGV-infected individuals. Among six chronic hepatitis patients with GBV-C/HGV RNA only, the histology of the liver samples revealed chronic active hepatitis in one patient and chronic persistent hepatitis in five others (Fiordalisi *et al.* 1996). All patients with chronic hepatitis had elevated ALT levels between 89 and 478 U/l. In contrast, among the 11 acute hepatitis cases positive for GBV-C/HGV RNA, the ALT levels varied between 615 and 2477 U/l. Colombatto *et al.* (Colombatto *et al.* 1997) studied GBV-C/HGV in 67 patients with liver disease without any markers for hepatitis A–E. They reported an association between nonspecific inflammatory bile duct lesions and elevated cholestatic enzymes (gamma glutamyl transpeptidase and alkaline phosphatase) in 50% of patients. Ross *et al.* (Ross *et al.* 1997) showed that GBV-C/HGV infection might affect the clinical course and outcome after orthotopic liver transplantation (OLT) by the development of severe cholestasis, which could result from bile duct damage and bile duct loss. In a preliminary study, an association between recurrent or

de novo GBV-C/HGV infection and severe post-transplant cholestasis and ductopenia was also observed in the grafts of GBV-C/HGV-positive liver organ transplant patients (Dhillon *et al.* 1996). However, in many studies no correlation between GBV-C/HGV infection and elevation of cholestatic enzymes were noted. Further investigations are needed to substantiate these findings.

Manolakopoulos *et al.* (Manolakopoulos *et al.* 1998) found an association between GBV-C/HGV and HCV-viremia and portal and periportal inflammation. They reported that the duration of HCV/GBV-C/HGV co-infection may be an important factor in the progression of liver disease and that inflammation with necrosis in the portal and periportal tracts was significantly higher in patients with combined viremia compared to those with HCV infection alone. The authors suggested that GBV-C/HGV in patients with HCV infection might accelerate liver injury toward more severe fibrosis in patients with dual infection. Diamantis *et al.* (Diamantis *et al.* 1997) reported that mild fibrosis correlated with GBV-C/HGV whilst Francesconi *et al.* (Francesconi *et al.* 1997) observed subtleties in histological appearance in HCV co-infected patients. However, numerous studies have shown that in HCV co-infected individuals, GBV-C/HGV does not affect HCV replication, HCV RNA concentration, and liver disease (Tanaka *et al.* 1996; Bralet *et al.* 1997; Enomoto *et al.* 1998; Pawlotsky *et al.* 1998; Petrik *et al.* 1998; Slimane *et al.* 2000).

During OLT, pre-transplant GBV-C/HGV has been reported to be associated with post-transplant viraemia (Fried *et al.* 1997; Feucht *et al.* 1997; Haagsma *et al.* 1997). In the absence of HBV or HCV in liver transplant recipients, the prevalence of GBV-C/HGV infection has no influence on the graft (Haagsma *et al.* 1997). Berg *et al.* (Berg *et al.* 1996) found a significantly higher percentage of hepatocellular carcinoma in patients with pre-OLT GBV-C/HGV co-infection compared with patients with HCV infection alone (5/6 *vs.* 16/68; $P < 0.01$). Bizollon *et al.* (Bizollon *et al.* 1998), on the other hand, showed that the prevalence of hepatocellular carcinoma was not different in patients with pre-transplantation GBV-C/HGV co-infection or with HCV infection. In addition, GBV-C/HGV co-infection did not seem to have a significant impact on the course of HCV infection after transplantation.

