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Genotypes of Hepatitis B and C Viruses in Nigeria

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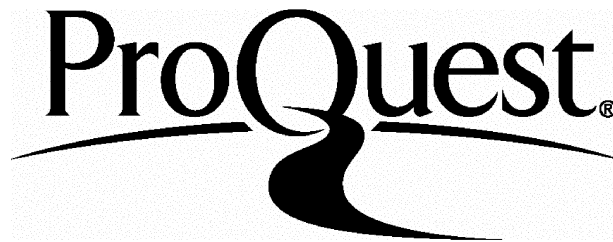
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DEDICATION

This thesis is dedicated to my brother Dr. Olubunmi Abiola Oni, for believing in

my future, and to the memory of my late parents, Chief Joshua Abereoje Oni and

Chief (Mrs) Emily Aina Afolaye Oni.

ABSTRACT

A small scale epidemiological survey of hepatitis B and C virus infection amongst healthy adult blood donors and children of pre-school age in Nigeria was carried out using various serological markers of hepatitis B virus (HBsAg, anti-HBs, HBeAg, anti-HBe), and hepatitis C virus infection (anti-HCV ELISA and RIBA tests). Between 10 and 11 % of the adults, and 16% of the children were HBsAg positive. A viraemia rate of 23.8% was found among the HBsAg positive adults, and 25% among the HBsAg positive children. Evidence of past exposure (anti-HBs) among adult blood donors was 41.24%. The subtype of HBV present in Nigeria was determined by amplifying and sequencing HBV DNA, encoding the antigenic determinant *a* and surrounding region. Two subtypes of HBV are present in Nigeria, *adw* & *ayw*. The subtype *ayw* was in the majority (7/10 of HBV sequenced). A multiple infection with both subtypes of HBV was found in one donor, another donor was infected with both wild type and a variant of *ayw*. HBV DNA encoding the pre-core and part of the core region of 7 donors was sequenced. One donor was infected with a precore mutant and another was infected with a further variant of HBV. The prevalence of anti-HCV detected by a second generation ELISA was 8% (16/200) of the adult donors, but most of them were negative on supplementary testing. None of the children tested was anti-HCV positive. 25% (4/16) of the anti-HCV positive sera were RIBA test positive and 12.5% (2/16) were indeterminate. Four RIBA positive samples and one negative were HCV-RNA positive, thus 31.25% (5/16) of the ELISA-positive sera were HCV RNA positive by reverse transcriptase polymerase chain reaction (RT-PCR). Three regions of HCV were sequenced, the 5'untranslated region (5'UTR), core and NS5. At least two HCV genotypes are present in Nigeria, type 1 and type 4. Phylogenetic analysis and pairwise comparison confirmed two new subtypes which were provisionally designated 1d and 4i.

Acknowledgement

He who Dwells in the Secret Place

of the Most High,

Shall Abide Under the Shadow

of The Almighty God,

I will say of the lord,

He is my refuge and my fortress,

My God in whom I will Trust.

Psalm 91 vs 1-2

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ABBREVIATIONS

ATP	=	Adenosine 5' triphosphate
cccDNA	=	Covalently Closed Circular DNA
dATP	=	2'-Deoxyadenosine 5'-triphosphate
dCTP	=	2'-Deoxycytidine 5'-triphosphate
DEPC	=	Diethyl Pyrocarbonate
DHBV	=	Duck Hepatitis B Virus
dGTP	=	2'-Deoxyguanosine 5'-triphosphate
DTT	=	Dithiothreitol
dTTP	=	2'-Deoxythymidine 5'-triphosphate
ELISA	=	Enzyme Linked Immunosorbent Assay
ER	=	Endoplasmic Reticulum
GSHV	=	Ground Squirrel Hepatitis Virus
HAAg	=	Hepatitis A Antigen
HAV	=	Hepatitis A Virus
HBV	=	Hepatitis B Virus
HCV	=	Hepatitis C Virus
HDV	=	Hepatitis D Virus
HEV	=	Hepatitis E Virus
LHBs	=	Large Hepatitis B Surface Antigen
MHBs	=	Middle Hepatitis B Surface Antigen
mRNA	=	Messenger Ribonucleic Acid
PCR	=	Polymerase Chain Reaction
RIBA	=	Recombinant Immunoblot Assay

RNA   =   Ribonucleic Acid

RT-PCR   =   Reverse Transcriptase Polymerase Chain Reaction

SHBs   =   Small Hepatitis B Surface Antigen

SDS   =   Sodium Dodecyl Sulphate

S-ORF   =   Surface Open Reading Frame

WHV   =   Woodchuck Hepatitis Virus

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CHAPTER 1

(*1.1*) GENERAL INTRODUCTION

Viral hepatitis is a cause of considerable morbidity, both from acute infection and the chronic sequelae. During the last 20 years there has been a dramatic improvement in the understanding of viral hepatitis, the consequences of which will affect hundreds of millions of people throughout the world. It is now recognised that most cases of viral hepatitis are caused by five totally unrelated human viruses. Hepatitis A (HAV) and hepatitis E (HEV) viruses cause epidemic hepatitis and are transmitted by the faecal oral route (Forbes *et al.*, 1990, Bradley, 1990). HEV is also responsible for a high mortality during pregnancy (Tsega *et al.*, 1993). Hepatitis B virus (HBV), and the recently identified hepatitis C virus (HCV), are spread by various parenteral routes and are endemic or hyper endemic in most human populations (Alter, *et al.*, 1990b, Aach *et al.*, 1991), both are associated with chronic hepatic disease and also with primary liver cancer (Beasley, 1988, Hasan *et al.*, 1990). Hepatitis D virus (HDV or the delta agent) characteristically co-infects or super-infects patients with HBV, and it is an important cause of severe chronic liver damage in hepatitis B surface antigen (HBsAg) carriers (Rizzetto *et al.*, 1977). The viruses causing chronic hepatitis B & C (and D) are the most important in terms of causing major morbidity and mortality throughout the world.

(*1.1.2*) **Hepatitis A Virus (HAV)**

Hepatitis A virus is a small symmetrical RNA virus, a member of

the Picornaviridae. The genome is a single stranded RNA molecule of around 7,500 nucleotides, and is linear, and of plus-strand polarity (Najarian *et al.*, 1985). HAV has a unique nucleotide sequence homology, there is remarkably little genetic variation, with apparent common antigen to all isolates from different sources. It shares the structural and functional characteristics of the picornavirus family (Najarian *et al.*, 1985). The virus is usually stable, resisting heat (up to 60 °C for 1 hour, or 25 °C for 3 months); indefinite cold storage (5 °C); acidic conditions (pH 3); and non-ionic detergents (Siegl *et al.*, 1984). Man is probably the only natural host. Transmission of the virus is by the faecal-oral route and viral replication probably occurs in the jejunum prior to transmission via the portal vein to the liver. Electron-microscopy, and immune electron microscopy can be used to identify viral particles in stool, but concentrations are usually low, viral concentrations of more than 10^6 per ml are required for immune electron microscopy (Feinstone *et al.*, 1973).

HAV antigen (HAAg), usually is detectable in the stools during the early course of illness, and immunoassay can provide a specific diagnosis of acute infection (Hollinger *et al.*, 1975). However, the level of HAAg in stool falls rapidly with the onset of symptoms, the sensitivity of this test is therefore poor. Molecular hybridization and the Northern blotting can be used to detect HAV RNA in faeces (Tassopoulos *et al.*, 1986).

Virus appears in stool during the prodromal phase, which begins 2-6 weeks after oral inoculation, and can remain detectable for up to 2 weeks after the onset of clinical hepatitis. The median incubation period from inoculation to onset of symptoms is about 4 weeks, although specifically hepatitic symptoms may be

delayed for a further 4-10 days.

The development of a serological test for hepatitis A (IgM anti-HAV), has confirmed that the faecal/oral route of spread is the common source of infection. IgM antibody to HAAg appears in serum 3-7 weeks after oral inoculation (Lemon *et al.*, 1980); the larger the inoculum the shorter the incubation period, the antibody is invariably present by the time of clinical presentation. Therefore, spread from one individual to another by close contact within a family or a community may occur at the onset of jaundice. Spread by urine, nasopharyngeal secretions and blood may occur uncommonly and homosexual spread is rare (Tassopoulos *et al.*, 1986).

(1.1.3)   **Epidemiology of Hepatitis A Virus**

HAV has a world wide distribution, and is responsible for both epidemic and sporadic disease (Bradley, 1990). The spread can occur sporadically or in epidemics. Epidemics have been traced to contaminated food (particularly seafood), and water supplies (contaminated by sewage; Graff *et al.*, 1993). Infection usually occurs in childhood and adolescence, but with the advent of good hygiene and sanitation in Western communities there is a decline in childhood of hepatitis A infection, and this has resulted in an increase in susceptibility in adults. This, however, has three main consequences: an increase in the average age of infection may possibly lead to an increase in morbidity; normal immunoglobulin may become less protective against hepatitis A; the risk of transmission through blood products contaminated by viraemic blood donors may rise (Gay *et al.*, 1994). The outcome of infection with HAV depends on the age at which infection occurs

and, perhaps, the infectious dose. The pattern of infection has changed dramatically in some countries, but the disease still remains a public health problem in many parts of the world. The disease is still a problem in some developed countries, and endemic in some developing countries, where above 80% are antibody positive by age of 3 years. Unlike hepatitis B, hepatitis A does not progress to a chronic carrier state and chronic liver disease.

(*1.1.4*)   Hepatitis A Vaccine

Both inactivated and attenuated hepatitis A vaccines have been developed. Inactivated hepatitis A vaccine was first described in 1978 (Provost and Hilleman, 1978), and was demonstrated in marmoset. The hepatitis A attenuated vaccine was demonstrated at Merck Institute in 1982 (Provost *et al.*, 1982). Protective efficacy trials of two different killed virus vaccines (Merck & SKB) in human beings were carried out in 1991. The inactivated hepatitis A vaccine is currently licensed.

(*1.1.5*)   Hepatitis E Virus

Hepatitis E virus (HEV), is an enterically-transmitted virus which spreads predominantly by faecal contamination of drinking water (Naik *et al.* 1992). HEV is an RNA virus, the genome is a positive-sense RNA, 7.5kb in length (Tam *et al.*, 1991), and the particles measuring 27 to 34nm were regularly recovered from stools of monkeys.

Hepatitis E has symptoms from a self-limited, to acute, and icteric disease, which are similar to those of hepatitis A. Jaundice is usually accompanied by malaise, anorexia, abdominal discomfort and liver enlargement. A conspicuous

feature of hepatitis E is the high mortality rate among pregnant women (approximately 20%), primarily those in their third trimester (Tsega *et al.*, 1993)

(1.1.6)   **Epidemiology of Hepatitis E**

The source of the disease is usually contaminated water (Naik *et al.*, 1992), and epidemics are frequently observed after raining season. The incubation period averages 6 weeks, with a range of 2 to 9 weeks and the highest attack rate among cases is observed between ages of 15 and 40 years, however, sporadic antibody positivity among children of 2 months to 15 years have been reported in Sudan and Egypt (Hyams *et al.*, 1992).

Both epidemic and sporadic HEV infection have been reported from various part of the world. Epidemics of water borne infections with HEV have been reported from India, Pakistan, Burma, Soviet Union, Central and South America (Naik *et al.*, 1992, Bryan *et al.*, 1994). Sporadic cases of hepatitis E along with other virus have also been reported.

(1.1.7)   **Hepatitis B Virus (HBV)**

Hepatitis B virus, is a member of the hepadnaviridae, this family comprises a number of small DNA viruses with a similar genome structure. HBV was the first human hepatitis virus from which the proteins and genome were identified and characterized. In 1963 Blumberg while searching for polymorphic serum proteins, discovered a previously unknown antigen in the blood of an Australian aborigine (Australia antigen). Soon thereafter, it was recognized that this antigen was related to type B hepatitis (Blumberg *et al.*, 1967). In 1970, D.S. Dane eventually

discovered virus like particles in the serum of hepatitis B patients that carried this antigen on their surface (Dane *et al.*, 1970). These particles were subsequently confirmed as hepatitis B virion.

For several years HBV was considered to be a unique virus, but later similar viruses were found in three different animal species. The first was found in 1978 in sera of the eastern woodchuck (*Marmota monax*), a member of the Sciuridae or squirrel family (Summers *et al.*, 1978). The most frequent cause of death in these animals was hepatocellular carcinoma accompanied by a particular form of hepatitis with degenerative changes. The sera of animals with chronic hepatitis were found to have an HBV-like virus containing a DNA polymerase activity and a circular, partially double stranded DNA similar in size to that of HBV. The virus was called woodchuck hepatitis virus (WHV).

The third member of this virus family, ground squirrel hepatitis virus (GSHV), was discovered in 1980 (Marion *et al.*, 1980). This virus was found in the Beechey ground squirrel (*Spermophilus beecheyi*), another genus of the Sciuridae family, during a search for an HBV-like virus in Californian relatives of the eastern woodchuck. The observation of the frequent hepatomas in a species of domestic ducks (*Anas domesticus*) in the People's Republic of China led to the discovery of the fourth member of this virus family, duck hepatitis B virus (DHBV). Sera from this duck species were found to contain an HBV-like virus. Attempts to passage this virus in eggs from Pekin ducks from the United States (USA) led to the discovery that approximately 10% of Pekin ducks in some commercial flocks in that country carry a similar virus (Mason *et al.*, 1980). Heron hepatitis B has been described (Sprengel *et al.*, 1988), the DNA sequence of this

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virus shows a considerable homology to that of DHBV.

Comparatively, there is no great difference in the genomic size of hepadnavirus genomes. The genomes of GSHV and WHV are approximately of equal size and are the largest of all hepadnaviruses around 3.3kb. HBV is between 3.1kb and 3.2kb, while DHBV is smaller, around 3.0kb. The X-ORF is conserved in similar form in the hepadnaviruses with the exception of avian hepadnaviruses where it is absent and the core gene is larger. DHBV has a similar virion morphology to the mammalian hepadnaviruses (Mason *et al.*, 1980). The serum from DHBV-infected ducks contains an excess of DHBsAg particles but their morphology is different in that the DHBV surface antigen has a variable diameter of between 40-60 nm. No filaments are detectable. All mammalian hepadnaviruses are associated with chronic hepatitis which frequently leads to HCC (Beasley *et al.*, 1981, Marion *et al.*, 1986), however, chronic DHBV infection has not yet been proven to carry an increased risk of HCC (Cullen *et al.*, 1990). Among mammalian viruses, WHV is by far the most potent tumour inducer; all chronic WHV carriers develop one or multiple HCC after 3 years of experimental infection on a carcinogen-free diet (Popper *et al.*, 1987). The frequency of HCC is lower in GSHV and HBV; nevertheless, chronic HBV carriers have an approximately 200-fold increased lifetime risk of developing primary liver cancer.

(*1.1.9*) **Epidemiology of Hepatitis B Virus**

The development of serological tests for HBV antigens and their respective antibodies made possible the recognition that HBV has a worldwide distribution (Courounce-Pauty *et al.*, 1983, Nasidi *et al.*, 1986), and that infection rates in some

parts of the world are extremely high. Hepatitis B causes an estimated 1 to 2 million deaths a year worldwide (W. H. O., 1992). The global prevalence of hepatitis B has considerable economic implications, because some of its chronic complications, such as cirrhosis, make great demands on the health care system. It is estimated that at least about 2,000 million people have been infected by HBV worldwide. Of these, more than 300 million are chronically infected carriers, of whom 25% are at risk of serious illness and eventual death from cirrhosis or hepatocellular carcinoma. Three-quarters of the world's population lives in areas where there are significant levels of infection (W. H. O., 1992). Hepatitis B virus is associated with chronic persistent hepatitis and chronic active hepatitis, also anicteric infection is very common and may usually lead to chronic disease and finally lead to the development of hepatocellular carcinoma (Beasley *et al.*, 1988).

Vertical transmission by blood or blood products following blood transfusion or the use of plasma concentrates, contaminated syringes or needles, is one of the potential ways for the spread of this disease, other methods of spread include tattooing, scarification and sexual contact, particularly homosexual. The role of spread by other body fluids such as saliva and faeces is still unclear, there is, however, a high rate of infectivity in certain groups such as dialysis and immune suppressed patients, and children with Down's syndrome.

(*1.1.10*)　　　　**Hepatitis B vaccines**

(a)　　　　**Plasma Derived Hepatitis B Vaccine**

One of the problems associated with development of vaccine for hepatitis B virus, was the lack of a cell culture system in which to propagate the virus. This

problem has directed attention to the use of other preparations for active immunization. The 22nm hepatitis B surface antigen induced protective antibody, this antigen has been purified from the plasma of symptomless carriers and developed into a vaccine. The vaccine is quite safe, as no known report of transmission of acquired immune deficiency syndrome (AIDS) or any other disease has been associated with the vaccine.

(b)   **Recombinant Hepatitis B Vaccine**

The advent of molecular biology and progress in nucleic acid chemistry eventually led to the identification of genes coding for biologically active substances and allowed for the cloning and purification of gene products. HBV DNA (S-gene) region encoding the surface protein was cloned and expressed in yeast. The protein product was purified and used as vaccine.

(c)   **Hepatitis B Vaccine Non-responders**

Immunization against HBV infection has been effective in preventing the establishment of chronic HBV infections, but not every subject responds to vaccination. The mechanisms causing non responsiveness to hepatitis B surface antigen (HBsAg) vaccines in man remain largely unknown, the antibody responses to the *a* determinant are presumed to be regulated by the major histocompatibility complex (MHC; Desombere *et al.*, 1995). The currently licensed recombinant hepatitis B vaccine consists only of the S-gene product. The increased incidence of non-responsiveness in subjects with HLA-DR3 or DR7 haplotype suggests that immune response mechanisms are governed by genes of the major

histocompatibility complex (MHC). However, Desombre *et al.* (1995) showed that HLA-DR2+, DR7+, and DPW4+ non-responders to the vaccine are able to take up, process and present HBsAg to allogenic, haplo-identical T cell lines *in vitro*. The fact that the majority of non-responders are mostly subjects of above 30 years of age, has raised the suggestion that age may be one of the determining factor(s), the reason for this is not yet clear. The problem of non-responsiveness to major surface proteins may be circumvented in future by the combination of S and pre-S2 proteins, since the pre-S2 region appears to be significantly more immunogenic than the S region of HBsAg (Milich *et al.*, 1985, Itoh *et al.*, 1986). In a classical experiment by Milich *et al.* (1985), two groups of non-responder mice were immunized intraperitoneally with pre-S2 encoded protein or a tenfold of S region encoded protein, produced from Chinese hamster ovary (CHO) cells. After ten days, all the pre-S2 protein immunized mice contained immunoglobulin G (IgG) specific for the pre-S2 region polypeptide but none for the S region polypeptide, even though they had received a tenfold greater dose of S protein than of pre-S2 region protein. The same result was obtained at a 46-fold greater dose of S region antigen compared to the pre-S2 region protein. Also, there is evidence that the antibody response to the pre-S2 region protein can stimulate the response to the S region protein (Milich *et al.*, 1985). The 26 amino acid residues at the amino terminal end (NH_2-terminus) of the 33kD polypeptide was found to represent a dominant antibody binding site on the pre-S2 protein, and also the immune response to pre-S2 seems to be regulated by genes which are distinct from those presumed to regulate the response to the S region (Milich *et al.*, 1985). The fine specificity of T-cell recognition of the pre-S2 region was dependent on the H-2

haplotype of the responding strain. All the 17 recognition sites present were subtype specific, which is consistent with the fact that the C-terminal sequence is highly polymorphic between the *d* and *y* subtypes of the pre-S2 region (Milich *et al.*1990).

(*1.1.11*)   **Delta Hepatitis (HDV)**

Rizzetto *et al.*, in 1977, reported the observation of a new viral antigen present in the nuclei of hepatocytes in patients infected with hepatitis B virus. Subsequent studies confirmed the original observation and revealed that the new antigen was related to a new agent, later termed hepatitis delta or HDV. The viral genome is coated with hepatitis B surface antigen inside of which are, the RNA genome and the only protein of HDV, delta antigen (HDAg). The diameter of the particle is about 38-41 nm (He *et al.*, 1989). The genome is a single stranded RNA of about 1.75 kb in length (Chen *et al.*, 1986). It is dependent on pre-existing or concomitant HBV infection for propagation. Super infection of hepatitis B carriers with HDV frequently is associated with chronic infection in about 70-90% of cases and deterioration of the underlying HBV chronic hepatitis. Co-infection is usually followed with clearance of both HDV and HBV, although in some instances the infection results in a fulminant course of the disease (Rizzetto *et al.*, 1990). HDV infection is normally associated with the presence of delta antigen in both liver and serum and viral RNA in serum followed by the development of antibodies to delta antigen (Smedile *et al.*, 1986). Serological findings can be used to distinguished acute from chronic delta hepatitis. The acute self-limiting course is characterized with low titre of IgM and IgG anti-HDV, with an early antigenaemic phase fol-

lowed by raised IgM antibody which can last for about 2-3 weeks before the appearance of IgG (Shattock *et al.*, 1989), while in chronic delta virus infection, the IgM persist throughout the disease.

(*1.1.12*)   **Epidemiology of Delta Hepatitis**

Transmission of HDV is mainly via the parenteral route and is generally associated with contact with blood or blood derived products, vertical transmission being a rare occurrence. Screening for HBsAg in potential blood donors minimizes but does not abolish the risk of post-transfusion delta hepatitis. In Northern Europe and U.S.A., HDV infection is practically confined to intravenous drug abusers and homosexuals (Weisfuse *et al.*, 1989). There is a high prevalence of HDV infection in Italy, and it is also very common elsewhere in Southern Europe (Sagnelli *et al.*, 1992).

The HDV infection has been reported throughout the world and its distribution tends to follow the prevalence of HBV (Omata *et al.*, 1985, Amazigo & Chime, 1988). However, there are regions of high HBV endemicity where a very low HDV prevalence have been reported, for example, in some parts of far East (el Guneid *et al.*, 1993), South Africa (Abdool *et al.*, 1991) and the Alaskan Eskimos. Marked differences of HDV incidence have also been reported in some European countries, Romania, Bulgaria and former Yugoslavia, and in different regions of some countries such as Kenya (Greenfield *et al.*, 1986), suggesting the possible involvement of additional factors in the spread of the infection.

(1.1.13)   Hepatitis C Virus (HCV)

(1.1.14)   **Discovery of HCV**

Despite the introduction of more sensitive tests for the detection of HBsAg

and the universal screening of blood donors, which virtually eradicated the

transfusion associated hepatitis B (TAHB) ironically TAH per se did not

disappear. With the discovery of hepatitis A virus (HAV) in 1973, and the devel-

opment of serological assays for this virus (Feinstone *et al.*, 1973), it was possible

to demonstrate that an agent other than viruses that cause hepatitis A or B is

responsible for the majority of cases of post-transfusion hepatitis (Feinstone *et al.*,

1975). Thus in 1975, the existence of a third type of hepatitis was recognized and

named non-A, non-B (NANB) hepatitis. The etiological agent of parenterally

transmitted non-A, non-B hepatitis is hepatitis C virus (HCV). The genome was

discovered using a recombinant complementary DNA (cDNA) approach (Choo *et*

al., 1989).

(1.1.15)   **Epidemiology of HCV Infection**

The prevalence of type C hepatitis in blood donors has been ascertained in

many countries. The positive immunoassay rate ranges from 0.18 to 1.4%. The

prevalence is generally low in western countries, ranging from 0.3 to 0.7%; is but

slightly higher in Japan and southern Europe, between 0.9 and 1.2%. (Alter *et al.*,

1989a). A higher prevalence has been found in southern Italy and eastern Europe

(Esteban *et al.*, 1989). There is a higher prevalence rate in the Middle east (Ayoola

et al., 1992) and in Africa, where a prevalence rate of up to 4.2% anti-HCV

positivity have been reported (Coursaget *et al.*, 1990, Delaporte *et al.*, 1993). The

highest prevalence worldwide is found in haemophiliacs, 50-90% of whom are anti-HCV positive, depending upon age, the duration of infection, factor VIII requirement and the source of factor VIII. The prevalence of anti-HCV is also high in multiply transfused patients with thalassaemia major, but varies geographically according to the source of the blood administered to patients (Wonke *et al.*, 1990).

The prevalence in intravenous drug users is extremely high (70-90%), because of repeated exposure to carriers of HCV through shared, contaminated needles. Anti-HCV is apparently also common in transplant patients requiring frequent blood transfusion, including renal, liver and bone marrow transplant recipients (Ponz *et al.*, 1991b, Poterucha *et al.*, 1992). Transmission after needle stick injury may also occur. Health care workers appear to be at relatively low risk (Kiyosawa *et al.* 1991). There are however, well documented instances of needle stick transmission of HCV, and of NANB hepatitis after surgery, or even hospitalization without transfusion (Kiyosawa *et al.*, 1991).

(1.1.16)   **The GB Hepatitis Agent**

The detection of various known hepatitis viral agents, through very sensitive, reliable immunoassays and molecular hybridisation probes, has not eliminated completely transfusion associated hepatitis and related diseases in man. One of the least known additions to the hepatitis agent family is the GB agent, which was originally isolated from the serum of a 34 years-old surgeon in Chicago, with acute hepatitis. The agent was obtained on the third day of the jaundice (Deinhardt *et al.*, 1967). GB were the initials of the surgeon from whom

the virus was isolated, hence the virus was called in his name. Inoculation of tamarins (*Saguinus* species) with the serum from this patient induced hepatitis in these animals (Deinhardt *et al.*, 1967).

The subtractive polymerase chain reaction method known as representational difference analysis (RDA; Lisitsyn *et al.*, 1993) was used to clone specific nucleotide sequences present in infectious plasma from a GB-infected tamarin, allowed for a detail examination of the GB genome (Simons *et al.*, 1995a, Simons *et al.*, 1995b). The genome is an RNA from all sources, and Northern blot analysis predicted the genome to be approximately 8.3kb long. Extensive sequence analysis identified two viruses, GB virus A and GB virus B (GBV-A and GBV-B), the genome is RNA molecule about 9kb long, both has a limited sequence identity with various isolates of HCV, and they were related to members of the *Flaviviridae* (Schlauder *et al.*, 1995, Simons *et al.*, 1995b). These two RNA molecules were identified as GBV-A and GBV-B (Simons *et al.*, 1995a). The third of these agents, GBV-C, was recently identified from a West African patient (Simons *et al.*, 1995b), this agent (GBV-C) has been suggested to be responsible for some cases of non-A, non-B, non-C, non-D, and non-E hepatitis.

CHAPTER 2

PART I : HEPATITIS B VIRUS

(2.1.1) MOLECULAR BIOLOGY OF HBV

The detailed understanding of the molecular biology of this virus, like all other viruses has only developed in the last twenty or more years. Among the problems faced by early researchers, were the lack of a cell culture system in which to propagate the virus. In 1973, the virion like particles discovered by Dane (Dane *et al.*, 1970), were confirmed as hepatitis B virus (HBV) by the detection of an endogenous, DNA-dependent DNA polymerase within their core (Kaplan *et al.*, 1973). The polymerase activity was dependent on all four deoxynucleotide triphosphates and divalent cations, but no exogenous primer or template was necessary, the polymerase reaction is possible because one of the HBV-DNA strands is incomplete (Landers *et al.*, 1977). The remaining gap is filled by viral DNA polymerase if the dNTPs are added *in vitro* (Kaplan *et al.*, 1973). The reaction could be inhibited by actinomycin D, which intercalates into DNA templates. The enzyme activity was precipitable with antibodies against hepatitis B core protein (HBc) if the hepatitis B surface (HBs) envelope was removed from the virion by treatment with a non-ionic detergent. The DNA template was not accessible to DNase unless the core protein was lysed by sodium dodecyl sulphate (SDS) or digested by proteinase.

This enzyme and advent of molecular biology cloning techniques allowed Robinson (Robinson & Greenman, 1974), to detect and characterize the HBV genome. The HBV genome is a small, circular, partially double stranded (figure 2.1.1) and is about 3200 nucleotides in length (Robinson & Greenman, 1974), one

of the strand is of full length, and as it is complementary to the viral mRNAs, is designated to be of minus polarity. The complete minus strand has defined 5' and 3' end, with a terminal redundancy of nine bases (Will *et al.*, 1987). The duplex DNA genome is held in a circular conformation by a cohesive overlap between the 5' ends of the two DNA strands (Sattler *et al.*, 1979). The cohesive overlap falls within the boundaries of a short repeat sequence that is 10 to 12 bases in length (Molnar-Kimber *et al.*, 1984, Will *et al.*, 1987). The copy flanking the 5' terminus of the minus strand is designated direct repeat 1 (DR1), and that flanking the 5' terminus of the plus strand is direct repeat 2 (DR2). A protein is covalently bound, via a tyrosine-linked phosphodiester bridge, to the 5' end of the minus strand (Gerlich *et al.*, 1980). This linkage causes extraction of virion-derived DNA to the phenol phase during DNA extraction, unless the sample is thoroughly digested with proteinase (Gerlich *et al.*, 1980). The other strand, termed the plus strand, is shorter than full length (Landers *et al.*, 1977), and has at its 5' end a sequence of 18 ribonucleotides that are capped in the same manner as an mRNA (Lien *et al.*, 1986). The incomplete plus strand has a defined 5' end but a variably situated 3'end (Delius *et al.*, 1983). The genome structure is typical for all hepadnaviridae, but slightly different in the avian hepadnaviruses where the overlap sequence and the distance between DR1 and DR2 is shorter. The structure of the hepadnaviral genome does not abide by the usual classification criteria for viruses in two respects: it contains both DNA and RNA and its genome contains single and double stranded regions (Delius *et al.*, 1983) These asymmetries result from the unique strategy for genome replication employed by the hepadnaviruses (Summers & Mason, 1982).

(2.1.2)   **Organization of viral genome**

Determination of the nucleotide sequences revealed that HBV genomes are between 3182 and 3221 nucleotides long (Tiollais *et al.*, 1981). The numbering of the bases starts in most publications at the cleavage site for the restriction enzyme *EcoRI* or at the homologous position if a particular genome type does not have such a site (figure 1). There are four overlapping open reading frames (ORFs), or genes (Ganem & Varmus, 1987), these are core (pre-C/C), Pol (P), surface (S) genes (analogous to the retroviral gag, pol, and env) which encode the HBV nucleocapsid, polymerase and envelope proteins, respectively. In addition there is an X gene, so called because the function of the X gene product was unknown when the X-ORF was identified. The HBV genome is not much longer than its longest P-ORF, which encodes the viral polymerase and its accessory functions. The S-ORF is completely located within the P-ORF, C-ORF and X-ORF overlap partially with P-ORF. HBV encodes more than one protein from some ORFs using internal ATG codons as a starting sites for protein biosynthesis. Thus, nested sets of proteins with different amino termini and a common carboxyl terminus are synthesized. The S-ORF encodes the three HBs proteins (Heermann *et al.*, 1984). The C-ORF encodes the HBe protein and the HBc protein (Ou *et al.*, 1986). The X-ORF also appears to encode more than one protein (Kwee *et al.*, 1992).

(2.2.1)   **Replication of HBV**

Genomic replication is via reverse transcription (Summers & Mason, 1982), which indicates that viral RNA must serve as both template for genomic DNA synthesis and messenger for the synthesis of viral proteins. The replication of the

hepadnavirus genome is unusual in several respect. Firstly, the DNA strands are synthesized successively, the minus strand must be completed before synthesis of the plus strand can proceed (Will *et al.*, 1987). This is in contrast to semi-conservative DNA replication, where both nascent strands are synthesized simultaneously. Secondly, the virus polymerase function as a reverse transcriptase and primes the RNA-directed DNA synthesis from an internal site of the polypeptide (Wang & Seeger, 1992). The priming of the plus strand synthesis is by an oligoribonucleotide derived from viral pre-genomic RNA (Lien *et al.*, 1986).

Four major steps are fundamental to the replication of hepadnavirus genomes (figure 2.1.2 & 2.1.3).

(*1*) Conversion of the asymmetric DNA found in the virion to covalently closed circular DNA (cccDNA) within the nucleus of infected hepatocytes.

(*2*) Transcription of the cccDNA by the host RNA polymerase to generate an RNA template (the pregenomic RNA) for reverse transcription, with encapsidation of the pre-genome into viral cores.

(*3*) Synthesis of the first (or minus) strand of DNA by copying pregenomic RNA using the polymerase as a primer.

(*4*) Synthesis of the second (or plus) strand of the DNA by copying the minus DNA strand and using an oligomer of viral RNA as a primer.

Figure 2.1.1: *Structure and genetic Organization of HBV genome.* The inner circles represent the partial dsDNA genome with the incomplete (+) strand and the position of important genetic elements indicated. p = promoter sequence, E = enhancer; GRE = Glucocorticoid responsive element. DR1 = Direct repeat 1, DR2 = direct Repeat 2. Bars in the middle represent the location of expressed open reading frames on the viral (+) strand, pre-c = precore; c = core, pol = reverse transcriptase/polymerase; pre-S1/pre-S2; pre-surface; s ; surface X, = x; gene. Outer lines indicate the extension of the viral transcripts (modification based on Tiollais *et al* ., 1981).

Figure 2.1.2: *Hepadnaviral life cycle at the molecular level.*

(1) Pregenomic RNA is transcribed from the template cccDNA in the nucleus. (2) After packaging of pregenomic RNA into pre core particles a terminal protein derived from the 5' region of the pol-ORF, acts as a specific primer for (-) polar DNA formation by attaching to the 3' DR1 sequence of the pregenome (3) Reverse transcription of (-) strand DNA by the HBV polymerase/reverse transcriptase (pol/RT) proceeds and simultaneously the pregenome is degraded by the RNaseH activity of the pol/RT. (4) After completion of the (-)strand a short RNA primer derived from the 5' terminus of the RNA pregenome is translocated and hybridizes to the DR2 region in order to initiate (+) strand synthesis. (5) After circularization of the viral DNA, (+) strand DNA replication proceeds, but remains incomplete. (6) After viral DNA is released from nucleocapsids into the nucleoplasm the incomplete dsDNA is converted by the viral polymerase into cccDNA and a new cycle begins. (See text section 2.2.3 and 2.2.4 for further details).

Fig 2.1.3: *Hepadnaviral viral life cycle at the cellular level*

(i) Complete virion carrying the incomplete dsDNA genome circulate within the bloodstream. (2) Enters the liver hepatocyte cell, and it is released from the lipid bilayer containing the viral surface antigens. (3) Core particles are transported into the nucleus, where the genome is released. (4) cccDNA is completed by the viral polymerase. (5) cccDNA acts as the template for transcription of pregenomic RNA. (6) Then it is packaged into nucleocapsids together with the viral polymerase. (7) Reverse transcription of (-) polar ssDNA is initiated within nucleocapsids and simultaneously the pregenomic RNA is degraded by the RNaseH activity of the viral pol/RT. (8) After completion of (-) strand DNA synthesis the (+) strand synthesis is initiated. (9) Maturation of the nucleocapsid. (10) Dane particles are released into the serum. (11) Or recycled into the nucleus. Modified from Tiollais *et al.*, 1985

Fig. 2.1.2
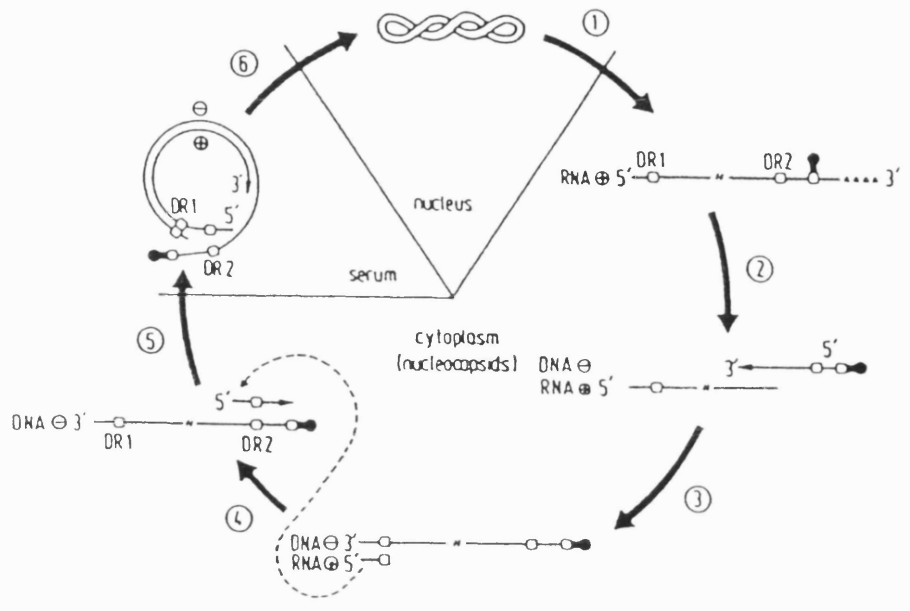
Fig. 2.1.3
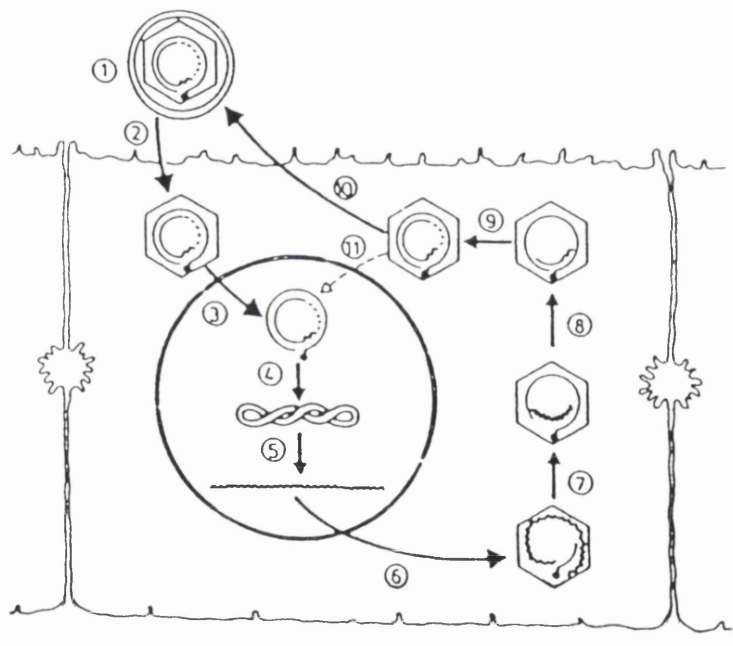
(2.2.2)   **Formation of ccc-DNA & pre-genomic RNA**

Covalently closed circular DNA (ccc-DNA) is the first form of viral nucleic acid which can be detected after infection (Ruiz-opazo *et al.*, 1982). Viral DNA enters the cell within the nucleocapsid and is thought to be transported to the nucleus within this particle (Ruiz-Opazo *et al.*, 1982), from where the relaxed circular viral DNA is converted to a ccc-DNA molecule (Mason *et al.*, 1982).

Transcription of the cccDNA yields several classes of hepadnavirus RNA, but only the large 3.5kb RNA (pregenomic RNA) contains all the sequence information present on the virus DNA and has the capacity to function as a template for reverse transcription. The pregenomic RNA is terminally redundant, (Ganem & Varmus, 1987), there is a repeated region at the ends of the molecules which varies from 130 to 270 nucleotide among the hepadnaviruses.

(2.2.3)   **Minus strand synthesis**

After packaging of the pregenomic RNA into viral precore particles in the cytoplasm, the polymerase primes the RNA-directed DNA synthesis of the minus strand from an internal site of the polypeptide (Wang & Seeger, 1992). As a strategy for this reaction, a protein is covalently linked to the 5' end of the reverse transcribed minus DNA strand (Gerlich & Robinson, 1980, Molnar-Kimber *et al.*, 1983). The priming reaction probably occurs in two biochemically distinct steps (Wang *et al.*, 1993). In the first, the reverse transcriptase binds to the 5' end of pregenomic RNA through the *epsilon* or RNA-packaging signal (fig.2.1.2 step 1; Junker-Niepman *et al.*, 1990), the sequence of which contain a palindrome, and has the potential to form a stem-loop structure with a bulge and a loop. The hydroxyl

group of the tyrosine residue at position 96 of the polymerase reaches into the active site of reverse transcriptase and provides the substrate for the formation of the phosphodiester linkage with deoxy guanidine monophosphate (dGMP; Zoulim *et al.*, 1994b). The complex then provides the substrate for the continuation of DNA synthesis. In the second step, the G residue is extended through the addition of three nucleotides, the resulted four nucleotide long DNA strand act as a primer for minus strand DNA synthesis. The synthesized nascent DNA strand then switches template to reanneal with the complementary sequences at the 3' end of the pre-genomic RNA (Zoulim *et al.*, 1994b). As the synthesis progresses, the RNA template is simultaneously degraded by the RNaseH activity.

(2.2.4) **Plus strand synthesis**

After the completion of the minus strand a short RNA primer is translocated and hybridizes to the DR2 region on the minus strand and initiates plus strand synthesis. This capped RNA is generated by the RNaseH from the 5' end of the pregenomic RNA (Lien *et al.*, 1986, Seeger *et al.*, 1986, Will *et al.*, 1987). Physical mapping of the 5' end of the plus strand DNA placed the initiation site at the 3' end of DR2 (Lien *et al.*, 1986, Will *et al.*, 1987). The translocation of the primer to the DR2 sequence on minus-strand DNA, appears to be facilitated by base-pairing between the 3' regions of plus and minus strand DNA, which provides a complementary binding site (Lien *et al.*, 1986). After the transfer of the primer to the DR2, the plus-strand DNA synthesis proceeds up to the 5' end of the DNA minus strand (Will *et al.*, 1987). For further elongation, a template switch probably takes place, in which the short terminal redundancy of the DNA minus strand is

likely to be involved (Seeger *et al.*, 1986). The last 5 to 8 nucleotides of the 3' end of the plus strand DNA are homologous to the first 5 to 8 nucleotides of the 5' end of the minus strand DNA, this homology allows the predicted intramolecular template switch to take place and results in the circular DNA conformation of the HBV genome. After the circularization of the viral DNA, plus strand DNA can be elongated, which will stabilize the circular structure.

(2.3.1)   Viral transcription

Transcription of hepadnaviral genomes is believed to be by the host RNA polymerase II (Rall *et al.*, 1983). All major hepadnavirus-specific mRNAs have been identified, the large genomic mRNA 3.5kb, two subgenomic mRNAs 2.4kb, and 2.1kb (Ganem & Varmus, 1987). Transcription of the pregenomic (mRNA 3.5kb) initiates at the core gene promoter (Ganem & Varmus, 1987), these mRNAs contain all the genetic information coded by the virus, and function both as intermediates in genome replication and as templates for translation of viral gene products. The 2.4kb RNA is probably the mRNA for the expression of the pre-S1 protein, and 2.1kb is the mRNAs for the pre-S2 and S protein (Cattaneo *et al.*, 1984). The 0.7kb is the mRNA for X-protein (Suzuki *et al.*, 1990).

Several classes of transcriptional promoter regions have been identified in the HBV genome (Liu *et al.*, 1991). They initiate transcription of various HBV genes, and are regulated by enhancers (Hu *et al.*, 1991). Of all the identified HBV promoters, none has a TATA box-like sequence, except the LHBs promoter (SPI) RNA. TATA-less promoters usually have multiple initiation sites, and this is indeed the case for the HBc/e, M/SHBs and X mRNAs.

(2.3.2)   **Viral envelope**

In the blood of HBV-infected patients, besides the 42nm virion, non infectious 22nm spherical or filamentous particles are also present. The envelope of virion and subviral particles is composed of proteins of three species of surface antigens (HBsAg) in varying amounts (Stibbe & Gerlich, 1982). They are translated from a single open reading frame (S-ORF) by means of three different in-frame start codons at pre-s1, pre S2, and S region of the S-ORF (Fernholz *et al.*, 1993), resulting in a common carboxyl terminus. According to their sizes and amounts present, HBsAg are designated as the large (LHBs), middle (MHBs), and major or small surface proteins (SHBs), each with two forms that differ in the extent of glycosylation. Within these particles, monomeric surface antigen subunits are extensively cross-linked by inter disulphide bonds (Vyas *et al.*, 1972)

(2.3.3)   **Small hepatitis B protein (SHBs)**

The SHBs contain 226 amino acids encoded by the S region, and exists in either unglycosylated (p24) or glycosylated (gp27) forms (Ganem & Varmus, 1987). The SHBs protein is synthesized initially as a transmembrane protein using the first stretches of hydrophobic domains as a signal (Eble *et al.*, 1986,). The synthesis begins at the conserved ATG of S-ORF (Fernholz *et al.*, 1993), and ends at its stop codon (fig.2.1.1). The amino acid sequences at the amino and carboxyl terminal ends were determined by Edman degradation (Peterson *et al.*, 1977). They are very rich in hydrophobic amino acids, and contain an unusual high number of cysteine (14) residues, which are all cross linked with each other (Nassal *et al.*, 1992). There is an N-linked glycan at asparagine 146 (Peterson *et al.* 1977), this

glycan has two complex antennas with sialic acid termini (Madalinski *et al.*, 1991).

(2.3.4) **Subtypes of hepatitis B virus**

Antigen reactivities that were present on all known HBs isolates were considered *a* determinant which is encoded between amino acid residue 124 and 147 of the S gene (Hopp & Woods 1981). Small hepatitis B virus antigen (SHBs) occurs in a stable subtypes that were originally defined by antibodies. Most of the SHBs epitopes depend on the presence of disulphide bonds (Vyas *et al.*, 1972). Mild treatment with DTT first destroys subtype specific epitopes, and the *a* epitopes is destroyed at a higher concentration (Sukeno *et al.*, 1972). Completely reduced HBs particles are almost non-immunogenic, suggesting that the HBsAg epitopes are dependent on conformation (Okamoto *et al.*, 1989). Thus, SHBs is not normally detected in immunoblots in which reduced and denatured proteins are reacted with antisera against HBs particles (Neurath *et al.*, 1984).

The antigenic determinants defining the different subtypes of HBV reside on the surface protein (Le Bouvier, 1971). HBV isolates have until recently been confined to the main four subtypes. The best known subtype determinants are *d* or *y* (Le Bouvier, 1971) and *w* or *r* (Bancroft *et al.*, 1972). Determinant *d* has a lysine at position 122 and *y* an arginine at the same position (Peterson *et al.*, 1984). Likewise, determinant *w* has a lysine at position 160, *r* an arginine (Okamoto *et al.*, 1988), a further subtype that has either isoleucine or threonine in position 126 of SHBs has been identified. These type-specific amino acid exchange may, however occur in quite a divergent HBV genomes. Additional determinants designated sub determinants of *w* (*w1* to *w4*) have allowed the definition of four

subtypes of *ayw* (*ayw1*, *ayw2*, *ayw3*, and *ayw4*) and two subtypes of *adw* i.e *adw2* and *adw4* (Courouce-Pauty *et al.*, 1983). The determinants *w1* to *w4* were once designated sub-determinants of *a*, because they were considered to be of the major *a* determinant (Courouce-Pauty, *et al.*, 1978). The *q* determinant, originally described as present on all subtypes apart from *adw4* (Norder *et al.*, 1993), was later also found to be absent from *adr* strains in the Pacific region, thus defining *adr* as either q^+ or q^-.

A classification of HBV genomes into six groups, A to F, based on the degree of similarity in the nucleotide sequence has been suggested (Okamoto *et al.*, 1988). Strains specifying *adw* are found in groups A, B, C and F, and those specifying *r* have so far only been found in group C (Okamoto *et al.*, 1988).

The occurrence of HBV subtypes follows a geographical pattern (Courouce-Pauty *et al.*, 1983). The strains of hepatitis B virus from the Pacific, north China and Korea can be expected to be *adr*, strains from Middle east and south east Europe to be *ayw* and southern Far East, sub Africa Saharan to be *adw* or *ayw*, North America and north Europe to be *adw*.

(2.3.5) Middle protein (MHBs)

The pre-S2 gene together with S gene encodes the 281 amino acid middle protein (MHBs) or minor component of the hepatitis B virus (Stibbe & Gerlich 1983). Since MHBs proteins is made up of the 226 amino acid encoded by the S domain, and the 55 amino acid encoded by pre-S2, the only difference between the amino acid sequence of the two is the 55 amino acid sequence at the amino terminal end of MHBs, the preS2 domain (Stibbe & Gerlich, 1983). The term pre-

S is due to the fact that this sequence is upstream of S. It does not mean that the larger pre-S proteins (MHBs) are precursors of the smaller S proteins (SHBs). The pre-S2 domain is hydrophilic and does not contain cysteine. It is very sensitive to protease. The MHBs protein appears in either a one-chain-glycosylated (gp33) or two-chain-glycosylated (gp36) form (Sheu & Lo 1994). No unglycosylated MHBs protein (p30) is observed in either virion or subviral particles (Tiollais *et al.* 1985, Ganem & Varmus, 1987). Two glycosylation sites of MHBs protein have been identified, one at the amino acid 146 near the carboxyl end of S region and the other in the pre-S2 domain (Tiollais *et al.*, 1981). The biological significance of glycosylation of MHBs proteins is unclear, since the avian HBV does not contain this corresponding sequence (Sprengel *et al.*, 1988).