Hepatocarcinogenecity of GBV-C/HGV

GBV-C/HGV RNA was detected in 11/111 (10%) of cases of hepatocellular carcinoma (HCC) (Kanda *et al.* 1997). The authors concluded that GBV-C/HGV was unlikely to be a major aetiological agent of non-B non-C HCC. In a large series of 503 patients with HCC in Europe, Brechot *et al.* (Brechot *et al.* 1998) demonstrated a major impact of HBV (19% positive) and HCV (40%) but not GBV-C/HGV (7%) in HCC. In a study of 167 Black South Africans with HCC and 167 matched controls, Lightfoot *et al.* (Lightfoot *et al.* 1997) showed that patients infected with GBV-C/HGV did not have an increased relative risk of developing HCC. In addition, co-infection with GBV-C/HGV did not further increase the risk of HCC in patients chronically infected with HBV and HCV. In a retrospective study of GBV-C/HGV in formalin-fixed, paraffin-embedded (FFPE) tissues of HCC patients from various geographical areas (Japan, Spain, Korea, United States, Japanese Americans in Hawaii), GBV-C/HGV was neither detected nor was there any evidence of any association of GBV-C/HGV with HCC (Abe *et al.* 1998). In this study HCV genotype II/1b and HBV were significantly associated with HCC.

In a population-based study of non-Asian patients with HCC and community controls in Los Angeles, California, Yuan *et al.* concluded that GBV-C/HGV infection may account for approximately 8% of HCC (Yuan *et al.* 1999). GBV-C/HGV RNA was detected in 12/144 (8.3%) non-Asian patients with HCC and 5/225 (2%) community controls. The presence of GBV-C/HGV RNA was associated with a statistically significant 5.4 fold risk, which was independent of the effects of HBV and HCV infections (Yuan *et al.* 1999). In a hospital-based case-controlled study the relative risk factor suggested a fair association between GBV-C/HGV infection and HCC (Tagger *et al.* 1997). However, GBV-C/HGV did not seem to be a major aetiological agent of HCC because the population-attributable risk was lower (4%) than those for HbsAg (52%), HCV RNA (36%) and excessive alcohol intake (52%) (Tagger *et al.* 1997). Among subjects with GBV-C/HGV exposure (RNA and anti-E2 positive) a greater proportion of cases (40%) than controls (14%) had a transfusion history (Tagger *et al.* 1997). Hepatocarcinogenicity of GBV-C/HGV is an important key question that remains controversial.

Extrahepatic manifestations of GBV-C/HG infection

Hepatitis-associated aplastic anaemia is a rare but well-documented phenomenon, unlikely to be caused by any of the known hepatitis viruses (Byrnes *et al.* 1996; Brown *et al.* 1997a). In some cases of hepatitis-associated aplastic anaemia, GBV-C/HGV was the only aetiological agent detected, even if the patients had not received any transfusions before diagnosis (Crespo *et al.* 1999; Kiem *et al.* 1997; Zaidi *et al.* 1996). Moriyama *et al.* (Moriyama *et al.* 1997) detected GBV-C/

HGV RNA in 5/18 (27.7%) patients with aplastic anaemia who received blood transfusions before diagnosis but not in eight patients who did not receive transfusions. Similarly, Brown *et al.* (Brown *et al.* 1997b) detected GBV-C/HGV RNA in 26.3% and 23.1% of patients with aplastic anaemia and multi-transfused control patients, respectively. Kiem *et al.* (Kiem *et al.* 1997) detected GBV-C/HGV RNA in 26.1% of patients with hepatitis-associated aplastic anaemia and idiopathic aplastic anaemia who did not receive transfusions. The authors concluded that although transfusions are a major source of GBV-C/HGV infection, the high prevalence in those who did not receive transfusions suggests an association of GBV-C/HGV with aplastic anaemia, whether associated with hepatitis or not. Further studies in serial serum samples and meticulous evaluation of the disorders associated with the infection will be needed to prove or disprove a causal association of GBV-C/HGV and aplastic anaemia.