All previous studies to ascertained the possible role of MHBs protein in virion formation are inconclusive (Bruss & Ganem, 1991), but the high conservation of the glycosylation signal in the N-site in all serotypes of HBV (Lo *et al.*, 1986), the highly immunogenic reaction of the amino acids in the pre-S2 region of the MHBs protein, (Itoh *et al.*, 1986), might reflects its importance. The pre-S2 domain binds to polymerized human albumin (Machida *et al.*, 1983). The pre-S2 domain appears to be immunodominant (Itoh *et al.*, 1986), the amino terminal peptide encoded by pre-S2 region was found to induce protective immunity (Itoh *et al.*, 1986)

(2.3.6)   Large hepatitis B surface antigen (LHBs)

The largest HBs protein contains 389 to 400 amino acids depending on the serotype of the virus, it encompasses the three domains, pre-S1, pre-S2, and S

(Heerman *et al.*, 1984). The LHBs protein, is present in unglycosylated (p39) or glycosylated (gp42) forms (Tiollais *et al.*, 1985, Ganem & Varmus, 1987). In the mature virion or HBs particles the pre-S domains are accessible to antibodies (Heerman *et al.*, 1984), for receptors (Neurath *et al.*, 1992) and also to protease (Heermann, 1987). The S domain and parts of the pre-S2 domain are hidden by the pre-S1 domain of LHBs (Heerman *et al.*, 1984). During biosynthesis, the entire pre-S domain of LHBs seems to stay initially at the cytoplasm, thus, the asparagine 4 of the pre-S2 domain is not glycosylated in LHBs (Heermann *et al.*, 1984). The pre-S1 (sequence 21-32) domain contains a binding site for hepatocyte membrane (Pontisso *et al.*, 1992). The amino end of the pre-S1 domain carries the sequence methionine-glycine which, together with other less well defined neighbouring amino acids, serves as a signal for the replacement of the methionine by myristic acid (Persing *et al.*, 1987). During virion or HBs particle maturation, the pre-S1 domain is reconfigured and translocated to the surface of the particle. The over expression of LHBs relative to expression of SHBs prevents secretion of HBs particles (Persing *et al.*, 1986). Only hepatocytes that express LHBs, SHBs, and the other viral components in a well balanced manner are able to assemble and secrete virion (Bruss & Ganem, 1991).

The pre-S1 domain is one of the most variable regions of the HBV genome. One reason for this may be that the part of the polymerase protein that is encoded by the same DNA region as pre-S1 is not essential for replication. The other reason is that this may be the surface structure that is most intensively selected for by immune pressure.

(2.3.7)   **Hepatitis B surface mutants**

(2.3.7a)   **Antibody escape mutants**

The most important, but rare mutations of the hepatitis B surface gene is

the type abnormalities that results in HBV humoral escape mutant. These variants

of HBV which escape neutralisation by vaccine-induced antibody (vaccine escape

mutants) were originally described as vaccine induced (Carman *et al.*, 1990,

Harrison *et al.*, 1991). So far it has not been a widespread clinical problem,

however, there are reports of isolated cases from Italy (Carman *et al.*, 1990)

Singapore (Harrison *et al.*, 1991), Japan (Okamoto *et al.*, 1992b), and in The

Gambia (Karthigesu *et al.*, 1994). In a vaccination programme in southern Italy,

subjects comprising infants born to carrier mothers and family contacts of carriers

were vaccinated, over 50 cases of HBs antigenaemia in the presence of good levels

of anti-HBs were seen in the infants of the carrier mothers. This kind of situation

does occur, but not frequently in most vaccination trials. These cases were unusual

in that, many developed markers of HBV infection such as anti-HBc or anti-HBe.

These neonates of carrier mothers became chronically infected despite circulating

anti-HBs following combined immunoprophylaxis with B immune globulin (HBIG)

and vaccine. Monoclonal antibody binding studies were performed on the

circulating HBsAg, however, in none of the cases, but all of the contacts was the

a determinant detected (Carman *et al.*,1990). This could have been due either to

blocking of the epitope by circulating anti-HBs or to a changed epitope. The

mapped set of anti-*a* mAbs of mother-infant pair revealed that the infant had

developed chronic HBeAg positive hepatitis. There was reduced binding in the

infant, but not in the mother, implying that the infants harboured a variant strain

of HBV. The type of situation reported in Italy has also been reported from The Gambia (Karthigesu *et al.*, 1994). Studies of vaccination efficacy in The Gambia, revealed post vaccination serum of some vaccinee contained protective levels of anti-HBs antibodies (greater than 10mIU/ml) and some were also positive for anti-HBc antibodies. One of the children with anti-HBc antibodies was found to be HBV DNA positive by DNA hybridization. In all the cases, nucleotide sequence analysis of the HBV genome encoding the major antigenic domain of the surface protein revealed a point mutation in the highly conserved *a* determinant of the HBsAg in the vaccinee who developed adequate levels of anti-HBs (Carman *et al.*, 1990, Harrison *et al.*, 1991, Karthigesu *et al.*, 1994). This substitution resulted in a coding change from a glycine to an arginine in the surface protein at position 145 (Carman *et al.*, 1990, Harrison *et al.*, 1991) or lysine to glutamic acid at position 141 (Karthigesu *et al.*, 1994), which is part of an epitope known to bind a large proportion of anti-HBs antibodies (Howard *et al.*, 1984). The amino acid substitution from glycine to arginine has also been observed in liver transplant patients (McMahon *et al.*, 1992). Monoclonal antibody was given to the graft recipient after transplantation to suppress reinfection. However, the patient became HBV DNA positive and sequence analysis of the isolates before and after transplantation revealed emergence of arginine from glycine. There are other important changes in other regions, which could affect the binding of anti-*a*, for example in pre-S1, another important antigenic region may lie in close proximity to the *a* determinant. Large numbers of people have now been vaccinated, escape mutation may become prevalent in areas where there is a high uptake of vaccine, however the pressure on the antigenic region is polyclonal, so mutants could be

neutralised.

(2.3.7b)　　**Other variants of HBV (Surface)**

Reports of infections in Senegal and other countries in which were characterised by low levels of serum HBV marker, either at the time of infection or after recovery, was HBsAg, led to the speculation about a new strain of HBV called HBV2 (Coursaget *et al.*, 1990, Echevarria *et al.*, 1991). The HBsAg reactivity was unlikely to have been a cross reaction as a neutralisation tests were performed and some, though not all, mAbs bound to the circulating antigen. Particles of different morphology were seen in serum, but HBV-DNA tests were only weakly positive in one person, and it was not shown that any patient was infectious. Three patients were negative for all HBV markers yet HBV DNA was detected by PCR. However, nucleotide sequence analysis of the pre-core and the core region reveals that the virus involved are not significantly different from HBV (Valliammai *et al.*, 1995a). The unusual serological patterns observed in these patients appear to be attributable to atypical immunological responsiveness on the part of the host rather than to a variant virus. Although, some random point mutations were present but expression of pre-S2 and S in transfected cells gave normal results. Thus, the significance of these isolates remains unclear.

(2.3.8a)　　**Hepatitis B core antigen (HBcAg)**

The major product of ORF C is the HBcAg, (it also encodes HBeAg) which can either be 183 amino acids in length in subtype *ayw* (Galibert *et al,.* 1979), or 185 amino acids in subtype *adw* (Seifer & Standring, 1994). HBcAg is a basic

protein of approximately 21kD containing many hydrophilic and charged amino acids. It does not contain lipid or glycan, but, if expressed in eucaryotic cells, it becomes phosphorylated (Roossinck & Siddiqui, 1987). In most isolates from highly viraemic carriers, ORF C has 212 or 214 codons, but translation of the HBc protein starts only at the AUG, 29 codons downstream of the first AUG (Ou *et al.*, 1990). The region upstream is termed pre-C for historical reasons (Tiollais *et al.*, 1981).

HBV packages its own RNA, the viral polymerase, and assembles into core particles. The ability to assemble to particles resides in the first 147 amino acids (Gallina *et al.*, 1989), while the packaging of the nucleic acid is believed to involve the four arginine clusters of the last 36 or 38 amino acids (Nassal, 1992). A critical concentration of HBcAg is necessary for the assembly to take place: firstly, dimers of HBcAg are formed when this concentration is attained (Zhou & Standring, 1992), phosphorylation of serine at position 170 or 172 between the arginine clusters 3 and 4 may interfere with nucleic acid packaging (Birnbaum & Nassal, 1990). The core particle consist of 180 subunits of the core protein (Birnbaum & Nassal, 1990). Once assembled, the structure is stabilized by disulphide bonds (Zhou & Standring, 1992). Packaging of the pregenome depends on the *epsilon* motif present in the RNA (see section *2.2.3*).

(2.3.8b)   **Hepatitis e antigen (HBeAg)**

The precursor of HBeAg is a p25 protein, which is the translation product of the entire C gene. p25 is different from the HBcAg in having an additional 29 hydrophobic alpha helix amino acid at the amino terminal end (Takahashi *et al.*,

1992). The 29 amino terminal residues act as a signal sequence and direct the translocation of p25 into the lumen of the endoplasmic reticulum (Ou *et al.*, 1986, Bruss & Gerlich, 1988). The protein (p25) undergoes cleavage at the N-terminus in the endoplasmic reticulum, 19 out of the 29 amino acid signal protein are removed (Bruss & Gerlich, 1988). It is suggested that the remaining 10 amino acids may probably prevent assembly of the HBeAg to core particles by interaction with the HBcAg protein sequence. It is believed that another 34 amino acid residues are further removed from the C-terminus of p25 in the endoplasmic reticulum (Ou *et al.*, 1990). This cleavage resulted in the soluble 17kDa (p17e) HBeAg protein (Bruss & Gerlich, 1988). HBeAg consists of 149 amino acids that are identical to the first 149 amino acids in HBcAg (p21c) preceded by 10 amino acids remaining from the signal protein (Takahashi *et al.*, 1983). It circulates with plasma proteins such as albumin and immunoglobulin (Takahashi *et al.*, 1983). Although both p17e and p21c shared a large stretch of identical primary sequence, the p17e is non particulate, whereas the p21c self-assembles into core particles, and both proteins act as a distinct antigens during HBV infection (Milich 1988). Core particles (HBcAg) elicit a rapid and strong humoural immune response in almost all infected individuals with no apparent protective effect, while the HBeAg induces a distinct, weaker, and delayed response, which frequently correlates with virus elimination (Hoofnagle *et al.*, 1981). Furthermore, there is absolutely no cross-reactivity of anti-HBc and anti-HBe, which indicates that the two antigens are in different physical structure states, however denaturation of the HBcAg exposes HBeAg epitopes (MacKay *et al.*, 1981).

During persistent hepatitis B virus (HBV) infection, carriers may

seroconvert from HBeAg to the corresponding antibody (anti-HBe). Some variants of HBV (precore mutants) are unable to synthesise HBeAg (see section *2.3.8*). HBV replicates more actively in carriers than those with anti-HBe. Carriers with HBeAg therefore have a higher probability of transmitting HBV than those with anti-HBe, typically in perinatal transmission of carrier state from mother to baby (Okada *et al.*, 1976). Expression of HBeAg probably helps induce persistent infection (Milich *et al.*, 1990), but this protein is not essential for virus replication or infectivity. High levels of secreted HBeAg are found in highly viraemic virus carriers with few symptoms. Disappearance of HBeAg seems to indicate that HBcAg protein expressing cells are no longer tolerated by the immune system (Bonino *et al.*, 1986), however, a state of low-level viraemia may often develop after elimination of HBeAg from the blood while HBsAg persists to circulate in the blood. Once most of the HBV-producing cells are eliminated, the state of asymptomatic HBsAg carrier is reached.

(2.3.9)   **Pre-core mutation**

The observation that some patients in the southern Mediterranean and the Far East have HBV DNA measurable in their serum by dot-blot hybridization but are negative for HBeAg (Hadziyannis *et al.*, 1983, Chu *et al.*, 1985), led to the discovery of a variant virus that was unable to synthesize the precore protein from which HBeAg is derived (Carman *et al.*, 1989, Brunetto *et al.*, 1989). Their disease was remarkable in that progression occurred, often rapidly, in the presence of antibody to HBeAg (anti-HBe). Because of the known contribution of the pre-C gene in the synthesis of HBeAg, this region was targeted for genetic analysis in

these patients. The HBeAg abrogating mutations detected so far include (a) the stop codon mutation described below, which is the most common (Brunetto *et al.*, 1989, Carman *et al.*, 1989, Fiordalisi *et al.*, 1990), (b) elimination of the precore AUG codon by single nucleotide substitutions (Okamoto *et al.*, 1990, Santantonio *et al.*, 1991), (c) frameshift mutations due to single or double nucleotide insertions or deletions (Li *et al.*, 1990, Tong *et al.*, 1990, Okamoto *et al.*, 1990, Santantonio *et al.*, 1991) and (d) mutation in the core promoter (Okamoto *et al.*, 1994).

A single base substitution (G to A) was found in the second to the last codon (codon 28) of the precore gene at nucleotide 1896 (numbered from the single *EcoRI* site; Carman *et al.*, 1989, Brunetto *et al.*, 1989, Fiordalisi et al., 1990). This give rise to a translational stop codon, explaining their apparent serological contradiction. In a large number of the Mediterranean patients, a second mutation in codon 29 (G to A at position 1899 A_{1899}) was found (Carman *et al.*, 1989), some Chinese patients show a G to A mutation in 1898 (A_{1898}).

The presence of two HBV distinguished by their capacity (or defect) to encode the production of HBeAg is of major interest. Pre-core mutants are not always detectable as a major species in the HBeAg phase of the chronic hepatitis but they emerge during, or some time after seroconversion to anti-HBe (Okamoto *et al.*, 1990). During the course of acute hepatitis the wild type virus (HBeAg positive) diminishes in titre in the peripheral blood, and small quantities of the HBeAg negative mutant become detectable (Carman *et al.*, 1993). In some patients the HBeAg-negative virus becomes dominant at the time of HBeAg/anti-HBe seroconversion, perhaps by immune selection through antibody or cytotoxic T-cell activity directed toward HBeAg bearing hepatocytes. In some of these patients,

HBeAg negative virus continues to replicate in association with the rapid development of progressive liver disease, but in others the HBeAg negative virus persists without inflammatory liver disease (Okamoto et al., 1990).

Evidence has been gained that the pre-core stop mutation at nucleotide 1896 exists inside the stem loop structure of the pregenomic RNA, which is involved in encapsidation of hepadnaviruses, and may interfere with the replication of HBV. Three naturally occurring HBe-abolishing mutations located within the hairpin structure of the packaging signal have been described. All are contained in the proposed 13bp stem region. A TAG stop codon mutation at precore codon 28 (described above) is the most common HBeAg eliminating mutation found in nature and has no detectable effect on replication of the viral genome (Tong et al., 1992). The second mutation is TGA, which also occurs at codon 28 and is a stop codon instead of TGG, the codon for tryptophan in wild type (Blum et al., 1991). Codon 28 can hence change to a stop codon by either of the alternative point mutations (TGA or TAG). However, TGA mutation (discussed above) is more common than the TAG. The change at codon 28 depends on the codon 15, because base pairing must be maintained. TAG (UAG) mutation occurs in HBV isolates bearing a CCU sequence at codon 15, while a TGA (UGA) mutation occurs in HBV with CUC sequence at codon 15 (Blum et al., 1991). The high mutational propensity at nucleotide 2 of codon 28 (see fig 2.3.9) is related to the viral pregenome encapsidation epsilon signal, overlapping the HBeAg coding sequence (Junker-Niepman et al., 1990), and forms a hairpin structure essential for the packaging of viral pregenomic RNA (Junker-Niepman et al., 1990, Tong et al., 1992). The common UGG to UAG mutation at codon 28 converts a wobble U-G

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base pair formed between nucleotide 3 of codon 15 (CCU) and nucleotide 2 of codon 28 (UGG) into U-A pair. A third natural precore mutant has, besides a nucleotide insertion at codon 29, produces a novel *NcoI* site (Li *et al.*, 1990). A silent TGT to TGC mutation of codon 14 was also found.

The pathological significance of the precore mutation is not yet very clear, because a definite function for HBeAg has still to be assigned. It has been proposed to be a tolerogen, particularly in infants (Milich *et al.*, 1990) and to divert the immune effector cells away from infected hepatocytes (Thomas *et al.*, 1985). It has also been suggested that the emergence of mutation in codon 28 of the precore region, helps the virus to survive the anti-HBe immune response of the host (Li *et al.*, 1993). Studies in patients with fulminant hepatitis (Kosaka et al., 1991, Carman *et al.*, 1993), showed these patients are often anti-HBe positive, clusters of cases have also been reported (Oren *et al.*, 1989). It is unclear whether the patients select this variant during a period of unusual aggressive clearance of HBeAg positive virus or whether it constitutes a major fraction of the initial inoculum.

```
                        G U
Loop               U       G
                     C       C
                      G-C
                      A-U
                      A-U
                      C-G
                      C-G
stem II               U:G
                      C-G  U
                      C-G
                      G-C
                      A-U
                    C
                 U
bulge       U
               G
                 U
                   C
                   A-U¹
             |     ³U:G²   |   1896 codon 28
codon 15     |     ²C-G³   |
             |     ¹C-G
                   U:G         1899
                   G-C
stem I             U-A¹ |
                   A-U² |       core initiation
                   C-G³ |
                   U:G
             |     ³U-A
codon 12     |     ²G-C
             |     ¹U-A
                 5'     3'
```
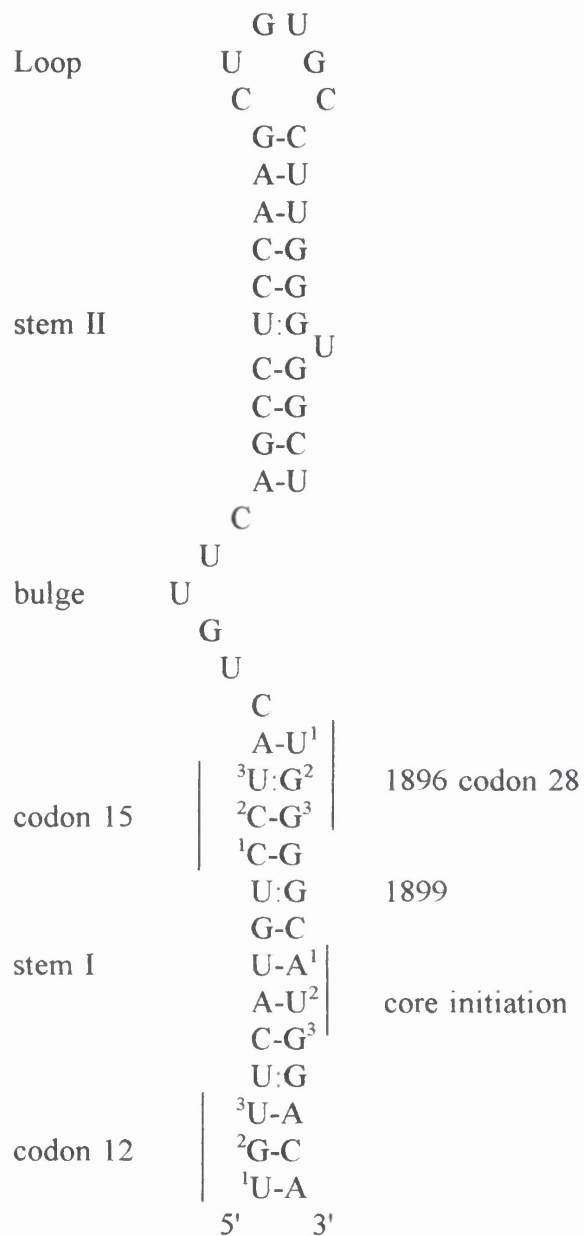
Fig. 2.3.9: Predicted secondary structure of the encapsidation signal, starting from amino acid codon 12 of the precore region and ending at the first seven nucleotides of the core gene. U:G (wobble) pairs are connected by colons, while C-G and A-U pairs are connected by hyphens. The nucleotides 2 and 3 of codon .15 pair with nucleotides 3 and 2 of codon 28.

(2.3.10)   **P-protein (P-ORF)**

The HBV DNA polymerase is the largest HBV gene encompassing approximately 80% of the genome and overlapping every other viral gene (Tiollais *et al.*, 1981). P-ORF gene is expressed as a single translational unit and independent of the core gene and the product is freely diffusible and not processed before core assembly (Radziwill *et al.*, 1990). The polymerase protein is only packaged together with the pregenomic RNA within core particles (Bartenschlager & Schaller, 1992).

The HBV polymerase has reverse transcriptase and RNaseH activities (Radziwill *et al.*, 1990). The amino acid sequence comparison between the translation product of P-ORF and the reverse transcriptase (pol gene product) of several oncogenic retroviruses reveals a great deal of homology (Toh *et al.*, 1983). The hepadnaviral P-protein is organized into four clearly distinguishable functional domains (Radziwill *et al.*, 1990), arranged from the amino- to the carboxyl terminus. The first domain contains the terminal protein (TP or primase) which is linked to the 5' end of the minus strand of virion DNA and primes the minus strand synthesis (Bartenschlager & Schaller, 1988), followed by the non-essential protein domain which has no specific function but acts as a spacer or tether. The third domain is the DNA polymerase or reverse transcriptase and the fourth domain is an RNaseH activity, which forms the carboxyl terminal of the polymerase protein. RNaseH cleaves the pregenomic RNA from the RNA-DNA hybrid (Radzilwill *et al.*, 1990).

Two novel proteins have been identified as P-ORF gene product the 93kd and 72kd proteins, however the 93kd seems to be the most abundant. Both proteins

are recognized by an anti polymerase monoclonal antibody (McGlynn *et al.*, 1992).

(2.3.11)   **HB-x Protein**

The HBx gene encodes a 16kd protein, a polypeptide of about 145 to 154 amino acids, depending on the subtype of the virus. The 3' end of X-ORF of the *ayw* contains a six nucleotide insertion (Galibert *et al.*, 1979), and a deletion of 27 nucleotides at the 3' end is common in the *adr* genome (Valenzuela *et al.*, 1979). The amino acid sequence of the HBx protein is highly conserved in the mammalian hepadnaviruses, but is absent from the avian hepadnaviruses. The amino acid sequence suggests that HBx is a cytosolic protein without a specific intracellular transport signal. HBx protein is known to transactivate a variety of cellular and viral promoters (Spandau *et al.*, 1988), including various enhancer/promoter combinations, HBV enhancer and core promoter (Spandau *et al.*, 1988). This phenomenon has led to the suggestion that it may be involved in the development of HBV-associated HCC (Diamantis *et al.*, 1992). The role of the HBx in hepatocarcinogenesis has also been studied with antibodies against the HBx-protein in histological sections of liver tissues from patients with HCC. It was found that 84% of the HCC cases expressed HBx gene product compared to 19% expressing HBsAg and 11% HBcAg (Wang *et al.*, 1991a). This finding suggested that among the different viral gene products, the HBx protein appeared to play an important role in the development of HCC (Seifer *et al.*, 1991). HBx protein upregulates HBV replication through activating transcription, this gave an indication that it does not participate in the mechanism of genome replication or in the virion assembly. HBx protein can be detected in the livers of many HBV

infected individuals (Wang *et al.*, 1991b). The significance of the X gene in the

viral life cycle remains largely obscure. HBx protein appeared not to be essential

in virus replication, because X-deficient mutants remain viable (Colgrove *et al.*,

1989), however, transfection of WHV DNA without a functional X-ORF did not

cause any infection in woodchucks (Zoulim *et al.*, 1994a).

(*2.4.1*)   **Prevalence   of   hepatitis   B   virus**

Asia is an hyperendemic area for hepatitis B. In some countries more than

70 % of the population over the age of 40 years shows signs of previous infection.

The Chinese account for about 80 % of the world's HBV carriers. In Africa,

prevalence of the disease is almost universally high, with a chronic carrier rate of

around 10% (Botha *et al.*, 1984, Amazigo & Chime, 1990). Hepatitis B is much

less prevalent in the industrialised world, but there are scattered areas of high

endemicity (Stroffolini *et al.*, 1989). Slightly higher rates are found in the Mediter-

ranean countries and eastern Europe. In Italy, for example, a reported overall

prevalence of 2.4% among pregnant women conceals geographical variations from

less than 0.5% in northern and central Italy to more than 5% in the south, and up

to 10% in the Naples and some isolated areas of the north (Stroffolini *et al.*, 1989).

A significant correlation exists between the prevalence of HBV and the incidence

of hepatocellular carcinoma. HBV causes up to 80% of the world's primary liver

cancer, which is one of the three most frequent causes of cancer death in Asia, the

Pacific Basin and Africa. Nigeria is endemic with hepatitis B infection, the

prevalence of between 9 and 10% carrier rate has been reported (Fakunle *et

al.*,1981, Kulkarni *et al.*, 1986, Amazigo & Chime, 1990).

(2.4.2)   **Transmission of hepatitis B virus**

The hepatitis B virus is transmitted via :

(i)   Perinatal exposure

(ii)   Sexual exposure

(iii)   Exposure to blood and blood products

(vi)   Drug abuse and parenteral exposure

(v)   Organ/tissue transplantation

(iv)   Exposure of unknown origin

(i)   *Perinatal exposure*

Transmission from mother to neonate (through contact with maternal blood and other infectious fluids during labour, colostrum and rarely through breast milk or placental transmission) is a common route of infection in areas of high hyperendemicity (Botha *et al.*, 1984). Between 90% and 95% of male neonates infected by this route and a proportion of the females will themselves go on to become carriers and, in turn, infect their offspring. Moreover, a significant proportion, particularly the males, will develop the serious sequelae of chronic hepatitis B infection, cirrhosis and hepatocellular carcinoma.

(ii)   *Sexual contact*

Hepatitis B viral DNA (HBV DNA) has been detected in seminal fluid, vaginal secretions and saliva (Karayiannis *et al.*, 1985), suggesting that these fluids are likely to be infectious. Studies in patients attending clinics of sexually transmitted diseases have demonstrated a link between promiscuous sexual activity

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and the risk of hepatitis B infection (Bello *et al.*, 1992). In terms of population risk, sexual transmission represents the most important route of transmission in the developed world. Hepatitis B is a sexually transmitted disease predominantly related to homosexual activity, in recent years, however, there are changes in sexual practices among the homosexual community, prompted by health concerns over human immuno deficiency virus, have slowed the spread of HBV among this population.

(iii)   *Blood and blood products*

Acute hepatitis B infection often results from contact with contaminated blood or blood products (Karayiannis *et al.*, 1985). However, with the advent of screening in many countries, blood transfusion is no longer a major route of transmission, it presently accounts for less than 2-3% of cases.

(vi)   *Drug abuse and parenteral exposure*

Of increasing importance, especially in the industrialised world, is the sharing of needles and equipment between intravenous drug users, and needle stick injuries among health care workers are an important route of transmission.

(v)   *Exposure of unknown origin*

In spite of the well known various routes of HBV transmission, in many cases the apparent routes of transmission remained unknown.

(2.4.3)   **Groups at risk to hepatitis B infection**

The single largest group of individuals at risk of infection are infants under three years of age in areas of hyperendemicity. This risk is not only of vertical transmission from mother to infant and intrafamilial contact in the home, but also through other points of close contact, for example at nurseries (Alter *et al.*, 1981a). An interesting development is the risk of spreading hepatitis B infection to non-endemic areas through the adoption of babies from areas of hyperendemicity such as Romania, Korea and Indochina. Health care workers, and the staff and inmates of prisons and residential institutions, are recognised as high-risk groups for hepatitis B because of their exposure to blood and body fluids (Hadler, 1990). Hospital and laboratory staff in haemodialysis units, surgical wards, cancer therapy units and institutions for the mental retarded, where chronic carrier rates among patients may be high, appear to be particularly vulnerable.

(2.5.1)   **Immune pathogenesis**

Human hepatitis B virus was recognized as a pathogen associated with liver disease, ranging from benign to cirrhosis and hepatocellular carcinoma. Clinical and experimental data suggests that the liver damage observed during acute and chronic HBV infection is due to the immune reaction of the host to viral antigens rather than to a direct cytopathic effect of the virus itself. The existence of patients with evidence of hepatitis B virus (HBV) replication, but no evidence of liver disease, both at early phase of acute infection, and in some chronic infections (Lok *et al.*, 1985), has led to the belief that this virus is not (directly) cytopathic, certainly not to the extent of causing massive cell death. Immunologically

immature or deficient individuals do not develop a typical hepatitis, but they become chronic carriers of the virus. In this case, the virus continues to replicate in hepatocytes without destroying tissue or severely impending organ function, studies have shown that there is no general decrease in immune functions in HBV chronic carriers. Among HBV infected humans with normal immune systems, several percent never develop an effective cytotoxic immune response and proceed directly to persistent infection with high level viraemia and antigenaemia.

Epidemiological studies have shown convincingly that persistent hepatitis B virus infection is closely associated with hepatocellular carcinoma (HCC) (Beasley *et al.*, 1988). There can be no doubt that hepadnaviruses have oncogenic potential, because HCC occurs in about 5% of individuals chronically infected with hepatitis B virus (Beasley *et al.*, 1988). However, definitive proof for a direct oncogenic role of HBV is still lacking as no common mechanism for HBV-induced hepatogenesis has been discovered. Virus specific mechanisms and (or) host response to viral infection, may be critical in the process of HBV-induced hepatocarcinogenesis. It is however, not yet very clear which of these possibilities operates.

Integration of HBV DNA and oncogenesis is not an essential step in the replication of HBV, however, it does occur (Rogler & Summers, 1984), therefore HBV may act as a non-selective, insertional mutagenic agent. Integration takes place during the premalignant phases and integrations frequently are present in a non-clonal pattern in chronically infected HCC-free liver tissue (Rogler & Summers, 1984, Takada *et al.*, 1990). There are several possibilities of how hepadnaviral integration may contribute to HCC development. Among these are:

The secondary chromosomal rearrangements associated with HBV DNA integration such as inverted duplications and translocation (Koike *et al.*, 1983, Tokino *et al.*, 1987) may play a major role in the oncogenic effect of HBV integration. The HBV genome contains at least four different promoters and two enhancer elements, the insertion of these genetic elements may deregulate expression of the gene (Dejean *et al.*, 1986). Transactivating proteins such as HBx protein also contribute to the HBV associated HCC. One of the most significant side effects of HBx protein may be its tumorigenic activity (Kekule *et al.*, 1993).

(*2.6.1*)   Aims and the objectives of the study

(a)   To investigate the present level of hepatitis B infection amongst healthy adult blood donors and children of pre-school age in Nigeria.

(b)   To find the relationship between the carrier rate and viraemia rate amongst healthy adult blood donors and children of pre-school age in Nigeria.

(c)   To determine the subtype of HBV present in Nigeria.

(d)   To set up a pilot survey for hepatitis C virus infection among healthy adult blood donors and children of pre-school age in Nigeria.

(e)   To determine the genotype of HCV present in Nigeria.

CHAPTER 3

MATERIALS AND METHODS

(3.1.1)   **Blood Samples**

(A)   Blood samples were collected from 2690 healthy adult blood donors at five

transfusion centres in Nigeria. These centres are:

(a)   Lagos University Teaching Hospital, Lagos.

(b)   Nigerian Institute For Medical Research, Yaba - Lagos,

(c)   University College Hospital, Ibadan.

(d)   University Of Nigeria Teaching Hospital, Enugu.

(e)   Federal Public Health Laboratories, Lagos.

(B)   Blood samples from 100 children of pre-school age were collected from

the paediatric unit of the University College Hospital, Ibadan.

(3.1.2)   **Separation of serum samples**

Five ml of blood sample was spun at 4°C at 3,000 x g for 30 min. The

serum (supernatant) was removed into a clean sterile Nalgene tube and stored at

-20°C until required.

(3.2.1)   **Serological tests**

(3.2.2)   **HBsAg Test (Wellcozyme)**

Fifty μl of reconstituted conjugate was dispensed into each mirowell, 150μl

of controls (negative & positive) were placed in their appropriate wells, followed

by 150 μl of serum samples under test. A new disposable microtip was used for each sample. The plate was covered and incubated at 37°C for 1 hr. At the end of the incubation, the micowells were aspirated and washed five times by filling the wells with wash fluid (0.1M piperazine, 0.1mM magnesium ions, 0.01mM zinc ions, 0.1% protein, with bronidox as preservative) followed by aspiration, making sure that each column of wells were soaked for at least 15 seconds with wash fluid before the aspiration cycle. Immediately after the washing stage, 50 μl of reconstituted substrate solution was added to each well. The plate was covered and incubated at 37°C for 40 minutes, then 100μl of reconstituted amplifier was added to each well and incubated at room temperature for 10 min. The reaction was stopped with 50 μl stop solution (2M sulphuric acid).

The absorbance of each well was read at 492 nm (A_{492}) within 15 min in a microwell plate reader (Multiskan MCC / 340 P version 2.3). The cut-off value was decided by adding 0.10 to the mean (A_{492}) of the Negative Control replicates. Any sample with an absorbance value equal to or greater than the cut-off value is considered positive.

(3.2.3.) Anti-HBs test (Bioelisa)

The first column of the microtitre plate was used for blank, negative and positive controls. 100 μl of negative control was transferred into the first 4 wells and 100μl of high calibration positive control was transferred into the next 2 wells, while the remaining 2 wells were left blank.

100 μl of each sample under test was transfered into the appropriate well. The plate was covered with adhesive tape and incubated for 1 hour at 40°C. At

the end of the incubation period, the adhesive tape was removed and discarded. The microwells were aspirated, and washed 5 times with washing solution (1 x phosphate buffer, 0.1% Tween, and 0.001% thimerosal) making sure that the microwells are soaked with wash solution for at least for 30 seconds before the next aspiration cycle. Then, 100 µl of reconstituted conjugate was added to each well with the exception of the two blank wells. The plate was covered with adhesive tape, and incubated for 30 min at 40°C. At the end of the incubation period, the adhesive tape was removed, and plate washed (as before), then 100µl of reconstituted TM/substrate solution was added to all the wells (including the blanks). The plate was incubated uncovered for 30 min at room temperature and reaction was stopped with 100 µl of stop solution (2M sulphuric acid). The absorbance was read within 30 min at 450 nm (A_{450}) in a microtitre plate reader (Multiskan MCC / 340 P-verson 2.3). The spectophotometer was blanked at 450 nm with the blank wells.

The cut off point was the average of the negative control plus 0.040 [(NCX + 0.040), where NCX = Average of the negative control]. Any sample with absorbance value equal or greater than the cut-off value is considered positive for anti-HBs.

(3.2.4)   Anti-HBe / HBeAg Test (Wellcozyme)

The first 2 colums (columns 1 & 2) of the mirotitre plate were used for the positive and negative controls. The first two wells of the first column were used for anti-HBe-positive control and the next two wells for the negative control. The same path was used for the HBeAg test in the second column of the plate. 100 µl

of each control were used. 100 μl of each sample under test was dispensed into the remaining microwells using a new microtip for each sample. 50 μl of neutralising antigen was added to the wells for anti-HBe tests, then 50 μl of reconstituted conjugate was added to all wells. The plate was covered with lid and incubated in water bath at 45°C for 1 hr. At the end of the incubation, the plate was aspirated and washed five times by filling the wells with wash fluid, followed by aspiration, making sure that the microwells are soaked for at least 30 seconds before the next aspiration cycle. 200 μl of reconstituted substrate was added into each well and incubated for 30 min at room temperature. The reaction was stopped with 50 μl stop solution (2M Sulphuric acid). The absobance was read within 30 min at wavelength of 450nm (A_{450}) using a microtitre plate reader.

(A)   Cut off   (Anti-HBe):-

Cut off = 0.5 x (Mean A_{450} -ve control + Mean A_{450} +ve control)

Any sample which gives an A_{450} equal to or less than the cut-off is considered positive for anti-HBe.

(B)   Cut off   (HBeAg test):-

Cut-off = Mean A_{450} -ve control + 0.1

Any sample which gives an A_{450} equal to or greater than the cut-off is considered positive for HBeAg.

(3.3.1)   **HBV DNA Screening**

(3.3.2)   **HBV DNA labelling (Probe)**

One µl (approximately 80ng) of HBV DNA purified from pEcob6 following EcoRI digestion and agarose gel electrophoresis preparation, was diluted with sterile distilled water to 7 µl. It was denatured by boiling for 5 min in a water bath, quenched immediately on ice. 5 µl DTM [DTM = 100 µM each of dATP, dGTP, dTTP, in 250 mM Tris-HCl (pH8.0); 25 Mm $MgCl_2$, 50 Mm 2 β-mercaptoethanol], 5 µl 1M of HEPES (pH6.6), 1.4 µl OL/2 [OL/2 = 45U/ml P(dN)^6 (Pharmacia 27-2166-01) in Tris-Hcl, 1 Mm EDTA (pH7.5)], 1 µl BSA (10mg/ml), 5 µl of labelled α^{32}P-dCTP, and 1 µl Klenow fragment (2.5U) were added on ice. The reaction was gently mixed and spun down (10 sec) and incubated at 37°C for 1 hr. The level of incorporation of radioactivity was measured by trichloroacetic acid (TCA) precipitation, and the total incorporation and specific activity determined.

The unincorporated radioisotopes and dNTPS were removed by a spun column. A thin layer of siliconized glass wool was pushed to the bottom of a disposable plastic 1 ml syringe barrel. The syringe was filled with TNE-equilibrated Sephadex G-50. It was placed in a sterlin tube and spun for 5 min at 1,600 rpm, to remove the excess TNE. It was equilibrated with 100 µl TNE and spun for another 5 min at 1,600 rpm. The probe was diluted to 100 µl with TNE and a capless microfuge tube placed under the sephadex column at the bottom of the sterilin tube. The column was overlaid with the diluted probe (100 µl) and spun for 5 min at 1,600 rpm, the microfuge tube was retrieved and the probe stored at -20°C until required.

The total and the acid precipitable radioactivity (counts per minute) of the probe were determined. One μl of radiolabelled HBV DNA (from above) was diluted to 10 μl with sterile distilled water. Two Whatman GF/C glass-fibre filters (2.4 cm diameter) were used, 1 filter was used to measure the total amount of radioactivity in the reaction, and the other filter was used to measure only the acid precipitable radioactivity. 4.5 μl of the diluted radiolabelled DNA was spotted at the centre of the filter. The filters were kept at room temperature until the fluid evaporated. One of the filters was transfered with a blunt-end forceps to a sunction manifold and washed twice under suction with 5 ml of ice cold 5% TCA and 0.1 M Sodium pyrophosphate (PPi), twice with 5 ml methanol, and once with an equal volume of acetone. It was then transfered to a beaker and left to dry at room temperature. The filters (both washed and unwashed) were transfered into a separate scintilation vial and 4 ml cocktail T (BDH) was added to each. The radioactivity on each filter was measured in a Beckman liquid scintillation counter. A typical reaction contained 50 μci which has 1.11×10^9 counts per minute (cpm), depending on the reference date. In most cases, radioisotope incorporation of between 50 and 60% is obtained.

(*3.3.3*)   **Dot blot hybridisation**

Fifty μl serum samples under test, and equal volumes of positive and negative controls were each treated with 20 μl of 3% β-mercaptoethanol and 20 μl of 10% NP40, incubated for 5 min at room temperature, then 90 μl of 2 M NaCl was added, followed by 180 μl of 1M NaOH. The reaction was properly mixed and left at room temperature until required.

Reconstruction was prepared with 10 μl HBV DNA (approximately 9.2ng/μl) purified from pEcob6 by EcoRI digestion and agarose gel electrophoresis. The DNA was dilluted to 100 μl with TE (pH8), denatured with 10μl 3M NaOH, and incubated for 10 min at 37°C, quenched on ice. Then 100 μl of Tris-HCl (pH7.2), 10 μl 3M HCl and 20 μl 5NaCl were all added on ice (HBV DNA final concentration = 4ng/μl). Different volumes of the treated serum samples (200 μl, and 100 μl), and various concentration of the reconstructed HBV DNA (200ng, 80ng, 40ng, 20ng, 10ng and 4ng), were applied with the aid of a manifold under vacuum suction to a hybond-N-membrane (Amershan U.K), prewetted with 6xSSC [(1xSSC = 0.15M NaCl + 0.015M NaAc $(Na_3C_6H_5O_7)$]. HBV DNA were immobilized onto the nylon membrane. The membrane was washed with 6xSSC, dried in between 3 mm Whatman filter paper, and wrapped in Saran Wrap. The membrane bound DNA was cross linked under ultra violet (u.v.) light for 5 minutes, sealed in a plastic bag and 200μl/cm^2 of pre-hybridization mixture [6 x SSC/33 mM EDTA, 5x Denhardt's solution, 0.5% SDS and 100μg/ml denatured herring sperm DNA (gene block)] was added and was prehybridized overnight at 65°C. The next day, the labelled probe (1.0 x 10^7 cpm/filter) was denatured in a boiling water bath for 5 min, quench on ice. The denatured probe was briefly spun and added into a freshly prepared hybridization solution 50μl/cm^2 (same concentration as in pre-hybridisation), mixed properly and poured into the plastic bag containing the pre-hybridized membrane, sealed and incubated with the same conditions used for pre-hybridization. The next morning, the membrane was washed twice in 2 x SSC at 65°C for 15 min, once in 2 x SSC + 0.1% SDS at 65 °C for 30 min, dried, and wrapped in Saran Wrap. The membrane was exposed

to an X-ray film (Hyperfilm ™-MP, Amersham UK) overnight at -70°C in an x-ray cassette.

(3.4.1)   **Polymerase Chain Reaction (PCR)**

(3.4.2)   **Surface gene (PCR)**

The primers used for PCR reaction were chosen from the conserved region of the S gene MD14 (Larzul *et al.*, 1990) and HCO2 (Fagan and Harrison, 1994). Primer MD14   **(5' GCGCTGCAGCTATGCCTCATCTTC-3')** binds to nucleotides (nt) 418 to 433 of the plus strand of HBV DNA and has a mismatch at the 5' end containing a *PstI* site. Primer HCO2 **(5'-GCGAAGCTTGCTGTACAGACTTG-3')** binds to nucleotides 761 to 776 of minus strand of HBV DNA, and has a mismatched 5' end containing a *Hind III* site.

10 µl of serum sample was diluted with an equal volume of double distilled sterile water and denatured in a water bath at 90°C for 30 sec. It was allowed to cool down slowly at room temperature for 30 min, and briefly spun down for 45 sec. This was used directly for PCR reaction in a final volume of 100 µl containing 200 µM of each 4 dNTPs (dGTP, dATP, dTTP, dCTP), 50 picomoles of each primer, 2.5 units of thermostable DNA polymerase (Advanced Biotechnologies, Leatherhead), 10 µl 10 x polymerase buffer (10 mM Tris HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), and overlaid with 100 µl of white light mineral oil (Sigma, St Louis, MI).

PCR was performed in a thermocycler (Perkin Elmer Cetus), programmed for 35 cycles, each consisting of a denaturation step (1.5 min at 94°C), annealing

(1.5 min at 50°C), and extension (3 min at 72°C). The last cycle reaction at 72°C was allowed to continue for 9.9 min to ensure complete DNA extension. An aliquot (20 µl) of each PCR product was analyzed by electrophoresis on a 2% agarose gel, stained with 0.5 µg/ml ethidium bromide, and visualized on a U.V. light box.

(3.4.3)   **Pre-core / core gene (PCR)**

Four primers were selected from the conserved region of nucleotide encoding the precore / core region and were used in a nested PCR. Primers HDM3 and MDD2 [(outer primers), Fagan and Harrison, 1994], were used for the first round PCR. Primer HDM3

(5'-GCGCTGCAGGAGTTGGGGGGAGGAGATTA-3') binds to nucleotides 1735 to 1755 plus strand of HBV, and has a mismatch at the 5' end containing a *PstI* site, primer MDD2

(5'-GCGAAGCTTGAGAATAAAGCCCCGTAAA-3') binds to nucleotides 2506 to 2484, and also has a mismatch at the 5' containing a *Hind III* site. The same method of amplification and PCR program used for S gene was followed here. 5 µl of the first round PCR product were used for the second round PCR (nested PCR) with primers MDN2 and HDB2 [(inner primers) Fagan and Harrison, 1994].

Primer **MDN5** (5'-GCGCTGCAGGAGGCTGTGGCATAAAT-3') binds to HBV DNA at map position 1775 to 1794, and has a mismatch at the 5' end containing a *PstI* site. Primer

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HDB2 (5'GCG<u>AAGCTT</u>AGATCTCTGGATGCTGGA-3') binds to HBV DNA

at nucleotides 2134-2152, it also has a mismatch at the 5' end containing a *Hind*

III site. The same PCR progamme used for the first round PCR was used here. An

aliquot (20 µl) of each PCR product was analyzed by electrophoresis on 2 % on

agarose gel, stained with 0.5 µg/ml ethidium bromide, and vistualized on a U.V.

light box.

(3.5.1)   **Cloning of the PCR product**

(3.5.2)   **Purification of the PCR product**

The PCR product was purified with gene clean II kit (BIO 101, La Jolla,

Ca). The mineral oil, on top of the PCR product was completely removed and 3

volumes 6M NaI (Sodium iodide) was added. It was vortexed, and 15 µl of glass

milk suspension (silica matrix in water) was added. It was vortexed and incubated

at room temperature for 5 min. The mixture was spun for 1 min in a micro

centrifuge. The supernatant was removed and discarded. The pellet was washed 3

times with 100 µl of ice cold gene clean II wash fluid. The pellet was re-

suspended in 50 µl of double distilled water and incubated at 45°C for 10 min. It

was spun down for 1 min and the supernatant (purified DNA) was removed into

a clean, sterile Eppendorf tube and stored at -20°C until required.

(3.5.3)   **Ligation**

Ten µl of the purified HBV DNA (from above) was digested with two

restriction endonuclease enzymes (*PstI* and *Hind III*) in a 20 µl reaction, containing

2 µl 10 x enzyme reaction buffer (React 2), 2 µl each of the restriction

endonuclease enzymes and 4 µl double distilled water. It was incubated in water bath at 37°C for 2.5 hr, and spun down briefly. The digested DNA was denatured at 70°C for 10 min and quickly quenched on ice for 10 min and then spun down (30 sec). The same enzyme reaction and procedures was applied to digest the vector [M13 (mp18, reverse sequence, and mp19, forward sequence)]. The ligation reaction was performed in a 10 µl reation containing 5 µl of the digested HBV-DNA, 1 µl of the vector (mp18 or mp19), 2 µl of 5 x ligation buffer [0.25M Tris-HCl (pH 7.6), 50 mM MgCl$_2$, 5 mM ATP, 5mM DTT, 25% (w/v) polyethylene glycol-800] and 1 µl T4 DNA ligase (Gibco). It was mixed properly and incubated at 12°C overnight. The next morning it was briefly spun down and used for transfecting *E. coli* (TG-2 strain) competent cells.

(*3.5.4*)   **Preparation of the competent cells**

One hundred µl of overnight fresh culture of E. coli (TG-2 strain) was used to inoculate 100ml YT broth, and incubated at 37°C with shaking (225 rpm) until an optical density (OD) of between 0.35 and 0.4 at 260nm was obtained. The culture was aliquotted into two centrifuge tubes (45ml each), 100 µl fresh YT broth was added to the remaining bacterial culture and returned to incubator and left to grow under the same conditions as specified above.

The culture (aliquot in centrifuge tubes) was incubated on ice for 30 min, and centrifuged in cold (4°C) at 7,000 rpm for 5 min. The supernatant was discarded, and pellet suspended in 10 ml of 50 mM CaCl$_2$ and 10 mM Tris-HCl (pH 8.0). The above procedure was repeated again and cells incubated on ice for 30 min, and centrifuged under the same conditions as before. The cells were

finally suspended in 5 ml (50 mM $CaCl_2$ and 10 mM Tri-HCl (pH 8.0) and kept on ice for 5 hr.

(3.5.5) **Transformation**

Five μl of the ligated HBV DNA / vector was mixed with 300 μl of competent cells and incubated on ice for 30 min, heat shocked at 42°C for 2 min, and returned on ice for 2min. 200 μl, 100 μl, and 50 μl aliquots were dispensed into 4 ml top agar containing 2% 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), 100 mM isopropylthio-β-D-galactoside (IPTG), and 200 μl of fresh TG-2 culture was added to the top agar and spread on a prewarmed YT agar plate. The plates were left on the bench for 15 min, and then incubated at 37 °C overnight.