It is interesting to note that GBV-C/HGV replication has been consistently shown in bone marrow and spleen, and not in the lymph nodes and tonsils (Laskus *et al.* 1998; Radkowski *et al.* 1999; Tucker *et al.* 2000), suggesting a haematological cell tropism. Because the genomic organization, structural and biological characteristics of GBV-C/HGV are similar to that of HCV, GBV-C/HGV has been investigated as a possible aetiological agent in the development of haematological disorders. Ongoing GBV-C/HGV infection was detected in 29 of 60 (48%) multi-transfused patients with haematological malignancies (Skidmore *et al.* 1997). GBV-C/HGV prevalence in patients with B-cell non-Hodgkin's lymphoma was significantly higher than in healthy controls (Zignego *et al.* 1997; Ellenrieder *et al.* 1998). All patients were asymptomatic and without clinical or sonographic signs of chronic liver disease (Ellenrieder *et al.* 1998). GBV-C/HGV prevalence in lymphoma or cryoglobulinemia patients do not support the hypothesis that this virus also may play a major role in lymphomagenesis or in the production of mixed cryoglobulinemia (Cacoub *et al.* 1997; Nakamura *et al.* 1997; Ellenrieder *et al.* 1998). Pavlova *et al.* (Pavlova *et al.* 1999) investigated two groups of patients, one with clonal stem cell disease with long latency period (myelodysplasia, myeloproliferative disease) and one with malignant haematological diseases (Hodgkin's lymphoma, non-Hodgkin's lymphoma, acute leukaemia, multiple myeloma). The prevalence of GBV-C/HGV RNA in the group of oncological cases (72%) was significantly higher ($P = 0.02$) than in the patients with clonal stem cell diseases (28%). A correlation could not be confirmed between GBV-C/HGV and liver enzyme levels, blood transfusions, chemotherapy, or viral co-infection (Pavlova *et al.* 1999). GBV-C/HGV infection in these patients is most likely to have originated from exposure to blood products, and to persist because of deficient immune surveillance. However, the clinical significance of these findings with respect to liver dysfunction is not yet clear. The pathogenetic consequences of GBV-C/HGV infection in lymphoproliferative disorders must be conclusively proven in additional studies.

Viral infections are presumed to trigger auto-immune processes. Heringlake *et al.* (Heringlake *et al.* 1996) observed that the prevalence of GBV-C/HGV in autoimmune hepatitis (AIH) type I-III was higher (9.8%) than in blood donors (4.7%). In contrast, patients with viral hepatitis B, C, and D were more frequently infected with GBV-C/HGV (16%, 20%, 36%, respectively). In contrast, Tribl *et al.* (Tribl *et al.* 1999) found a significantly increased prevalence of GBV-C/HGV in patients with AIH (11%), HBV (16%), and HCV (21%) than in healthy controls (2%). However, it remains unclear whether infection with GBV-C/HGV has an impact on the course of disease in patients with AIH. Persistent GBV-C/HGV RNA detected in 7/36 (19.4%) thalassemic patients was not associated with significant biochemical evidence of liver damage (Zemel *et al.* 1998). Patients with common variable immunodeficiency (CVID) are prone to unexplained chronic hepatitis whilst patients with X-linked agammaglobulinemia (XLA) who have a similar primary antibody deficiency are not prone to hepatitis (Morris *et al.* 1998). In their study of 78 CVID and 28 XLA patients, Morris *et al.* (Morris *et al.* 1998) concluded that the high prevalence of GBV-C/HGV viremia is due to the long-term exposure to blood products and that GBV-C/HGV does not cause chronic hepatitis in immunocompromised XLA patients. In addition, the authors suggested that, in the majority of CVID patients, GBV-C/HGV is not the cause of chronic non-B or -C hepatitis. In Japanese leprous patients the prevalence of GBV-C/HGV was higher (5.2%) than in blood donors (1%) (Egawa *et al.* 1996). Tucker *et al.* (Tucker *et al.* 1998) suggested an association with glomerulonephritis, hinting that virus replication may occur in the kidney.

Interferon (IFN) treatment of GBV-C/HGV infection

There are conflicting reports concerning the sensitivity of GBV-C/HGV to interferon (IFN) therapy. In some studies it seems to be similar to HCV (Berg *et al.* 1996; Tanaka *et al.* 1996; Orito *et al.* 1997; Jarvis *et al.* 1999), but in others it appears to be independent (McHutchison *et al.* 1997; Nagayama *et al.* 1997; Umlauft *et al.* 1997). However, the response may be different (Saiz *et al.*