(3.6.1) **Sequencing**

(3.6.2) **Preparation of DNA templates**

A single white plaque was picked with a sterile tooth pick (or Eppendorf tip), used to inoculate 1.5 ml YT broth, and grown in a shaker at 37°C for 5.5 hr. The culture was spun for 5 min and the supernatant was transferred into a sterile Eppendorf tube containing 150 μl PEG (20% Polyethylene glycol-6000 and 14.6% NaCl in water), whirlmixed and left at room temperature for 30 min. It was spun down for 5 min, the supernatant tipped away and respun for a further 2 min, any remaining supernatant was removed with micropipette and the tube inverted to dry. The tube was wiped fully dried with a kimwipe. The pellet was resuspended in 100 μl of TE pH 8.0 and 50 μl buffered phenol, whirl mixed and spun for 5 min. The

top 90 μl above the interface was removed into a clean Eppendorf tube and 10 μl

3M NaAc, 300 μl cold absolute ethanol was added. It was kept at -20°C overnight.

The next morning it was pelleted in cold (4°C) for 15 min and the supernatant

discarded. The pellet was washed in 1 ml cold 70% ethanol and spun for 15 min.

The supernatant poured off, and tube inverted to dry. The pellet was resuspended

in 30 μl TE (pH 8.0) and stored at -20 °C.

(3.6.3) Sequencing Reaction

Dideoxynucleotide chain termination method (Sanger *et al.*, 1977), was used

for sequencing reaction with phage T7 DNA polymerase (Sequenase version 2.0,

United States Biochemical Corporation).

The sequencing reaction was performed in 3 steps:-

(a) Annealing Reaction

(b) Labelling Reaction

(c) Termination Reaction

(a) *Annealing Reaction*

Seven μl of DNA template was annealed to 0.5 picogram universal primer

(M13) (5'GTTTTCCCAGTCACGAC-3') in a 10 μl reaction containing 1 μl

primer, 2μl 5 x sequenase reaction buffer (200 mM Tris-HCl pH7.5, 100 mM

$MgCl_2$, 250 mM NaCl). It was incubated at 65°C (in waterbath) for 2 min, and

allowed to cool down slowly at room temperature for 30 min, spun down briefly

and kept on ice.

(b) *Labelling Reaction*

5.5 μl labelling mixture [(1 μl 0.1M DTT, 0.4 μl labelling mix (dGTP), 1.6 μl double distilled water, 0.5 μl [α-^{35}S] dATP, 1.75 μl enzyme dilution buffer (10 mM Tris-HCl pH7.5, 5 mM DTT, 0.5 mg/ml BSA), and 0.25 μl sequenase (version 2.0)], was added to 10 μl annealed primer-DNA template (from a), and incubated at room temperature for 5 min and returned on ice.

(c) *Termination Reaction*

Four Eppendorff tubes (0.5 ml) were labelled A, G, C, and T, (each set of four Eppendorf tubes for a sample). 2.5 μl of each Termination Mix was placed in their appropriate labelled tubes (ddATP into tube labelled A, ddCTP into tube labelled C, ddGTP into tube labelled G, and ddTTP into the tube labelled T).

The termination mixture was prewarmed for 1 min at 37 °C in a water bath, and 3.5 μl of the labelled DNA (from b) was added to each of them. It was incubated at 37 °C in water bath for 5 min, and the reaction was stopped with 4 μl of stop solution (95 % Formamide, 20 mM EDTA, 0.05 % Bromophenol Blue and 0.05 % Xylene Cyanol FF). It was stored at -20 °C or separated immediately by polyacrylamide gel electrophoresis.

(3.7.1) **Polyacrylamide Gel Electrophoresis**

6% polyacrylamide (Acrylamide : Bisacrylamide 19:1), 7M Urea, 153 μl 25% ammonium per sulphate and 65.6 μl N'N'N'N'-Tetramethelenethylenediamine (TEMED) in 70 ml 1 x TBE, mixed properly and poured slowly from pipette, down one side of the gel mould (two glass plates held together in position with

electrical tape and bulldog binder clips). The mould was laid at an angle by resting

the top on support of about 5cm high and the flat side of the shark's tooth comb

inserted into the gel (approximately 0.5cm into the gel). The comb was clamped

into position with bulldog binder clips and gel allowed to polymerize for 90 min.

After polymerization, damp paper towels were used to wipe away dried

polyacrylamide / urea from outside of the gel mould. The shark's tooth comb was

gently removed and the electrical tape at the bottom of the gel mold stripped off

with scalpel blade. The gel mould was mounted onto gel electrophoretic tank and

clamped into position. The top reservoir was filled with 600ml 1 x TBE and the

bottom reservoir with an equal amount of 1 x TBE. With the aid of a pasteur

pipette, the gel loading surface was washed with 1 x TBE to remove any dried

polyacrylamide / urea hanging to the surface. The shark's tooth comb was

reinserted with its teeth just sticking into the loading surface of the gel. The gel

loading surface was washed as before and the gel was pre run at 60 watts for 1hr.

3.5 µl of each sequencing reaction was denatured by immersing in water

bath at 85 °C for 1 min and loaded onto adjacent slots of the gel. The gel was run

at 60 watts for 3.5 hr or until second dye ran out of the gel. The gel was

transferred onto a 3 mm Whatman paper and covered with Saran Wrap, dried at

80 °C under vacuum suction for 2 hr or until gel was properly dried. The dried gel

was removed from dryer, and Saran Wrap peeled off.

(3.7.2) **Sequencing Analysis**

The gel was autoradiographed by exposure to an x-ray film (hyper film™-

MP, Amersham, UK) for 18-24 hrs at room temperature. The film was developed

and the DNA sequence were read. DNA sequence of each clone (6 or more clones)

from each donor were independently compared with each another, where there is

a consensus, the consensus sequence obtained from each donor was independently

compared with the EMBL database using BLAST sequence in the computer

network program, (Altschul *et al.*, 1990). When there is more than one virus, the

various consensus sequences obtained, are independentely compared with the

sequences in the EMBL programm. This program and its other parameters are

optimized to find nearly identical DNA sequences rapidly from the gene bank.

CHAPTER 4

(4.1) **RESULTS**

(4.1.1) **Survey of Adult Blood Donors for HBsAg**

2490 adult blood donors from 5 transfusion centres in Nigeria, were screened for hepatitis B surface antigen (HBsAg). **274** (11%), were positive. The HBsAg-positive sera were tested futher for HBV DNA by dot blot hybridisation. 42 (15.3%), were positive. The summary of this result is shown in table 4.1.1.

(Table *4.1.1*) **Summary of the Results of HBsAg, and HBV DNA of Adult Blood Donors**

	No. Screened	No. Positive	% Positive
HBsAg	2490*	274	11 %[a]
HBV DNA	274**	42	15.3%[b]

* = Total population   ** = No of HBsAg positive

a = % from total population   b = % from HBsAg positive samples
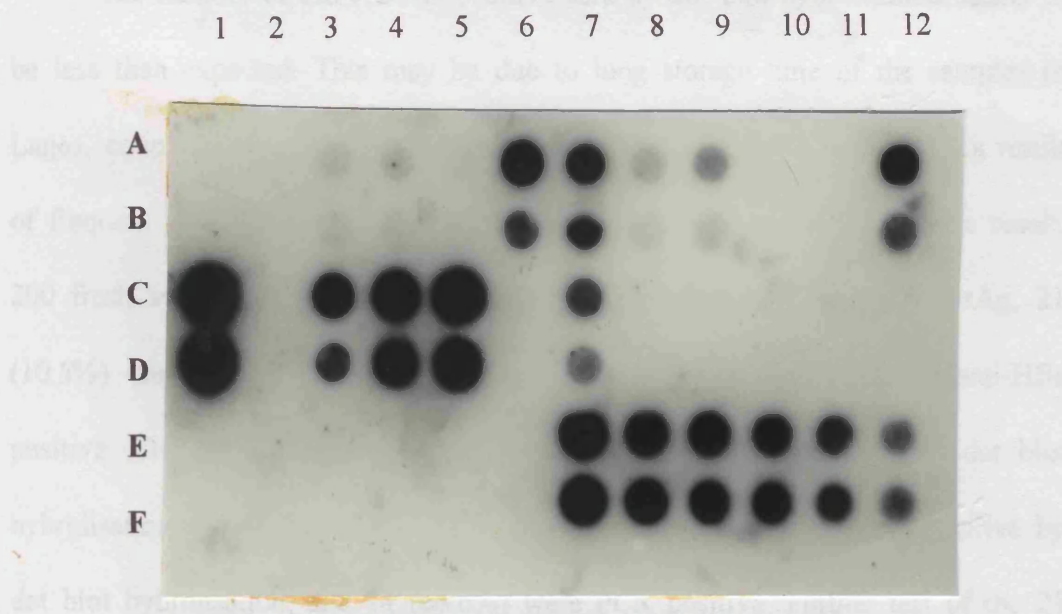
Figure 4.1.1: Results of the HBV DNA Dot Blot Hybridisation.

50μl of each serum sample was treated with 20μl of 3% β-mercaptoethanol and 20μl of 10% NP40 and 90 μl 2M NaCl was added. The DNA was denatured with 180 μl of 1M NaOH. 200 μl and 100μl from each were applied onto the N-bond membrane filter. (rows A and C = 200μl, rows B and D = 100μl, rows E7 to E12 and F7 to F12 are HBV DNA reconstruction in duplicate. E7 contains 200ng (HBV DNA), E8 contains 80ng, E9 contains 40ng, E10 contains 20ng, E11 contains 10ng, E12 contains 4ng. C1 and D1 = Positive controls in duplicate of 200μl and 100μl C2 and D2 = Negative controls in duplicate of 200μl and 100μl. The limit of detection by this method is estimated to be about 1ng.

The number of HBV DNA positive sera by dot blot hybridisation seems to be less than expected. This may be due to long storage time of the samples in Lagos, coupled with intermittent freezing and thawing of the samples, as a result of frequent power cuts in Lagos, during storage. To ascertained the above result, 200 fresh serum samples from the same sources were screened for HBsAg, 21 (10.5%) were positive, 73 (41.2%) of the 177 samples tested were anti-HBs positive. The HBsAg-positive sera were tested for HBV-DNA by both dot blot hybridisation, and polymerase chain reaction (PCR). 5 (23.8%), were positive by dot blot hybridisation, and 14 (66.6%) were PCR positive. Further test of the 21 HBsAg-positive sera with other hepatitis B virus infection markers (HBeAg, anti-HBe), showed 2 (9.5%) were HBeAg-positive and 7 (33.3%) anti-HBe-positive. Results are presented in table 4.1.2 and summarized in table 4.1.3.

(TABLE *4.1.2*)   **Results of The 21 HBsAg Positive Adult Blood Donors**

Screened for Anti-HBe, HBeAg & HBV-DNA (Dot blot & PCR)

Sample	Anti-HBe	HBeAg	Dot blot	PCR
T4	+	-	-	+
018	-	+	+	+
22	+	-	-	+
23	-	+	+	+
053	-	-	-	-
55	+	-	-	+
056	+	-	+	+
087a	-	-	-	-
087b	-	-	-	-
097	-	-	-	-
135	+	-	-	+
255	-	-	-	-
259	-	-	-	-
261	-	-	-	+
283	+	-	-	+
287	-	-	+	+
292	-	-	-	-
309	-	-	-	+
316	-	-	-	+
321	+	-	-	+
327	-	-	+	+

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

— 275bp
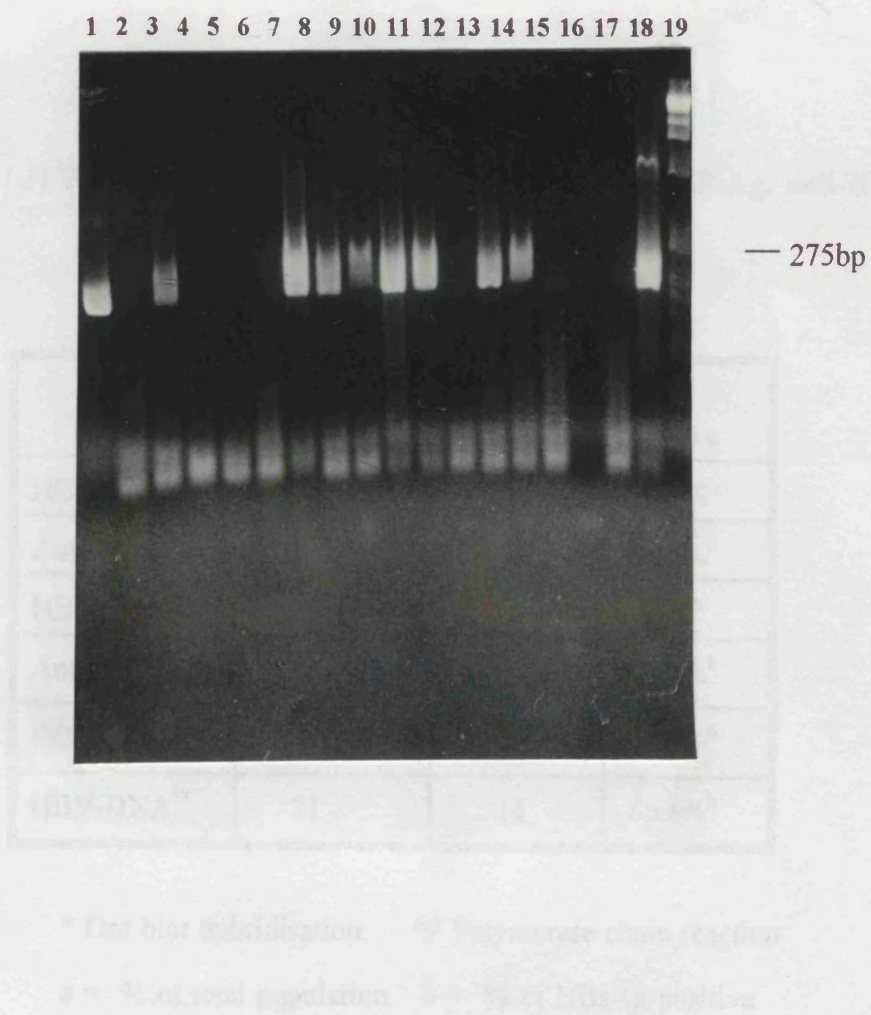
Figure 4.1.2: Results of Polymerase Chain Reaction of the HBV S-ORF (adult donors). Lanes 1 to 14 are samples under test, Lane 15 = Negative control, Lane 16 = empty, Lane 17 = negative Control, Lane 18 = positive control, Lane 19 = 1Kb Ladder marker* (1Kb Ladder fragment size in appendix).

(Table *4.1.3*) **Result of Adult Blood Donors Sceened for HBsAg, anti-HBe & HBV DNA**

	No. Screened	No. Positive	% Positive
HBsAg	200	21	10.5%[a]
Anti-HBs	177	73	41.2%[a]
HBeAg	21	2	9.5%[b]
Anti-HBe	21	7	33.3%[b]
HBV-DNA*	21	5	23.8%[b]
HBV-DNA**	21	14	66.6%[b]

* Dot blot hybridisation,   ** Polymerase chain reaction

a = % of total population   b = % of HBsAg positive

(4.1.4)   **Survey of Pre-School Children**

Sera from 100 children of pre-school age collected from the paediatric unit of University College Hospital Ibadan (U.C.H) were screened for HBsAg, HBV DNA (by dot blot and PCR) HBeAg, and anti-HBe. 16 (16%) were HBsAg-positive. 4 (25%) of the HBsAg-positive were HBV-DNA positive by dot blot hybridisation, 10 (62.5%) were PCR positive. 4 (25%) were HBeAg-positive while none tested was anti-HBe-positive. The results of these tests are shown in table 4.1.4 and summarized in table 4.1.5
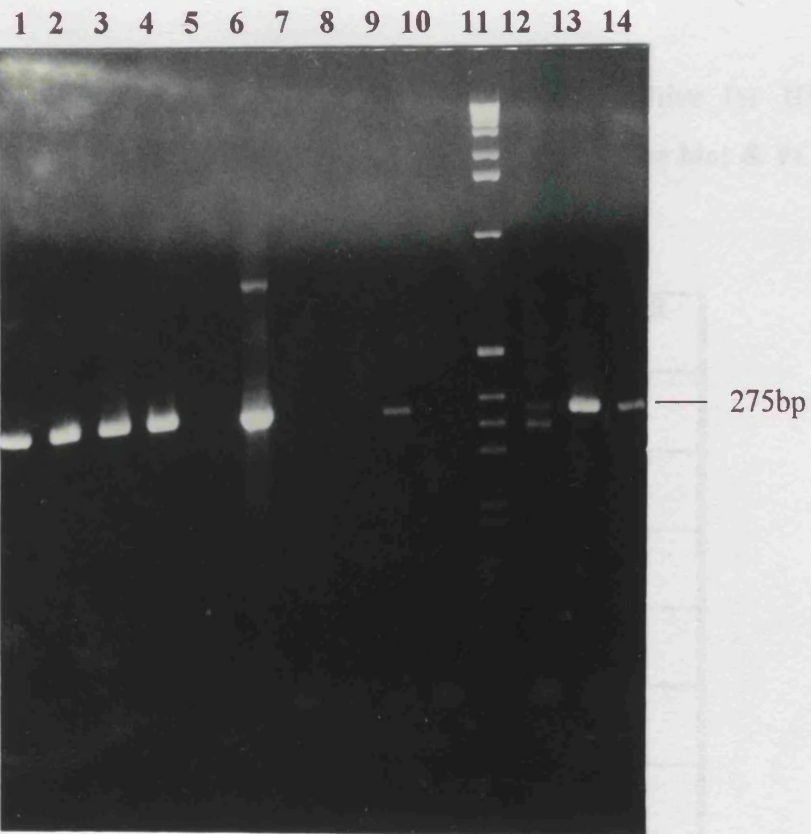
Figure 4.1.4: Results of Polymerase Chain Reaction of the

HBV S-ORF (Pre-School Age Children). Lanes 1 to 9, and 12 to 14 are samples

under test, Lane 10 = Negative control, Lane 11 = 1Kb Ladder Marker, Lane 13

= Positive Control. (1 Kb Ladder size in the appendix).

(TABLE *4.1.4*) Results of 16 Pre-School age children positive for HBsAg Screened for anti-HBe, HBeAg, & HBV DNA (Dot blot & PCR)

Sample	Anti-HBe	HBeAg	Dot blot	PCR
13	-	-	-	-
15	-	-	-	+
19	-	-	-	+
23	-	-	-	-
25	-	-	-	-
26	-	-	-	-
27	-	-	+	+
28	-	+	-	+
33	-	-	-	-
60	-	-	-	+
67	-	+	-	+
70	-	-	+	+
74	-	-	+	+
76	-	-	-	-
92	-	+	+	+
93	-	+	+	+

(Table *4.1.5*)   Summary of The Results of Pre-School age Children

Screened For HBsAg, HBV-DNA

	No. Screened	No. Positive	% Positive
HBsAg	100	16	16%[a]
HBV DNA*	16	4	25%[b]
HBV DNA**	16	10	62.5[b]
HBeAg	16	4	16%[b]
anti-HBe	16	NIL	0%[b]

* dot blot hybridisation   ** Polymerase Chain Reaction (PCR)

a = % of total population   b = % of HBsAg positive

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(4.2.1)   **Results of Sequence Analysis**

(4.2.2)   **Nucleotide Sequence of the Major Antigenic Determinant *a* Region**

and Surrounding sequence

Ten of the PCR amplified S-gene, encompassing the region encoding the antigenic

determinant *a*, spanning 255 nucleotides were selected and sequenced. At least six

clones were sequenced in each direction (forward and reverse) from each donor. The

sequences of each clone from each donor were compared with each another and a single

consensus file from each donor were independently compared with the sequences of S-

gene in the database. The highest homology were with the HBV from the Brazil

(subtype *adw4*), Angola, and HBV from the West Africa sub region (Senegal, Niger).

The Nigeria subtype *adw* showed a high percentage homology with that of the

Brazilian subtype *adw4*, above 90% homology. The nucleotide sequence comparison

of the Nigeria isolates with the Brazilian subtype adw4 is shown in fig 4.2.2a below,

the area of homology are shown in dotted line. Most of the changes observed are C to

A, G to A, C to T, and T to A, all these changes were commonly found among all the

HBV that were sequenced. The pattern of nucleotide changes observed were such that

if not common to all the sequenced, it will be common to a particular subtype.

However, none of these changes resulted in amino acid substitution in the open reading

frame, with the exception of a clone from donor AON28, which is one out of the 8

clones that were sequenced. The wild type were in the majority. This change was at nt

561 (CTC to CTT, codon 136) and it resulted in an amino acid substitution at residue

136 from serine to leucine. The significant of this change if any is not known as far as

the common subtypes are concerned, since this change was outside the two common

subtypes determinants for *adw* and *ayw* (position 122 & 160).

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Adw4	GTTTG	TCCTCTACTT	CCAGGATCCA	CGACCACCAG	CACGGGACCC	TGCAAAACCT
AON1S**	.....	......A..	........AT	.A........	T........A	...CG.....
AON1S*	.....	......A..	........TT	.A..A.....	T........A	....
AON2S	.....	......A..	........A.	.A..A.....	T........A	....
AON6S	.....	......A..	........A.	.A..A.....	T........A	....
AON8S	.....	......A..	........A.	.A..A.....	T........A	....
AON15S	.....	....G..A..	........AT	.A........	T.........	...CG.....
AON28S*	.....	......A..	.......AT	.A........	T.........	...CG.....
AON28S**	.....	......A..	.......AT	.A........	T.........	...CG.....
AON35S	.....	......A..	.......AT	.A........	T.........	...CG.....
AON36S	.....	......A..	.......AT	.A........	T.........	...CG.....
AON52S	.....	......A..	.......AT	.A........	T.........	...CG.....
AON56S	.....	......A..	.......AT	.A........	T.........	...CG.....

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adw4	GCACAACTCT	TGCACAAGGA	ACCTCTATGTT	TCCCTCCTGT	TGCTGTTCCA	AACCCTCGGA
AON1S**	....G.....	...T......	....	.....A...	........A.	....T.....
AON1S*	....G....C	...T.....C	.A.........	.....A...	.....A.A.	....TA....
AON2S	....G....C	...T.....C	.A.........	.....A...	.....A.A.	....TA....
AON6S	....G....C	...T.....C	.A.........	.....A...	.....A.A.	....TA....
AON8S	....G....C	...T.....C	.A.........	.....A...	.....A.A.	....TA....
AON15S	....G....T	...T......	....	.....A...	........A.	....T.....
AON28*	....G....T	...T......	....	.....A...	........A.	....T.....
AON28**	....G....T	...T......	....	....TA...	........A.	....T.....
AON35S	....G....T	...T......	....	.....A...	........A.	....T.....
AON36S	....G....T	...T......	....	.....A...	........A.	....T.....
AON52S	....G....T	...T......	....	.....A...	........A.	....T.....
AON56S	....G....T	...T......	....	.....A...	........A.	....T.....