1997). During IFN-α therapy, serum GBV-C/HGV RNA levels decrease in most patients treated, and it may become undetectable (Tanaka *et al.* 1996; Martinot *et al.* 1997; Nagayama *et al.* 1997; Saiz *et al.* 1997; Brandhagen *et al.* 1999; Jarvis *et al.* 1999). In only a small percentage of patients the response is sustained, and in most cases the GBV-C/HGV RNA concentration returned to pre-treatment levels after therapy was stopped (Berg *et al.* 1996; Tanaka *et al.* 1996; Karayiannis *et al.* 1997; Martinot *et al.* 1997; Saiz *et al.* 1997; Pawlotsky *et al.* 1998). Genotype, viral load, IFN dose, and the amino acid substitutions in the NS5A region (designated as the interferon sensitivity determining region (ISDR)) are considered to be some of the predictors for the efficacy of IFN therapy on HCV (Shiratori *et al.* 1997). However, most researchers detect no influence of GBV-C/HGV infection in response to IFN-α in patients with chronic HCV (Tanaka *et al.* 1996; Martinot *et al.* 1997; Orito *et al.* 1997; Saiz *et al.* 1997; Kato *et al.* 1999). No correlation between the amino acid sequence in the GBV-C/HGV NS5A region and response to IFN therapy was found, indicating that the GBV-C/HGV NS5A region does not act as the ISDR (Kato *et al.* 1999). A sustained response is predictable in patients with a low pre-treatment GBV-C/HGV viral load (Nagayama *et al.* 1997; Orito *et al.* 1997; Saiz *et al.* 1997; Enomoto *et al.* 1998; Jarvis *et al.* 1999).

Conclusions

GBV-C and HGV are closely related Flaviviruses of human origin. The detection methods for GBV-C/HGV need to be standardized and subjected to repeated quality control studies. GBV-C/HGV has a relatively high prevalence in the general population and a higher prevalence in certain high-risk groups. Transmission by blood transfusions, parenterally, sexually and from infected mothers to their new-born infants has been documented, and can induce persistent viraemia in humans. Most studies on GBV-C/HGV tropism have been limited to PBMCs and liver biopsies. Clearly, additional tissues from different organs of non-immunocompromised patients need to be studied using highly specific techniques to resolve the site(s) of replication for GBV-C/HGV. The role of GBV-C/HGV in human pathology needs to be examined more thoroughly in the absence of co-infection with other viruses. More information about its immune responses against the viral antigens and the virus pathogenicity is needed to better understand the clinical significance of GBV-C/HGV. However, some findings suggest that GBV-C/HGV is involved with some cases of acute and chronic hepatitis; that GBV-C/HGV may be pathogenic to primates considered to be appropriate non-human hosts for viral hepatitis studies; and that GBV-C/HGV does indeed replicate in human liver. It would be premature to screen blood donors for GBV-C/HGV and exclude a large proportion of blood donors from the donation pool without solid evidence that GBV-C/HGV is indeed pathogenic to humans.

Current evidence suggests that other viral agents or other factors may be responsible for a large majority of post-transfusion or community-acquired non-A–E hepatitis. A new pathogen, namely, Transfusion Transmissible Virus (TTV) has become a new focus of viral hepatitis research. This DNA non-enveloped virus has numerous similarities to GBV-C/HGV. Several independent studies have cast doubts on the pathogenicity of TTV. More recently, a novel hepatitis virus code named SEN-V has been isolated. SEN-V is considered by some leading researchers in hepatitis, to be the 'best candidate virus to account for previously unexplained hepatitis.' However, no data on SEN-V (The New York Times, 20th July 1999) has been presented in peer-reviewed publications.

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Brief Communication

Group 5: GBV-C/HGV Isolates From South Africa

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It was assumed initially that analysis of the 5′ NCR allowed the discrimination of three major groups that correlated with the geographic origins of the isolates and with the analysis of the complete genome sequences. Based on this assumption, sequence and phylogenetic analysis of the 5′ non-coding region (5′ NCR) of GBV-C/HGV isolates from the Western and Eastern Cape Provinces [Tucker et al., 1999] and the province of KwaZulu Natal [Sathar et al., 1999] of South Africa demonstrated the presence of new variants of GBV-C/HGV in South Africa. Unlike Tucker et al. [1999] neither major deletions nor additional bands to the predicted 344bp PCR fragment were observed in any of the isolates from KwaZulu Natal [Sathar et al., 1999].