Fig.4.2.2a. Nucleotide sequences of the major antigen determinant region of the 10 HBV isolates. The sequences are compared to the Brazilian HBV isolate (adw4). There are two Isolates from AON1S and AON28S (asteriked). AON1S is infected with both subtypes, adw (AON1S**) and ayw (AON1S*), While AON28S is infected with both wild (AON28S*) and a variant (AON28S**) of subtype ayw. adw4 = Consensus nucleotide sequence from Brazilian HBV isolate

```
      586
adw4    CGGAAACTGC ACTTGTATTC CCATCCCATC ATCCTGGGCT TTAGGAAAAT ACCTATGGGA
AON1S** ......T... .......... .......... ...A...... ..C...... T.........
AON1S*  T.....T... ..C....... .......... ...G...... ..C...... ..........
AON2S   T.....T... ..C....... .......... ...G...... ..C.C..... ..........
AON6S   T.....T... ..C....... .......... G......... ..C.C..... ..........
AON8S   T.....T... ..C....... .......... G......... ..C.C..... ..........
AO15S   ......T... .......... .......... ...A...... ..C...... T.........
AON28S* ......T... .......... .......... ...A...... ..C...... T.........
AON28S**......T... .......... .......... ...A...... ..C...... T.........
AON35S  ......T... .......... .......... ...A...... ..C...... T.........
AON36S  ......T... .......... .......... ...A...... ..C...... T.........
AON52S  ......T... .......... .......... ...A...... ..C...... T.........
AON56S  ......T... .......... .......... ...A...... ..C...... T.........


      646
adw4    GTGGGCCTCA GCCCGTTCTC CTGGCTCAGT TTACTAGTGC AATTTGTTCA GTGGTGCGTC
AON1S** .......... .......... .......... .......... .C........ ......T...
AON1S*  .......... .......... .......... .......... .C........ ......T...
AON2S   .......... .T........ T......... ....TC...G .C........ ......T...
AON6S   .......... .T........ T......... .......... .......... ......T...
AON8S   .......... .T........ T......... .......... .C........ ......T...
AON15S  .......... .......... .......... .......... .C........ ......T.A.
AON28S* .......... .......... .......... .......... .C........ ......T.A.
AON28S**.......... .......... .......... .......... .C........ ......T.A.
AON35S  .......... .......... .......... .......... .C........ ......T.A.
AON36S  .......... .......... .......... .......... .C........ ......T.A.
AON52S  .......... .......... .......... .......... .C........ ......T.A.
AON56S  .......... .......... .......... .......... .C........ ......T.A.


      706
adw4    GGGCTTTCCC CCACTGTTT
AON1S** .......... .........
AON1S*  .......... .........
AON2S   .......... .........
AON6S   .......... .........
AON8S   .......... .........
AO15S   .......... .........
AON28S* .......... .........
AON28S**.......... .........
AON35S  .......... .........
```

ctd. **Fig 4.2.2a** (see page 76 for legend)
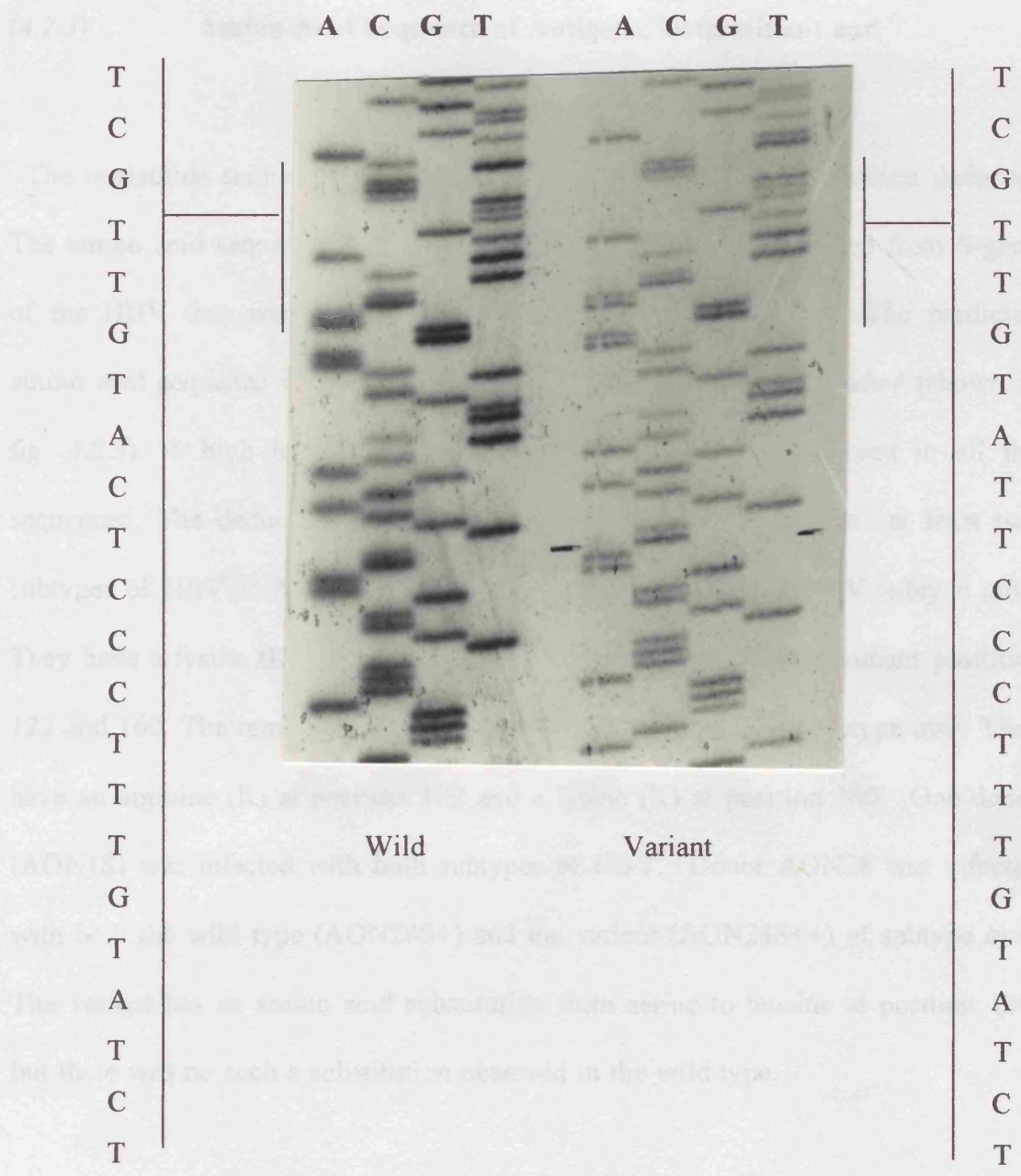
nt position		556	561	568
Wild type	AON28S*	T C C C T _C_ A T G T T G C		
Variant	AON28S**	T C C C T _T_ A T G T T G C		

Figure 4.2.2b: Nucleotide Sequence difference Observed in the S-ORF of two HBV isolates from donor AON28S. Isolate AON28S*, is a wild-type and AON28S** is a variant. The nucleotide change from **C** to **T** is at nucleotide position 561.

(4.2.3)   **Amino Acid Sequence of Antigenic Determinant and**

Surrounding Sequence

The nucleotide sequences were translated and the amino acid sequence deduced. The amino acid sequence comparison were made with those deduced from S-gene of the HBV that were used in nucleotide sequence comparison. The predicted amino acid sequence were also compared with Brazillian subtype *adw4* (shown in fig. 4.2.3). A high level of homology (above 95 %) was observed in all the sequenced. The deduced amino acid sequence predicted there are at least two subtypes of HBV in Nigeria. Three donors were infected with HBV subtype *adw*. They have a lysine (K) at the both common major antigenic determinant positions 122 and 160. The remaining 6 donors were infected with HBV subtype *ayw*. They have an arginine (R) at position 122 and a lysine (K) at position 160. One donor (AON1S) was infected with both subtypes of HBV. Donor AON28 was infected with both the wild type (AON28S+) and the variant (AON28S++) of subtype *ayw*. The variant has an amino acid substitution from serine to leucine at position 136, but there was no such a substitution observed in the wild type.

```
AMINO        111111111111111111111111111111111111111111
ACID         222222222233333333333444444444455555555556666
POSITION     01234567890123456789012345678901234567890123
CONSENSUS    PCKTCTTPAQGTSMFPSCCCTKPSDGNCTCIPIPSSWAFARFLW
             R   I   N Y     T              GKY

BRAZIL       ..K...CL.....F........S.............GKY..

W.AFRICA     ..R....L.....F........S.............GKF..

SENEGAL      ..R..M.L.....F........S.............GKF..

NIGER        ..R....L.....F........S.............GKF..

ANGOLA       ..R....L.....F........S.............GKF..

NIGERIA   SAMPLES

AON1S*       ..R..........Y........T.............GKF..

AON1S**      ..K..........F........T.............GKY..

AON2S        ..K..........F........T.............AKY..

AON6S        ..K..........F........T.............AKY..

AON8S        ..K..........F........T.............GKF..

AON15S       ..R..........F........S.............GKF..

AON28S+      ..R..........F........S.............GKF..

AON28S++     ..R..........F.L......S.............GKF..

AON35S       ..R..........F........S.............GKF..

AON36S       ..R..........F........S.............GKF..

AON52S       ..R..........F........S.............GKF..

AON56S       ..R..........F........S.............GKF..
```

Fig. 4.2.3. Predicted amino acid sequence of the major antigenic determinant region of the 10 HBV isolates aligned with HBV subtypes *adw* & *ayw*, from sub - Saharan Africa and Brazil. Donor AON1S (AON1S* and AON1S**) was infected with both *ayw* and subtype *adw*, while donor AON28S was infected with a mixture of wild-type (AON28S+) and mutant (AON28++) of subtype *ayw*.

The nucleotide sequence of the pre core, and part of the core region of HBV, was obtained from seven donors. 4 donors were HBeAg-negative and anti-HBe-positive (donors 55C, 135C, 283C, T4C), 2 donors were HBeAg-positive and anti-HBe-negative (donors 018C and 23C), and one donor was negative for both HBeAg and anti-HBe (donor 287C). The nucleotide sequences of each clone from each donor were compared with each another and a consensus file representing the HBV from each donor was then independently compared with the pre-core and core sequences of HBV in the EMBL data base. There was a great homology of all the sequenced in the pre-core region, compared with the *adw4* sequence. They all showed homology of above 95%. The pre-core region was highly conserved in all the sequences, only four nucleotide changes were observed. These changes were at positions 1850, 1858 and 1884. The nucleotide change at position 1850 (A to T) was observed in all the sequenced with exception of donor 018C, and the one at position 1858 (C to T) was observed in all the sequenced. The nucleotide change at position 1884, was observed in donor 287C (T to C), this change resulted in amino acid substitution from leucine to proline at codon 24. The fourth substitution was observed at position 1896 in donor 283C. This change converts the tryptophan codon (UGG) into a stop codon (UAG) at codon 28. There are however, some clones, where this mutation was not observed in this donor, hence 283C is infected with a mixture wild-type and mutant. The DNA sequence from the core region was not as conserved as in the pre-core region. This region showed a high degree of variability when compared with the *adw4*. Most of the nucleotide changes were commonly observed in all the that were sequenced from

this region, with exception of few cases. The PCR results and the purified products are shown on figures 4.2.4a and 4.2.4b, while the nucleotide sequence comparison with Brazilian isolate subtype *adw4* is shown in fig. 4.2.4c.

Figure 4.2.4a: Results of the Polymerase Chain Reaction of the HBV Core Region. Lanes 1 to 4, 6 & 7, and 10 to 12 are samples. Lane 5 = Negative Control, Lane 8 = Positive Control, Lane 9 = 1kb Ladder Marker.

350 bp

Figure 4.2.4b: Purified Polymerase Chain Reaction product of the HBV

core region. Lanes 1 to 4 and 6 & 7 = purified products, Lane 5 = 1kb Ladder

Marker.

```
Precore 1814                                                    |1873
adw4     ATGCAACTTT TTCACCTCTG CCTAATCATC TCTTGTACAT GTCCCACTGT TCAAGCCTCC
018C     .......... .......... .......... .......... .....T..... ..........
55C      .......... .......... .......... .....T... ....T..... ..........
23C      .......... .......... .......... .....T... ....T..... ..........
283C     .......... .......... .......... .....T... ....T..... ..........
135C     .......... .......... .......... .....T... ....T..... ..........
287C     .......... .......... .......... .....T... ....T..... ..........
T4C      .......... .......... .......... ......T... ....T..... ..........


Precore 1874                              core                 1930
adw4     AAGCTGTGCC TTGGGTGGCT TTGGGGC     ATGGACATTG ACCCTTATAA AGAATTTGGA
018C     .......... .......... .......     .......... .......... ..........
55C      .......... .......... .......     .......... .......... ..........
23C      .......... .......... .......     .......... .......... ..........
283C     .......... .......... ..A....     .......... .......... ..........
135C     .......... .......... .......     .......... .......... ..........
287C     .......... C......... .......     .......... .......... ..........
T4C      .......... .......... .......     .......... .......... ..........


         |1931,                                                 1990
adw4     GCTACTGTGG AGTTACTCTC GTTTTTGCCT TCTGACTTCT TTCCTTCCGT ACGAGATCTC
018C     .......... .......... .......... .......... .......A.. .A.......T
55C      .......... .......... .......... .......... .......A.. TA.......A
23C      .......... .......... .......... .......... .......... CA........
283C     .......... .......... .......... G......... .......AA. .A.......T
135C     .......... .......... .......... .......... .......A.. .A.......T
287C     .......... .......... .......... .......... .......A.. .A.......T
T4C      .......... .......... .......... .......... .......A.. TA.......A
```

Fig.4.2.4c. Nucleotide sequences of the pre-core and part of the core of the 7 HBV. The sequences are compared to Brazillian HBV ADW4 isolate. Donor 283C was infected with a precore mutant with a stop codon at codon 28, while donor 287C was infected with a further variant of HBV which has a proline substituted for leucine at codon 24.

```
Core ctd. 1991                                                          2050
adw4       CTAGACACCG CCTCAGCTCT GTATCGAGAA GCCTTAGAGT CTCCTAGAGT TTGCTCACCT

018C       ....T..... .......... ......G..T ........A. .......... ...TA.....

55C        ....T..... .......... ......G..T ........A. .......... ...T......

23C        .......... .......... ......G..T ........A. .......... ...T......

283C       .....T.... .......... ..T...G..T .....G..A. ..T....... ...T......

135C       .....T.... .......... ......G..T ........A. .......... ...TA.....

287C       .....T.... ....C..... ......G..T ........A. .......... ...T......

T4C        .....T..GA .......... ......G..T ........A. .......... ...T......


           2051                                                        2110
ADW4       CACCATACTG CACTCAGGCA AGCCATTCTC TGTTGGGGTG AGTTGATGAA TCTGGCCACC
018C       .......... .......... G........T ..C.....G. .AC.A....C ...A..T...

55C        ..T..C.... .......... .T.......T ..C.....G. .AC.A....C ...A..T...

23C        .......... .......... ...T.....T ..C.....G. .AC.A....C ...A..T...

283C       .......... .......... .........T ..C.....G. .CC.A....C ...A..T...

135C       .......... .......... .........T ..C.....A. .CG.A....C ...A..T...

287C       .......... .......... ..T......T ..C.....A. .AC.A....C ...A..T...

T4C        ..T....... .......... .........T ..C.....G. .AC.A....C ...A..T...


           2111       2130
ADW4       TGGGTGGGAA GTAATTTGGA
018C       ........TG TA........

55C        ........TG TA........

23C        ........TG TA........

283C       ........TG TA........

135C       ........TG TA........

287C       .......TG TA........

T4C        ........TG TA........
```

Fig. 4.2.4c. (see legend in page 85)

Nt. position	1886										1896		
Wild Type sequence	G	G	G	T	G	G	C	*T T T G*	G	G	G
Mutant sequence	G	G	G	T	G	G	C	*T T T A**	G	G	G

Figure 4.2.4d: Nucleotide sequence of the Pre-Core region Showing the Pre-Core mutation at nucleotide 1896. The nucleotide change from G to A is observed at position 1896 (asterisked), is the most common mutation associated with pre-core mutants.

(4.2.5) Predicted Amino Acid Sequence of the preCore/and part of the Core Region

The predicted amino acid sequence for both pre-core and part of the core region were deduced. The sequences were compared with the published amino acid sequences from the west African sub region, Angola and Brazil. There is almost 100 % amino acid sequence homology with HBV sequence from West Africa. With exception of 283C with a stop codon at precore codon 28 and the 287C with an amino acid substitution from leucine to proline at residue 24, there were no other significant changes observed in the pre-core region. All the isolates has a phenylalnine substituted by a serine at position 11. The amino acid sequence of the core region is not as conserved as those of the precore region. The level of variability were as predicted by the nucleotide sequence. The 283C (a pre-core mutant) is the most variable of all the sequenced. It showed amino acid changes at various positions, these changes are serine to alanine at position 21, tyrosine to phenylalanine at residue 38, proline to serine at amino acid residue 45. In T4C there is only one amino acid change observed at position 34 (alanine to threonine). Other significant changes observed were glutamic acid to aspartic acid in three of the isolates (283C, 135C, and 287C) at position 64 and leucine to valine at position 65 in 135C and 287C. All the isolates has histidine substituted for asparagine at position 51, and an asparagine been replaced by valine at position 74. The results of the amino sequence are shown in figure 4.2.5

```
              1         11        21        1         11        21
ADW4    MQLFHLCLII FCSCPTVQA SKLCLGWLWG MDIDPYKEFG ASVELLSFLP SDFFPSVRDL
                   T                                T          I
018C    .......... S........ .......... .......... .......... ..........
55C     .......... S........ .......... .......... .......... ..........
23C     .......... S........ .......... .......... .......... ..........
283C    .......... S........ ........*. .......... .......... A.........
135C    .......... S........ .......... .......... .......... ..........
287C    .......... S........ ....P..... .......... .......... ..........
T4C     .......... S........ .......... .......... .......... ..........


Core          31        41        51        61        71
ADW4    LDTASALYRD ALESPEHCTP NHTALRQIL CWGELMTLAS WVGNNL
              VA         S          N        S
018C    .......... .......... H....... ........T ...V..
55C     .......... .......... H....... ........T ...V..
23C     .......... .......... H....... ........T ...V..
283C    .......F.. ....S..... H....... ...D.....T ...V..
135C    .......... .......... H....... ...DV....T ...V..
287C    .......... .......... H....... ...DV....T ...V..
T4C     ...T...... .......... H....... ........T ...V..
```

Fig. 4.2.5. The predicted amino acid sequence of the precore and part of core region (single letter code) for the 7 HBV isolates. The derived amino acid sequences are compared with the consensus nucleotide sequence in fig.4.2.4c.

Table 4.2.6.   Serological data and HBV DNA analysis from 7 HBsAg carriers

sequenced in the precore and part of the core region

Sample	anti-HBe	HBeAg	DotBlot	PCR
T4C	+	-	-	+
018C	-	+	+	+
23C	-	+	+	+
55C	+	-	+	+
135C	+	-	-	+
283C	+	-	-	+
287C	-	-	+	+

Note : Four donors were anti-HBe (T4C, 55C, 135C, and 283C) only one (55C) was HBV DNA positive by dot blot hybridisation. Two were HBeAg positive (018C and 135C), and were both HBV DNA positive by dot blot. All were PCR positive.

CHAPTER 5

(5.1)   **DISCUSSION**

Numerous studies have shown that HBV infection is highly endemic in Nigeria (Nasidi *et al.*, 1986, Kulkarni *et al.*, 1986, Ayoola & Olubuyide, 1989, Bello *et al.*, 1992). In the current work, a small scale epidemiological survey of hepatitis B virus infection amongst the healthy adult blood donors and children of pre-school age in Nigeria was carried out. The HBsAg carrier rate was correlated with the viraemia rate. There have been no recent report on the epidemiology of hepatitis B infection among adult blood donors in Nigeria. The last report was in 1990 (Amazigo & Chime, 1990), they reported a 9.1% HBsAg carrier rate from the eastern part of Nigeria. Earlier reports were in 1981 and 1986, (Fakunle *et al.*, 1981, Kulkarni *et al.*, 1986). The first group reported 10% HBsAg carrier rate from Zaria, in the northern Nigeria (Fakunle *et al.*, 1981), and the second group reported 9.1% (Kulkarni *et al.*, 1986). In the current work the carrier rate of between 10.75 and 11 % was found among healthy adult blood donors, which is in agreement with the earlier reports. The little difference observed between the previous reports and the current work, may firstly be due to the size of donor population. In the current work, a large population of donors (2690) were screened, and unlike in the previous reports, were not restricted to a particular area of the country, efforts were made to have a well distributed samples, that spread across the major cities and ethnic groups. Secondly, the screening methods used in the current work were more sensitive than those used in the previous reports. Although there is no data, as to what were the sex of the donors screened in this work, however, about 90% of the blood donors in Nigeria are males, and hence, it is assumed that the

majority of the samples comes from the male donors. This must have effect on this result, to what extent is not known.

An HBsAg carrier rate of 16% was found among the children of pre-school age, from Ibadan, the second largest city in Africa. The high carrier rate observed among these children was not quite surprising, because it shows the effect of blood to blood contact in transmission. A vast majority if not all of the Ibadan population practice a religion and custom which involves a blood to blood contact as seen by the way of tribal marks and scarification. These practices have long been of great concern for the transmission of hepatitis B virus among this population, because most of these practices are often performed not in an hospital but at homes (scarification), or at the ancestral family homes (tribal marks), with unsterilized materials and by persons without any medical training. Many children undergo the scarification together or tribal marks applied to their faces in groups with the same knife, without sterilization thereby spreading the HBV infected blood to some who have not been previously infected through other routes. 10% HBsAg carrier rate among children of pre-school age has been reported from an area in Nigeria, where these practices are not practised frequently (Fakunle *et al.*, 1981). This route of transmission may account for the majority of hepatitis B infection in Nigeria. 41.2% donors were anti-HBs-positive which means 51.7% (10.5% HBsAg-positive & 41.2% anti-HBs-positive) of the tested population has one way or the other been exposed to HBV. The results of other hepatitis B virus markers also indicated a high degree of chronic infection among the tested population. Among the HBsAg-positive donors 9.5% were HBeAg-positive and 25% of the children. Anti-HBe-positivity of 33.3% was observed in the HBsAg-positive donors but none of the

children.

Numbers of the adult donors as well as the children that were HBsAg positive, but negative for all other HBV markers, the reason for this observation is not very clear, however, the fact that most of these samples were HBeAg negative, may be an indication that other HBV markers (anti-HBs, anti-HBe) are probably circulating at a very low level that could not be detected by the method used. It should have been appropriate to test these samples for anti-HBc, to confirm the reliability of the HBsAg test, serum were however not sufficient to carry out this test. This will be further investigated in the future.

The viraemia rate was determined by dot blot hybridisation (HBV DNA detection). The viraemia rate of about 24% was observed in the HBsAg-positive adult blood donors, and 25% of the pre-school age children. This is a very high rate, if extrapolated to the Nigeria population of 100 million, this indicates that around 11 million of the total population are carriers, 2.3 million possibly viraemic. It is also believed that 25% of the viraemic population will eventually become a life long carrier of the virus, with a high possibility of developing hepatocellular carcinoma (Popper et al., 1987). A 65% HBsAg-positivity, and over 84% anti-HBc-positive, were reported in an HBV marker survey in the sera of the primary liver carcinoma patients in Nigeria (Gashau & Mohammed, 1991). These data are an indication that a substantial number of the viraemic population in Nigeria may eventually develop hepatocellular carcinoma (HCC) later in life.

HBV DNA results, from the more sensitive polymerase chain reaction (PCR), showed 66.6% positivity among the HBsAg-positive adult donors, and 62.5% of the children. These were quite high, the results confirmed that Nigeria is highly

endemic with hepatitis B virus infection. Although, the number of adult donors screened (2690), is a large number, but when compared to the entire Nigeria population (100 million), may seems not large enough, however, this limitation has been taken into consideration, donors were collected in such a way that they cut across the population and hence the results, were believed to be the true picture of HBV infection in Nigeria.

The four commonest subtypes of HBsAg can be generated by the combination of either *d* or *y* (Le Bouvier, 1971) and either *w* or *r* (Bancroft *et al.*, 1972), these combinations are accepted as the phenotypic expression of the four major genotypes of HBV (*d/y*, *w/r*). These differences are the results of the substitution of amino acid residues within the 226 residue translation product of the S gene (Peterson *et al.*, 1984), the *d* strains have a lysine at position 122 and *y* strains have arginine, *w* strains always have lysine at position 160, whereas *r* strains have an arginine (Okamoto *et al.*, 1989). The HBV genotypes followed a geographical distribution throughout the world (Courouce-Pauty *et al.*, 1983, Norder *et al.*, 1993). The amino acid residues of the antigenic determinant *a* for ten HBsAg-positive sera from Nigeria were deduced. The results of amino acid sequence obtained from three donors (AON2S, AON6S and AON8S) showed that they belong to subtype *adw* and six donors (AON15S, AON28S, AON35S AON36S, AON52S and AON56S) were *ayw*. The remaining one donor (AON1S), was infected with both subtypes. Of the 2 subtypes of HBV present in Nigeria, *ayw* was in the majority. The *ayw* subtype has long been confirmed as the predominant indigenous subtype of western sub-Saharan Africa (Ayoola & Olubuyide, 1989, Norder *et al.*, 1993). The occurrence in Nigeria of subtype *adw*

which was until now regarded as an indigenous subtype of the Central and East of sub-Saharan Africa, Ethiopia, Somalia & South Africa (Norder *et al.*, 1993), in an appreciable proportion is not a surprise. This subtype has been in Nigeria all along, but not in an appreciable amount (Ayoola & Olubuyide, 1989), the increased in international travels and migration might have affected the proportion. The Nigerian oil boom of the late 1970s, probably might have been responsible for the increase in proportion observed in this subtype. The oil boom was accompanied by the inflow into Nigeria of many foreigners from far and near, it also created a lot of opportunities for many Nigerians, to travelled abroad for holidays and for business. The results of which might have been responsible for subtype of HBV that was formerly regarded as alien to the country being imported to the country. Again there are very few reports about the HBV subtype of Nigeria origin, most if not all of these reports, were from countries of western sub Saharan, which may not be a true situation of what is present in Nigeria. One of the donors was infected with HBV of both subtypes. The clinical significance of subtype of HBV multiple infection has not yet been documented. The source of infection might probably be through multiple blood transfusion with contaminated blood and each time with blood contaminated with different subtype of HBV, or has previously been infected with one subtype before been transfused with blood contaminated with other subtype of HBV. It may also be through contaminated syringes during sickness or by scarification. The sources, however, is not through sample contamination in the laboratory. One of the donors (AON28S) was infected with both wild and variant of HBV subtype *ayw*. The variant has a leucine substituted for cysteine at the highly conserved amino acid position 137, these

clones were however in the minority. The significance of this substitution is not known, because no report of such a substitution has been reported elsewhere.

The presence of hepatitis B e antigen in the serum may be generally regarded as an indication of active HBV replication, while sero-conversion to anti-HBe is accompanied by a decrease or cessation of viral replication (Realdi *et al.*, 1980). Five donors were HBeAg-negative (T4, 55, 135, 283 and 287) out of which 3 (T4, 55, 287) were HBV DNA positive by dot blot hybridisation, but all were PCR positive. The nucleotide sequence analysis showed only one (283) has a pre-core mutation, with a stop codon at codon 28, which abort synthesis of HBeAg (Carman *et al.*, 1989, Brunetto *et al.*, 1990). Others has no mutation of any sort in the pre-core region, except 287 and yet all were HBeAg-negative, there may probably be other factors rather than mutation in the pre-core region, that is responsible for the HBeAg-negativity observed in these donors, such as a mutation in the core promoter (Okamoto *et al.*, 1994). Donor 287 was infected with a variant which has a mutation at amino acid position 24 of the pre-core region, where phenylalanine was substituted for leucine. The significance of this mutation at the pre-core region is unclear. This donor also was anti-HBe-negative.

CHARTER 6

PART II: HEPATITIS C VIRUS (HCV)

(6.1.1)   MOLECULAR BIOLOGY OF HCV

Hepatitis C virus is an enveloped virus. The genome is RNA, single stranded and of positive polarity, and comprises approximately 9,400 nucleotides (9.4kb; Choo et al., 1991, Takamizawa et al., 1991). Comparative analysis of the viral nucleotide sequence reveals that HCV is a novel agent but shares both biophysical and genetic characteristics with that Flaviviridae family of viruses. The HCV genome is organized in a manner similar to that of the flaviviruses and pestiviruses but is more similar to the pestiviruses (Miller & Purcell, 1990). The polyproteins encoded by the positive stranded RNA genomes of these viruses are almost of equal size (HCV 3010, Flavivirus 3400, Pestivirus 4000 amino acids). The 5' terminus of the HCV genome has substantial primary sequence identity with the corresponding region of the pestivirus genomes (Han et al., 1991). The polypeptide also exhibits a significant sequence identity with nucleoside triphosphate (NTP)-binding helicases encoded by the pestiviruses and to a lesser extent, with the flaviviruses (Miller & Purcell, 1990, Choo et al., 1991). There are also numbers of small dispersed amino acid sequence homologies with the nonstructural proteins of flaviviruses (Miller and Purcell, 1990, Choo et al., 1991). Protease and replicase sequence motifs conserved among the pestiviruses and flaviviruses are also present within the HCV-encoded polyprotein, along with the more extensively conserved helicase sequence, which are all similarly collinear among the three types of viral polyproteins (Choo et al. 1991). The hydrophobicity of the HCV-encoded polypeptide is remarkably similar to those of the flaviviruses,

and to a lesser extent, to those of the pestiviruses, thus indicating similarities in their basic structures and functions (Choo *et al.* 1991). All these features indicate that, while HCV is a novel agent, it is closely related to both the animal pestiviruses (e.g bovine viral diarrhoea virus (BVDV), and hog cholera virus; HoCV) and to human flaviviruses (e.g yellow fever virus and dengue virus), hence, it has been assigned to a separate genus within the Flaviviridae. The genomic structure and organization of HCV shows a close similarity to the pestiviruses and flaviviruses in the 5' untranslated region (5' UTR) and 3' untranslated regions (3' UTR). The viral genome is made up of a large open reading frame (ORF) that extends throughout most of the genome, and encodes a single polyprotein of between 3010 and 3033 amino acids, depending on the source of the isolate (Takamizawa *et al.* 1991). It is then cleaved into several structural and non structural proteins (Houghton *et al.*, 1991). Distinct regions have been identified on the HCV encoded polyprotein from the 5' end to the 3' end. Schematically, four domains can be identified; two untranslated regions (UTR) located at the 5' (5' UTR) and 3' ends (3' UTR) of the genome (Han *et al.*, 1991). In the 5' UTR, there are several short open reading frames which are thought probably might be involved in the regulation of HCV expression and provide an internal ribosomal entry site (IRES; Tsukiyama-Kohara *et al.*, 1992). This is discussed further in section 6.1.2. The structural region includes three proteins; the capsid protein (with a basic region in its N-terminal part) and two putative envelope proteins which are glycosylated and referred to as envelope 1 (E1) and envelope 2 (E2) (Houghton *et al.*, 1991). The non-structural region encodes proteins of NS2, NS3, NS4, and NS5. NS3 has both protease and helicase activities, which are probably involved

in the replication of HCV RNA and in the cleavage of the polyprotein (fig.6.1.1 Houghton *et al.*, 1991). The NS5 encodes an RNA-dependent RNA polymerase (Houghton *et al.*, 1991). Further discussed in 6.1.7, the location of each region is shown schematically in fig 6.1.1

(6.1.2)   The 5 ' Untranslated Region (5 ' UTR)

The complete 5' UTR consists of 341 nucleotide (Han *et al.*, 1991, Chen *et al.*, 1992), but recently many shorter sequences have been reported (Okamoto *et al.*, 1991, Choo *et al.*,1991, Takamizawa *et al.*, 1991). The precise terminus of the native RNA genome has proved difficult to determine, because preliminary data suggested the existence of truncated RNA molecules in vivo (Han *et al.*, 1991). The 5' UTR of the flaviviruses is shorter (Brinton and Dispoto, 1988), and there are no reports of any significant sequence homologies with HCV. There are, however, several small collinear regions in HCV which show significant homology to the 5' UTR sequences from the pestiviruses BVDV and HoCV (Houghton *et al.* 1991). There are up to five small ORF's in HCV 5' UTR, however, it is not known if these are actually translated and what the function of the products might be (Yoo *et al.*, 1992).

The secondary structure of the 5' UTR has been determined by thermodynamic modelling and enzymatic cleavage of RNA by specific ribonucleases (Tsukiyama-Kohara *et al.*, 1992). These studies revealed the presence of a large conserved stem-loop structure in the proximal part of the 5' UTR, which serves as a putative IRES. An analogous is also present in the 5' UTR of piconaviruses (Pelletier and Sonenberg, 1988). Other studies, using mono and

dicistronic RNA constructs have also suggested the presence of an IRES in the HCV 5' UTR (Wang *et al.*, 1993), there are, however, other contradictory report (Yoo *et al.*, 1992). With the current knowledge about the HCV, it is believed, there is a great similarity in the translation of this virus and that of picornaviruses (Jang *et al.*, 1989). Thus, HCV polyprotein translation seems to be cap independent and initiates at an IRES within the 5' UTR, proximal to the initiation codon (AUG) of the polyprotein.

A complete understanding of the HCV replication mechanism is still lacking, however, it is assumed that like in flaviviruses and pestiviruses that HCV RNA is replicated by a direct RNA to RNA mechanism. It has not been possible to detect DNA intermediates in serum or liver of infected individuals. Antigenomic (minus) RNA strands have been detected in liver (Takehara *et al.*, 1992) and in plasma (Fong *et al.*, 1991) of patients. The detection of minus strand in plasma is quite surprising, because HCV replication is believed to be taking place in the liver. This report should therefore be interpreted with some caution, as there is at present technical difficulties with strand specific PCR, used in the detection of the HCV minus strand (Willems *et al.*, 1993). Antigenomic strand synthesis should normally start at the 3' terminus but, some small repeated 6-8 bp sequences, are found present at the both 5' and 3' UTRs, it is then not clear if this may be involved in secondary structure formation or cyclisation of the RNA genome (Takamizawa *et al.*, 1991). The precise mechanism of viral replication remained to be elucidated.

Fig.6.1.1; Schematic representation of the HCV RNA including the origin of several cloned antigens, used in the anti-HCV antibody detection systems. HVR1 indicate the position of the hypervariable region 1 in the N-terminus of E2/NS1
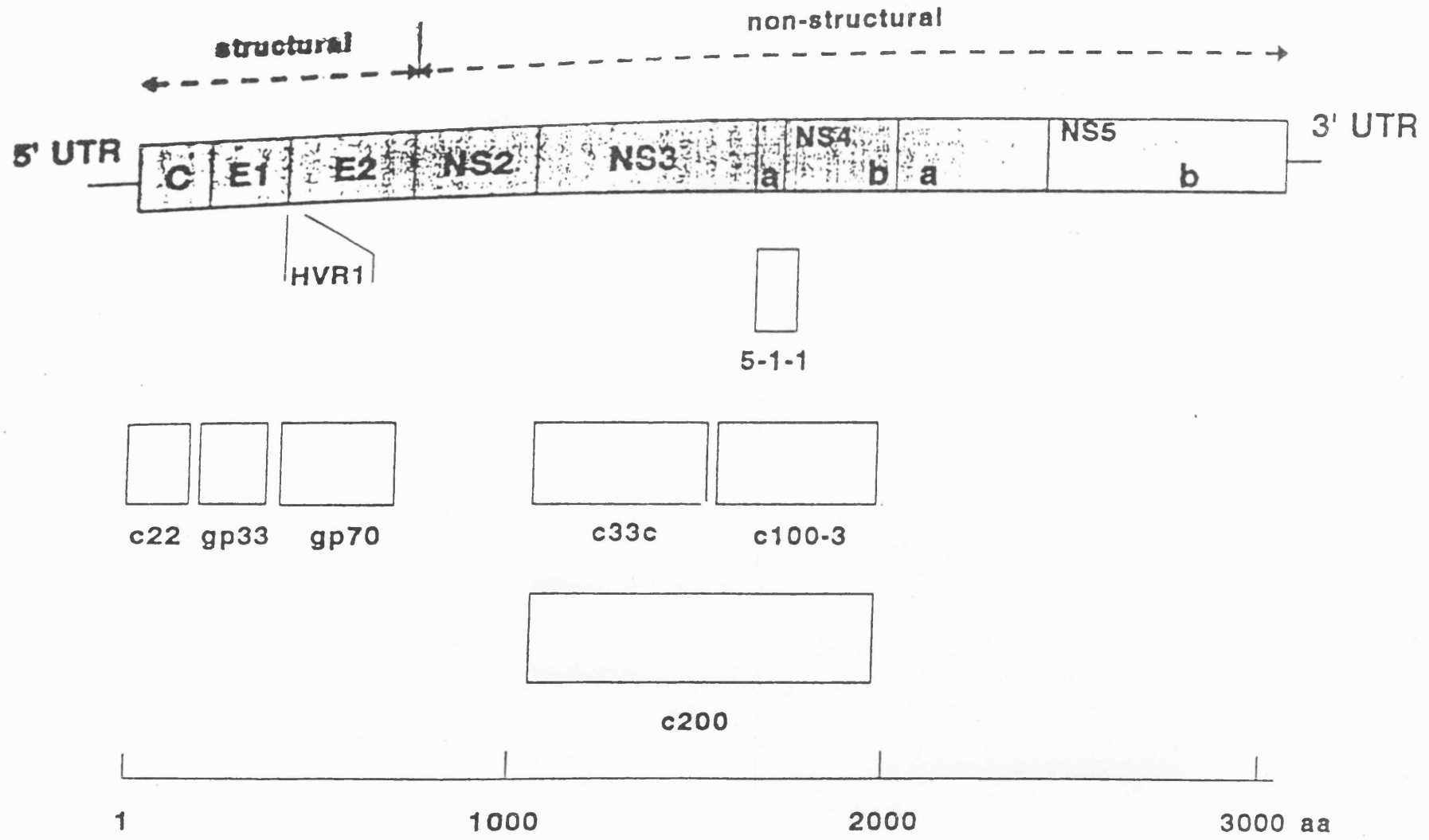
Fig.6.1.1 ; Schematic Representation of the HCV RNA Genome.

(6.1.3)   **The Core Region**

HCV core protein (p22) contains many proline residues (about 12% of the amino acids), and is highly basic because of the high content of basic amino acids (mostly arginine and lysine) residues (Houghton *et al.*, 1991, Choo *et al.*, 1991), but the 20 amino acid residues at the carboxyl terminus are highly hydrophobic and it has been suggested that they may probably act as a signal sequence (Takeuchi *et al.*, 1990). N-glycosylation sites have not been reported in this protein. There is every possibility that further processing of the p22 takes place to generate matured core which is incorporated in the virion (Hijikata *et al.*, 1991). Specific association between p22 and HCV genomic RNA accounts for the formation of nucleocapsid particles (Takahashi *et al.*, 1992), it also has an RNA binding capacity. P22 contains several highly conserved immunoreactive epitopes, therefore both recombinant core proteins and synthetic peptides, representing linear core epitopes, can be used for efficient detection of antibodies in most patient sera (Chiba *et al.*, 1991).

(6.1.4)   **The Envelope Regions (E1 and E2)**

The HCV E1 and E2 regions encode the putative viral envelope proteins. The envelope protein (E1) contains many potential N-glycosylation sites and is detectable in a glycosylated (gp33) form. The glycosylation has been demonstrated in a cell free system (Hijikata *et al.*, 1991). The envelope glycoprotein (gp33) of HCV corresponds to the envelope glycoprotein (E1) of the pestivirus BVDV and HoCV of the pestiviruses and the envelope proteins (M/E) of the flaviviruses (Weiner *et al.*, 1991), although there are no significant sequence homologies

observed among them. Transmembrane transport of gp33 is presumably facilitated by a 20 amino acid motif (referred to as N-terminal stretch), that probably functions as a signal sequence, recognized by cellular signalase, thereby cleaving the core protein C22 from the precursor protein. The stretch of hydrophobic amino acid residues between 350 and 390 is believed to act as a membrane anchor (Heinz, 1992), as seen in the corresponding sequence in flaviviruses.

The E2 region encodes the glycoprotein (gp72) which has a 38kDa protein as backbone (Hijikata *et al.*, 1991). A repeated amino acid motif has been observed between residues 471 and 511 (Pro-$(X)_5$-Pro-$(X)_6$-Pro-$(X)_6$-Pro-$(X)_6$-Pro-$(X)_5$-Pro-$(X)_6$-Pro; Kato *et al.*, 1991) which has a cystine residue in each of the last four intervening (X) sequences, their positions seem to be conserved. This complex organization suggest that probably the secondary and tertiary structures may play an important role in this glycoprotein. The gp72 of HCV is more heavily N-glycosylated than those observed in the corresponding flavivirus NS1 protein, and pestivirus gp55-53, and moreover a full length gp72 is not secreted from the cell, like the flavivirus NS1 protein, but remains membrane associated. This is probably made possible by the presence of a single transmembrane anchor, since C-terminally truncated E2 protein is rapidly secreted (Spaete *et al.*, 1992). It is most likely that while the C-terminus is membrane anchored, the N-terminus of E2 located at the outside, probably would allow surveillance by the immune system. This has led to a postulation that gp72 may probably be a structural envelope protein of HCV (Spaete *et al.*, 1992). Critical evaluation of all the available E2 sequences revealed the presence of a hypervariable region (Kato *et al.*, 1992), which is located directly downstream of the putative cleavage site between E1 and

E2, this region is within residue 380 to 385 and covers 30 amino-terminal residues (384 to 414) of the E2 protein (Weiner *et al.*, 1991). No conserved secondary structure has been observed in the hypervariable region (HVR1), but a close resemblance with the V3 loop of human immunodeficiency virus 1 gp120 has been established (Weiner *et al.*, 1992). Hypervariable sequence observed in the E2 may be as a result of sequential mutations leading to escape mutant. This is presumably due to the absence of selection pressure favouring new mutants caused by an inadequate immune response against the initial HVR1 epitope. HVR1 shows hypervariation mostly in chronic infections, and conservation has been observed (van Doorn *et al.*, 1994). That the N-terminal part of the E2 region may encode distinct antigenic variants, susceptibility to immune selection has been proposed (Weiner *et al.*, 1992). HVR1 can be used to specifically distinguish HCV variants, which provides an important tool for the study, treatment and epidemiology of HCV infections (Okada *et al.*, 1992). The most significant implication of gp72 variability is probably in development of a protective immune response and in vaccine strategies.

(6.1.5)   **The NS2 and NS3 Proteins**

The nonstructural protein 3 (NS3), is a non glycosylated protein (p72). The NS3 protein shows a very limited sequence homology with the corresponding flavivirus and pestivirus protein (Miller and Purcell, 1990). The N-terminal region (about one-third of NS3) resembles a trypsin-like serine protease, similar to that found in flaviviruses (Chambers *et al.*, 1990). The serine at position 165 is found to be critical for proteolytic activity, substitution of this serine with another amino

acid normally abolished the observed activity (Eckart *et al.*, 1993). The activity of this protease is found to be important for some of the processing steps of the precursor polyprotein into mature proteins. Any modification to the histidine at position 1083 and serine at position 1165 at the C-terminus of the protease domains results in abolition of all four cleavages (NS3-NS4a, NS4a-NS4b, NS4b-NS5a, and NS5a-NS5b), these cleavage sites show several common features, which probably determine the specificity of the NS3 protease (Grakoui *et al.*, 1993).

The cleavage of NS2-NS3, is mediated in cis by a Zn^{2+} requiring metalloproteinase. Cellular protease, such as the signal peptidase, are involved in sequential sites on N-terminus of E1, E2, and possibly NS2. NS3 can also acts in cis on its own C-terminus, but the remaining three sites only can be processed by NS3 in trans (Tomei *et al.*, 1993).

(*6.1.6*)   **The NS4 Protein(s)**

The function of the NS4 protein is not yet very clear. The protein is processed into NS4a and NS4b, which contain a highly immunogenic epitopes and several predicted transmembrane regions. The first cloned C100-3 antigen, which is used in most antibody detection assays contains, in addition to other NS regions, the N-terminal part of NS4b. NS4 protein has also been used for serotyping of HCV into various genotypes (Simmonds *et al.*, 1993b).

(*6.1.7*)   **The NS5 Protein(s)**

The nonstructural protein 5 (NS5) is processed into NS5a and NS5b. The NS5b show a significant amino acid sequence homology with the putative RNA-

dependent RNA replicase from carnation motile virus (CARMV; Miller and Purcell, 1990). This is very interesting, because CARMV is a member of the carmovirus group of plant viruses. Furthermore, the NS5b contains a Gly-Asp-Asp sequence (between residues 2737-2739), which is a characteristic sequence motif for RNA viruses (Koonin, 1991), it can be concluded that NS5b encodes the viral replicase. More important is the suggestion that, in some isolates a secondary structure may probably exist in the genomic RNA at the C-terminal part of the NS4 region which could possibly form an IRES (Okamoto *et al.*, 1992a), if so, this would allow an earlier increased production of NS5b encoded replicase, since the IRES so formed would be located just upstream of a common inframe initiation codon. There is, however, no experimental data to support this hypothesis. NS5a contains important B-cell epitopes but has not been associated with any specific function.

(*6.1.8*)   **The 3' UTR**

There are significant variations observed in both the length and sequence of the published nucleotide sequence from the 3' UTR. However, most important of these differences is the occurrence of poly(rA) tail in some isolates (Han *et al.*, 1992), and a poly(U) tail in others (Takamizawa *et al.*, 1991, Chen *et al.*, 1992). Much still remains unclear about this region.

(*6.2.1*)   **Morphology Of The HCV Virion**

Very little is known about the morphology of HCV viral particles, the little known was obtained from different indirect methods such as, buoyant density

determination of the HCV virion by gradient ultracentrifugation, followed by testing the fractions for infectivity and presence of core antigen or HCV RNA (Bradley *et al.*, 1991). The average density of the presumptive HCV particles is about 1.08g/ml, which is similar to that of BVDV (between 1.09 and 1.15g/ml), but slightly different from that of the flaviviruses (about 1.20g/ml; Bradley *et al.*, 1991).

The particle size was between 30 and 38 nm, as determined by microfiltration and HCV RNA detection (Yuasa *et al.*, 1991), it has a diameter of between 36 to 62 nm by gradient fractions, which interestingly resembles togavirus and flavivirus morphologically, with a unit membrane envelope and surface projections.

(6.3.1)   Genomic Variability of HCV

Different isolates of HCV show substantial nucleotide sequence variability distributed throughout the viral genome (Okamoto *et al.*, 1991). Nucleotide sequence variation has been observed in genomes amplified from serum of patients with HCV infection, this has provided an early indication of the heterogeneity of HCV (Okamoto *et al.*, 1990, Chan *et al.*, 1992). Coexistence of several closely related sequences in a single patient has been observed (Tanaka *et al.*, 1992). The replication of viral genome is by NS5-encoded RNA-dependent RNA polymerase, during this process errors may occur, resulting in the mutations. The nucleocapsid, and the 5' UTR are relatively conserved (Bukh *et al.*, 1992), but the region encoding the putative glycosylated envelope proteins are highly variable, and hypervariable domains (HVR) are also present in the N-terminal regions of the E1

and E2 regions (Weiner *et al.*, 1991). Sequence comparisons between isolates of HCV indicate that, HCV can be classified into a series of distinct genotypes which differ substantially in nucleotide sequence from one another and showed varied geographical distributions.

(6.4.1)   Classification of HCV

The first full length HCV sequences was derived from a chimpanzee infected with contaminated American Factor VIII concentrate (Choo *et al.*, 1989). This sequence became known as the HCV prototype, HCV-US or HCV-1, to which each new sequence is compared. Later, two highly similar full length sequences were reported from two Japanese HCV isolates (HCV-J, Kato *et al.*, 1990, HCV-BK, Takamizawa *et al.*, 1991). Comparison of nucleotide sequence of the two isolates from Japan (HCV-J & HCV-BK), showed 92% sequence homology, but only 79% homology with HCV-1 isolates from USA (Choo *et al.*, 1991). Sequences in the upstream 5' UTR are much more conserved, relatively few sequence differences exist between different HCV genotypes. This has enabled the derivation of a virus typing method based upon amplified sequences from this region (Table *6.4.1*; Simmonds *et al.*, 1993b, McOnmish *et al.*, 1993, Stuyver *et al.*, 1993a). Although existing genotypes can be recognized with this method, it is problematic to assign new genotypes on the basis of the sequence comparisons in this region alone, and it is therefore essential that comparisons of putative new genotypes, are analyzed in coding regions. Comparison of published sequences of HCV has led to the identification of a number of distinct virus types that may differ from each other by as much as 33% over the whole viral genome (Choo *et*

al., 1991, Chan *et al.*, 1992, Okamoto *et al.*,1992). This problem has compelled various laboratories investigating sequence variations among HCV isolates to propose schemes of classification and nomenclature (Okamoto *et al.*, 1992, Chan *et al.*, 1992, Simmonds *et al.*, 1993a). However, a proposal has recently been made for a unified acceptable system of nomenclature (Simmonds *et al.*, 1994). This proposal seems to be logical as the system recognises the problem of nucleotide variability among various HCV isolates, the new system proposed that nucleotide sequence homology less than 72% in the NS5 region can be classified as new type and nucleotide sequence homology of between 75-85% can be classified as subtype in a clusters of isolates (Simmonds *et al.*, 1994).