Recent evidence suggests that phylogenetic analysis of the 5′NCR may not always provide an accurate guide to the relationship of complete genome sequences [Smith et al., 2000]. In their thorough analysis of 33 epidemiologically distinct complete or near complete genomic sequences of GBV-C/HGV, Smith et al. [2000] provided evidence of the existence of four major geographically distinct phylogenetic groups that were equally divergent from the chimpanzee isolate, GBV-C_{trop} [Birkenmeyer et al., 1998]. Group 1 included isolates from Ghana, West Africa, and a single Japanese isolate; Group 2 included isolates from Europe, North and South America, and Japan; Group 3 included isolates from Japan and China; and Group 4 included isolates from southeast Asia [Smith et al., 2000].

Analysis of 5′ NCR (positions −388 to −1) and subfragments of this region, including those previously identified as reproducing the phylogenetic relationships of group 1 and 2 isolates, provided <70% bootstrap support for either groups 3 or 4 [Smith et al., 1997]. However, the variants from KwaZulu Natal grouped separately from the 5′-NCR sequences of complete genome sequences for the region −388 to −1 [Smith et al., 2000]. When 5′ NCR sequences of complete genome sequences for the region 143–442 were analyzed, Group 3 (bootstrap support 83%) and Group 4 (bootstrap support 71%) isolates are not clearly differentiated by the phylogenetic analysis of this region; only the South African isolates grouped separately (Fig. 1). In contrast, analysis of a 200-nt fragment from the center of the E2 gene (positions 1,344–1,543) provides>70% bootstrap support for all four phylogenetic groupings, while a 600-nt region (position 994–1,594) provided >98% bootstrap support. GBV-C/HGV isolates from KwaZulu Natal form an additional fifth group when E2 sequences for the 350 nt region (positions 1,146 to 1,495) are compared with the corresponding region from the complete genome sequences of all four phylogenetic groups [Smith et al., 2000]. With the sole exception of E2 gene, phylogenetic analysis of individual genes and subgenomic regions, including the 5′ NCR, failed to consistently produce congruent phylogenetic trees of the four major groups that correlate with the geographic origin of the isolates [Smits et al., 2000]. Phylogenetic analysis of the E2 gene segment of KZN isolates was shown to be consistent with previous analysis of the 5′ NCR [Sathar et al., 1999], suggesting that these belong to a fifth group [Smith et al., 2000].

In their proposal that South African GBV-C/HGV isolates be classified as "Genotype 5," Tucker and Smuts [2000] did not make reference to the findings of Smith et al. [2000] nor did they include in their analysis GBV-C/HGV isolates from the province of KwaZulu Natal, South Africa [Sathar et al., 1999]. Phylogenetic analysis of 5′NCR sequences of the "novel" South African GBV-C/HGV isolates from the provinces of the Western, and Eastern Cape (excluding the deletants) [Tucker et al., 1999] and from KwaZulu Natal [Sathar et al., 1999], grouped South African isolates as

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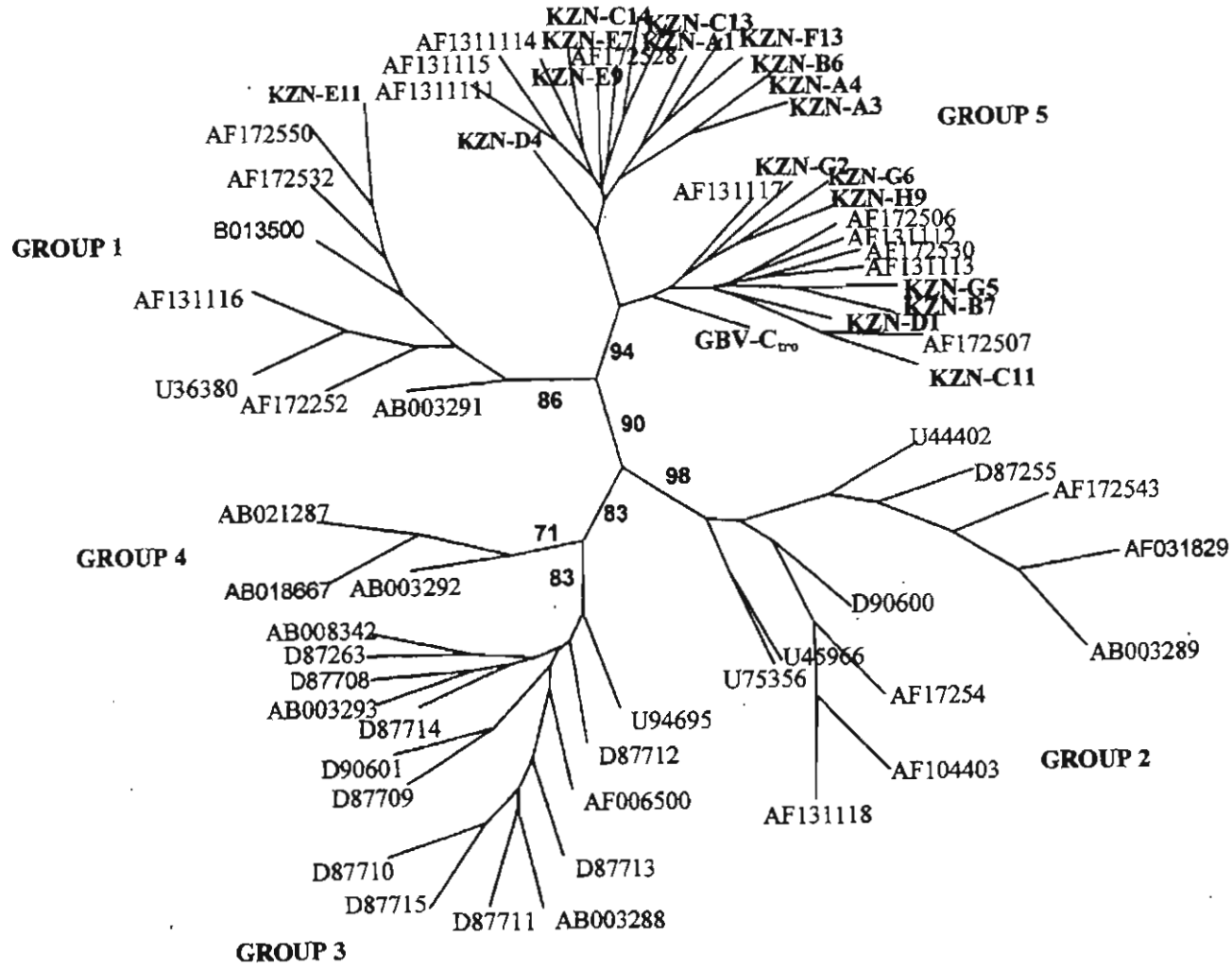

Fig. 1. Consensus phylogenetic tree of 5'NCR sequences of GBV-C/HGV isolates from South Africa and representative published sequences from GenBank/EMBL. Phylogenetic analysis was carried out on a 311-bp fragment, positions 143–442 in the GBV-C prototype isolate (U36380). Maximum likelihood distances between sequences were calculated using Phylip DNADIST (ts:tv = 3, assuming no rate variation between sites), and used to produce a neighbour joining tree using Phylip NEIGHBOR. Bootstrap values (100 replicates) were obtained with the SEQBOOT and CONSENSE options of Phylip. The tree was produced using Treeview version 1.5. KZN = KwaZulu Natal; A-H = 8 geographical health regions of KwaZulu Natal.

an additional fifth group (Fig. 1). The inclusion of GBV-C/HGV isolates from KwaZulu Natal in the proposal made by Tucker and Smuts [2000] that South African isolates be classified as Group 5 or Genotype 5, will be more representative of isolates from South Africa.

It would certainly be interesting to obtain the E2 sequences of additional South African isolates. However, the complete sequence of one or more of the novel South African isolates would indeed settle the question of whether or not this is in fact a new phylogenetic variant.

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