(Table *6.4.1*)   **Nomenclatures for HCV Genotyping**

Representative	Stuyver	Bukh/ Purcell	Chan/ Simmonds	Chan/ Urdea	Enamoto/ Date	Okamoto
HCV-1, H[a]	1a	1a	I	I	K-PT	I
HCVJ,HCV BK	1b	1b	1	II	K-1	II
HC-J6[a]	2a	2a	2	III	K-2a	III
HC-J8[a]	2b	2b	2	IV	K-2b	IV
E-bl, Ta[b]	3a	3a	3	IV	nc	V
Tb, Tr[b]	3b	nc	nc	IV	nc	VI
EG16, GB116, Z4[c]	4a	4a	4	nc	nc	nc
GB549, Z1[c]	4b	4b	nc	nc	nc	nc
GB809, Z6[c]	4c	4c	nc	nc	nc	nc
DK13[c]	nc	4d	nc	nc	nc	nc
SA1, PC[4]	5a	5a	5	V	nc	nc
HK1[a]	nc	6a	6	nc	nc	nc

Origin of Reported Isolates

[a] HCV-1; Choo *et al.*, 1991, HCV-J; Kato *et al.*, 1990, HCV-BK; Takamizawa *et al.*, 1991, HC-J6; Okamoto *et al.*, 1991, HC-J8; Okamoto *et al.*, 1992.

[b] Eb1; Chan *et al.*, 1992, Ta; Mori *et al.*, 1992, Tb; Mori *et al.*, 1992, Tr;

[c] EG-16; Simmonds *et al.*, 1993, GB116, 809; Stuyver *et al.*, 1993, Z1, Z4, Z6, DK13; Bukh *et al.*, 1993.

[d] SA-1; Bukh *et al.*, 1992, PC; Stuyver *et al.*, 1993

[e] HK-1; Simmonds *et al.*, 1993

Genotyping of HCV has provided an epidemiological tool for studying geographical differences in hepatitis C infection. There are clearly discernible patterns of genotype distribution found in those countries that have been studied so far. For example, both blood donors and patients with chronic hepatitis from countries in Western Europe and the USA are frequently infected with genotypes 1a, 1b, 2a and 3a, the frequencies of occurrence of each genotype, however, varies. There is a trend for relatively more frequent infection with type 1b in southern and eastern Europe. In many European countries genotype distributions vary with the age of patients, reflecting rapid changes in genotype distribution with time within a single geographical areas Simmonds *et al.*, 1993a, Bukh *et al.*, 1993, Stuyver *et al.*, 1993, McOmish *et al.*, 1994). In Japan and Taiwan and probably in some parts of China, the genotypes 1b, 2a and 2b are most frequently found. Infection with type 1a in Japan appears to be confined to haemophiliacs who have received commercial (US produced) blood products and concentrates (Kinoshita *et al.*, 1993). Type 3 is rarely found in Japan and is also infrequent in Taiwan, Hong Kong and Macau. This genotype is, however, prevalent in Singapore, and accounts for the majority of infections in Thailand and (possibly) in Bangladesh and Eastern India.

There occurs a striking change in genotype distribution between Europe and Turkey (type 1b), and countries in the Middle East and parts of North and central Africa, where infection with genotype 4 is highly prevalent (Bukh *et al.*, 1993, Simmonds *et al.*, 1993a, Stuyver *et al.*, 1993). Most of the genotypes in the Middle East and Central Africa can be confidently assigned as genotype 4 on the basis of

sequence comparison in the core, E1 and NS-5 regions (Bukh *et al.*, 1993, Simmonds *et al.*, 1993b). In Egypt as well as in the Middle East, at least three other subtypes of type 4 are identifiable, none of which correspond to subtypes of type 4 found in Zaire and Burundi (Bukh *et al.*, 1993, Stuyver *et al.*, 1993). South Africa has a lower prevalence of HCV infection, but in this region, 30-50% of anti-HCV positive blood donors in two areas (Johannesburg and Durban) are infected with type 5a, in contrast to type 4, there is relatively little sequence heterogeneity found within this genotype. The infection with this HCV variant is highly restricted geographically, being found only rarely in Europe or elsewhere (McOmish *et al.*, 1994). The 6a was originally found in Hong Kong (Bukh *et al.*, 1993, Simmonds *et al.*, 1993a, McOmish *et al.*, 1994), and has been shown to be a new major genotype by sequence comparisons in the NS5 and E1 regions. Data are yet not sufficient enough to show how extensively type 6a is distributed in south-east Asia.

(6.5.1)   Heterogeneity of HCV and Disease

The degree of sequence variability is sufficient to alter the antigenic and biological properties of members of the virus group significantly. The immuno active region of the NS-4 protein is highly variable and hence most of the epitopes are type specific (Simmonds *et al.*, 1993a). Whether changes in HCV during infection or mutation to a particular variant affect different episodes of disease is not yet established, although there is some evidence that the response of persistently infected patients to treatment with interferon may depend to some extent on the genotype of the virus (Kanai *et al.*, 1992). It has, however, been sug-

gested that sequence changes in the envelope protein may correlate with exacerbations in hepatitis (Gunji *et al.*, 1992). The differences in the rate of nucleotide sequence variation in hypervariable region (HVR) in patients with a course punctuated by exacerbations have been documented (Kurosaki *et al.*, 1993). This finding has not been consistent, and it is uncertain whether the observed genetic drift reflects inherent viral evolution, or whether the antigenic variation of E2 is driven by a cytotoxic immune response associated with hepatocyte lysis, or results from antibody selection (Kato *et al.*, 1993). There is currently a lot of interest to research into the possible differences in the course of HCV disease associated with different genotypes, this include such areas as the rate of development of cirrhosis and hepatocellular carcinoma (HCC), and whether certain genotypes are more or less likely to respond to interferon treatment. There is no substantial evidence available, mainly because there is yet no cell culture system established for HCV. However, it has been suggested that type1 HCV has an inherently greater pathogenicity in patients with cirrhosis, this may be due to the predominance of type1 infection in most patients in countries where studies have been carried out. Moreover, several clinical investigations have documented severe and progressive liver disease upon infection with each of the well characterized genotypes (1a, 1b, 2a, 2b, 3a, 4a) so far there is little evidence so far for variants of HCV that are completely non-pathogenic (Dusheiko *et al.*, 1994).

There is belief that response to interferon therapy among HCV infected patients depends significantly on the HCV genotype (Kanai *et al.*, 1992). A number of authors have since suggested that patients with type 1 infection are less sensitive to interferon treatment than patients with type 2 or 3, and are less to have

a sustained response to therapy (Kanai *et al.*, 1992, Dusheiko *et al.*, 1994). The mechanisms by which different genotypes might differ in responsiveness to treatment remains obscure, because most patients are sometimes infected by two or more viruses. Especially, materials derived from contaminated plasma pools, such as factor VIII preparations, may contain a large variety of HCV sequences.

(6.6.1)   Diagnostic Tests

HCV probably circulates in the serum at a concentration of between 10^2 and 5×10^7 particles per millilitre (Shindo *et al.*, 1994), and so far it has proved impossible to detect viral antigens by conventional methods. Therefore, the detection of antibodies to HCV has become important as an indication of past or present infection. A specific diagnostic tool for circulating HCV antibody (anti-HCV) was developed from purified viral polypeptide derived from recombinant yeast expressing a small fragment of the HCV genome (Kuo *et al.*, 1989). The development of serological assays for antibodies to HCV have transformed the diagnosis of NANB hepatitis from one merely based on exclusion into that of a specific disease hepatitis C. Most of the early epidemiological and diagnostic studies were carried out using the first generation enzyme-linked immunosorbent assay (ELISA), which was based on antibody capture. This assay detected antibodies to C100-3, a 360 amino acid fusion polypeptide representing part of the NS4 region of the HCV genome, and expressed in yeast fused with superoxide dismutase (Kuo *et al.*, 1989). The presence of antibody to c100-3 proved a good marker for infection with HCV in both post-transfusion and sporadic NANB hepatitis (Hopf *et al.*, 1990). Seropositivity was also shown to be associated with

chronic infections and correlated with infectivity in blood donations (Esteban *et al.*, 1991). There were, however, problems associated with the first generation ELISA, especially when screening low-risk groups, such as blood donors, in whom there was a substantial risk of false positivity (McFarlane *et al.*, 1990). This was possible because of non-specific binding of IgG or immune complexes to the solid phase (McFarlane *et al.*, 1990, Aceti *et al.*, 1990). The assay also lacked sensitivity in that anti-c100-3 was not readily detectable in the sera of all individuals with past or present HCV infection or during the early clinical phase of the illness and can take up to 1 year after elevation of transaminases to become detectable (Alter *et al.*, 1989a). Anti-c100-3 also disappears fairly rapidly after the resolution of HCV infection but may persist throughout chronic disease (Tanaka *et al.*, 1991).

The shortcomings observed in the first-generation ELISA assays were responsible for the development of the second-generation tests, which incorporated two extra HCV-derived recombinant proteins, which increased the sensitivity of the assay (McHutchison *et al.*, 1992). The first of these proteins is the (C22-3) which represents the majority of the 22-kDa nucleocapsid protein, the amino terminus of which contains an immunodominant epitope (Nasoff *et al.*, 1991). Antibodies to C22-3 occurred earlier and more frequently than those to c100-3 during the course of HCV infection (Katayama *et al.*, 1992). The second protein is derived from either the NS3 and NS4 regions (C200), or a smaller fragment of it (C33c) from NS3 alone. The C200 protein is the product of a large cloned fragments, combining both the C33c and c100-3 regions. Antibodies to C200 are more common than those to c100-3, presumably as a result of reactivity to epitopes in the C33c fragment (McHutchison *et al.*, 1992). Overall, the second-generation

ELISA shows enhanced sensitivity when compared with the first generation. The format of the second-generation assay is the same as the first-generation assay and therefore has the same risk of false positives.

As a result of false positivity associated with the ELISA assay, supplementary methods have been developed to further test the results obtained by ELISA. These supplementary methods are designed in such a way, that they use a different assay format and utilize different antigens. The most widely used method is the recombinant immunoblot assay (RIBA, Chiron Corporation, CA, USA), in which recombinant antigens of HCV are coated in bands on nitrocellulose strips, which are then incubated with serum at a dilution of 1:50. Individual reactivities are detected with peroxidase-labelled anti-IgG and 4-chloro-1-naphthol as substrate. The first generation RIBA used c100-3 and 5-1-1, fused to human superoxide dimustase (SOD) antigens to coat cellulose strips. The c100-3 and 5-1-1 were produced in E. coli, while the SOD was produced in yeast. The second-generation RIBA test (RIBA 4) is an advancement over the first generation (Marcellin et al., 1991), it utilizes altogether five recombinant antigens, inclusive of the three already present in the first generation RIBA. The five recombinant antigens are 5-1-1, c100-3, c33c C22-3 and SOD. 5-1-1 and c33c are produced in E. coli, whereas c100-3, c22-2 and SOD are produced in yeast. Samples are regarded RIBA positive if antibodies to two or more of the HCV proteins are present and indeterminate if only one antibody is found. The value of RIBA in excluding false positive result has been extensively demonstrated, and there is a correlation between RIBA positivity and viraemia (Ebeling et al., 1990).

Neutralization assays have been used to confirm positive results obtained

by ELISA. This technique employs the initial immunosorbent assay with and without the addition of another recombinant HCV antigen whose only difference is that it has been produced differently. Confirmation of positivity is justified when the initial result is neutralized by the second antigen, proving specificity for the viral antigen.

The serological assay have proved inadequate to confirm HCV infection, because of false positivity, and false negativity, an alternative approach for confirmation of HCV infection is at present provided by PCR-based methods for detection of viral RNA in serum (Okamoto *et al.*, 1990, Garson *et al.*, 1990, Kato *et al.*, 1990). This procedure begins with extraction of HCV RNA from a small volume of serum or plasma, under highly denaturation conditions that are likely to inactivate circulating RNases. Direct detection of HCV RNA by conventional nucleic acid hybridization techniques is not always possible, because of the low virus numbers. Complementary DNA is synthesized from the RNA using reverse transcriptase (RT) and a specific oligo-nucleotide primer. The cDNA product is then used as template for amplification of a double-stranded DNA by a conventional PCR, using pairs of HCV-specific primers, which are complementary to opposing strands of the viral cDNA. These primers are usually positioned several hundred bases distant from each other within the region of interest in the linear map of the HCV genome. The first round PCR is usually between 30 and 35 amplification cycles, a small fraction of the amplification product is removed and used for second round PCR with a second set of oligonucleotide primers that are nested within and do not overlap with those primers used for the first round PCR. RT-PCR has been very useful in confirming the diagnosis of HCV infection

in a large number of clinical studies (Cristiano *et al.*, 1991). However, PCR has

the limitation of not been quantitative, and incapable of providing an estimate of

titre of virus present without additional modifications, when dilutions of serum are

tested by RT-PCR, estimates of the titre of RNA correlate well with infectious

determined by chimpanzee inoculation (Shindo *et al.*, 1994).

A new method, called branched-chain DNA hybridization assay, has been

developed to quantitatively estimate HCV RNA directly from the serum specimen.

The assay was designed to detect the genome of the HCV in serum specimen from

infected patients (Urdea *et al.*, 1991). The technique is based on the specific

hybridization of synthetic oligonucleotides to the 5'UTR and core genes of HCV

RNA, which allow the RNA to be captured onto a solid surface. Synthetic

branched DNA molecules (bDNA) and multiple copies of an alkaline phosphatase-

linked probe are hybridized to the immobilized complex. Detection is achieved by

incubating the complex with a chemiluminescent substrate and measuring the light

emission generated by the bound alkaline phosphatase. Application of all these

assays to clinical practice has finally provided the best evidence that HCV is the

major aetiological agent of post transfusion NANB hepatitis around the world

(Esteban *et al.*, 1989, Katayama *et al.*, 1992), as well as being a major cause of

sporadic, and community-acquired NANBH (Aach *et al.*, 1991).

(6.7.1)   **Transmission of HCV**

There is every reason to believe that the mechanism of transmission of

HCV should be similar to that of HBV, however, there are some significant

differences. Available data suggest that there are many possible routes by which

HCV infection can occurred or may likely occur. Among these routes are;

(a)   Intravenous drug users

(b)   Recipients of blood and blood derivatives

(c)   Recipients of organ and tissue transplants

(d)   Sporadic or community acquired

(e)   Sexual transmission

(g)   Close contacts of individuals infected by one of the other routes.

(a)   **Transmission by intravenous drug use (IVDU)**

The problem of syringe or needle sharing among the IVDU is recognized as one of the potential source of parenterally transmission of HCV. The prevalence of anti-HCV in intravenous drug users in developed counties ranges from 48% to 80% (Esteban *et al.*, 1989). A higher prevalence of 55% was reported in intravenous drug users in USA within 6 weeks of the onset of illness, however this figure increased tremendous to 94% when these individuals were followed up for a period of 4 years. There is no evidence yet, as to whether this is also the case in other countries, although it has been reported that sero conversion may not likely occur for at least over a year from the point of exposure to HCV (Alter *et al.*, 1989a).

(*b*)   **Transmission by blood and blood products**

Transfusion of infected blood and blood products is particularly a very efficient route of transmission of HCV, and of other blood borne infectious agents. Evidence abound that approximately 85-90% of post transfusion non-A and non-B

hepatitis (PT-NANBH) is due to HCV infection (Alter *et al.*, 1989a), however, only a negligible percentage (about 0.5-10%) of unscreened blood (red cell or single-donor product) transfusions give rise to PT-NANBH (van der Poel *et al.*, 1989, Aach *et al.*, 1991). Blood transfusion remained as one of the most potential routes of transmission of HCV, but yet, it cannot account for the prevalence in the population, because most individuals never receive a transfusion during their life time. However, transfusion can be a significant source in certain groups of patient, such as haemophiliacs.

(c)   **Transmission by organ and tissue transplantation**

That HCV may be transmitted through organ and tissue transplantation has now been documented. Post-transplantation liver disease still remained an important cause of morbidity and mortality, especially in renal transplant recipients (Ponz *et al.*, 1991), in whom NANBH has been considered to be a significant cause. Survey of HCV infection among post-transplantation liver disease, confirmed that organ transplantation could transmit HCV (Poterucha *et al.*,1992). A case of transmission of HCV by a frozen bone graft has been reported (Locasciulli *et al.*, 1991).

(d)   **Transmission by undefined routes**

It has not been possible to identify the precise route of infection in at least 40-50% of cases of HCV infection (Alter *et al.*, 1991b). In some infected individuals with apparently no defined risks, sporadic or community-acquired infection can occur, raising the possibility of less obvious routes of infection.

(e)   **Sexual transmission**

As observed in HBV, sexual transmission (especially in homosexual) would seems a likely route of spread of HCV. The available data suggested that HCV can be transmitted sexually to a certain extent, but the route is uncertain, as the efficiency of transmission appears to be very low (van Doornum *et al.*, 1991). The sexual transmission of HCV may be dependent on the susceptibility of different populations to HCV infection, level of infectivity in individuals, strains of HCV, frequency of sexual contact, and the extent of condom usage (Tedder *et al.*, 1991). Comparatively, sexual route of transmission in HCV is not as efficient as that of HBV. Transmission patterns also differ to a great extent, as seen in the case of homosexual men, the transmission rates of HCV are quite low in this group, unlike the very high rates observed in HBV (Melbye *et al.*, 1990).

(f)   **Mother to Child Transmission**

Current studies on the routes of transmission of HCV has shown that a limited vertical transmission do occur but not as frequently seen with HBV or HIV infected mothers (Thaler *et al.*, 1991). It is not certain whether transmission of HCV from infected mother to child occurs in utero by infection via placenta during the development of the fetus, or during birth by direct infection with virus present in the cervical secretions or in the perinatal period breast milk.

(g)   **Transmission to contacts of individuals infected by one of the other routes**

(i)   **Occupational contact**

Some individuals are at high risk of infection due to the nature of their occupation, because of their exposure to a number of infectious agents, such as infectious body fluids and close contact with infected individuals or through needlestick accidents which is common among medical staff. Needlestick associated HCV infection have been reported in nursing and other medical staff (Kiyosawa *et al.*, 1991). The probability that HCV may be transmitted through saliva have been reported (Dusheiko *et al.*, 1990). This has been a point of concern in dental practice, where the degree of exposure of dental staff to saliva and blood of patients is high. However, the prevalence of HCV among dentists and dental surgeons varies from region to region (Herbert *et al.*, 1992).

(6.8.1)   **Biology of Hepatitis C**

That chronic post-transfusion hepatitis leads to an increased risk of liver cancer, has long been recognized even before the etiological agent was identified (Resnick *et al.*, 1983). HCV has now been recognized as the main cause of post-transfusion hepatitis (Choo *et al.*, 1989). Chronic type C hepatitis is a potential disease that can lead to cirrhosis and hepatocellular carcinoma (Kiyosawa *et al.*, 1990, Simonetti *et al.*, 1992). In other infected carriers, the disease remains relatively benign for a long period of time, with little mortality over 20 years (Farci *et al.*, 1991). There is currently no evidence to indicate that HCV replication or gene expression interferes specifically with the host genome. The HCV is believed to replicate in the cytoplasm by reverse transcription and viral RNA to DNA does not occur, and hence no integration of viral genomic sequence into the

host chromosomal DNA is possible. Surprisingly, HCV still causes chronic infection in at least 50% of cases, which then predisposes to the sequential development of cirrhosis and primary liver cancer (Kiyosawa *et al.*, 1990). Epidemiological surveys have revealed the presence of HCV structural and non-structural proteins in 15-80% of patients with HCC depending upon the patients population studied (Blum, 1994). Chronic hepatitis C is typically characterized by fluctuations in serum amino-transferase concentrations, which may be normal for months or years, and then unpredictably increase many fold. Whether changes in HCV during infection or mutation to a particular variant affect different episodes of the disease is also yet uncertain. The duration and the pattern of the antibody response vary according to the type of the antibody analyzed and, in some cases, are strictly dependent upon the outcome of HCV infection. The antibodies to certain non-structural proteins, such as anti-c100 disappear after an extremely variable period that may range from 1 week to several months in most patients with transient HCV viraemia (Abe *et al.*, 1992). The virological and serological profiles of chronic HCV infection differ from those seen in acute hepatitis. In chronic HCV infection, HCV viraemia usually follows two main patterns: persistent or intermittent (Abe *et al.*, 1992).

The contribution of NANB hepatitis to the aetiology of fulminant hepatitis has been the object of intensive investigation. With the discovery of HCV, it has become possible to investigate more accurately the contribution of NANB hepatitis to fulminant hepatitis. The evidence thus far accumulated suggests that the association between HCV and fulminant hepatitis differs markedly according to the geographical area considered. In Japan, HCV infection as determined by antibody

seropositivity or by the presence of viraemia, was documented in a high proportion of patients, ranging from 50% to more than 90% (Yanagi *et al.*, 1991). This was in contrast to studies conducted in USA and in Europe where a very low prevalence of HCV infection in fulminant hepatitis has been reported (Wright *et al.*, 1991). These discrepancies are likely to reflect geographical differences in the epidemiology of HCV. Alternatively, HCV may go unrecognized because factors, such as the timing of sampling or improper handling and storage of the clinical specimens, may result in damage to HCV virion with possible loss of detectable HCV RNA.

Since the development of diagnostic assays that detect antibodies to HCV structural and non-structural proteins, there has been an intensive interest in the possible role of chronic HCV infection in the pathogenesis of hepatocellular carcinoma (HCC). Epidemiological surveys have revealed the presence of such antibodies in 15-80% of patients with HCC depending upon patient population studied (Blum, 1994). The percentage of patients with HCC infected by HCV vary from population to population and this depend in part on the general population to the virus and the number of HCC cases attributable to chronic HBV infection. For example, HCV appears to be a major cause of HCC in Japan, Italy and Spain, whereas it seems to play a less important role in South Africa and Taiwan (Kiyosawa *et al.*, 1990, Simonetti *et al.*, 1992). Thus far there is no evidence to suggest that HCV has a direct role in the molecular pathogenesis of HCC but rather is associated with this disease by its ability to produce chronic liver injury, followed by degeneration and the eventual development of cirrhosis.

CHAPTER 7

MATERIALS AND METHODS

(7.1.1)   **Serum samples**

(a)   The **200** sera used for HBV screening (section 3.1.1.A), were used in this

work.

(b)   **100** serum samples from children of pre-school age, which were used for

the HBV work also were used for HCV screening (see section 3.1.1. B).

(7.1.2)   **SEROLOGICAL SURVEY**

(7.1.3)   **Anti-Hepatitis C (HCV) Test (Murex diagnostics)**

180 µl of sample diluent was added into each microwell, then 20 µl of

negative control in triplicate were added to microwells A1 to C1, and the positive

control was added in duplicate to microwells D1 and E1, followed by addition of

serum samples under test from F1 upwards. The microtitre plate was covered with

a lid and incubated for 1hr at 37°C under humid conditions. At the end of the

incubation, the plate was washed five times by soaking the wells with wash fluid

followed by aspiration, leaving enough time for the well to be soaked for at least

30 sec before aspiration. Then, 100 µl of conjugate was added to each well, the

plate was covered with the lid and incubated for another 30 min at 37°C under

humid conditions. The plate was washed as before and 100 µl of substrate added

into each well, the plate was covered and incubated for exactly 30 min under the

same conditions as before. 50 µl of stop solution was added to each well. The

absorbency of each well was read at 450 nm (A_{450}) in a microwell plate reader.

The cut-off point value was decided by adding 0.60 to the mean (A_{450}) of the

nenegative control replicates. Any sample giving absorbency less than cut-off value was considered negative. Samples that were positive in the first round ELISA, were retested to eliminate false positives.

(7.1.4) Recombinant Immunoblotting Assay (RIBA; Chiron Corporation)

Strips were placed in their respective tubes, one tube per test sample, and one each for positive and negative controls. 1 ml of working specimen diluent was added to each tube, and then 20 µl of test sample and controls were added to their appropriate tubes. The tubes were incubated with shaking for 4 hr at room temperature. At the end of the incubation, the tubes were aspirated and washed twice with 1 ml of wash buffer. The strips were then transferred into a beaker and washed with 60 ml of wash buffer, this procedure was repeated thrice. Then 1 ml of conjugate per strip was added and the beaker agitated on a rotary shaker for 30 min at room temperature. The conjugate was decanted, and the strips were washed thrice, each time with 60 ml wash buffer. Then 1 ml of working substrate per strip was added, and incubated with agitation for 15 min at room temperature. The working substrate was decanted and strips washed twice each time with 60 ml distilled water. The strips were transferred onto Whatman paper and excess water dried off. Strips were kept in darkroom for 20 min at room temperature, and result was interpreted within 1 hr.

(7.2.1)   **HCV RNA SCREENING**

(7.2.2)   **HCV RNA Extraction**

RNA was extracted from 100 μl of anti-HCV positive serum using the guanidine isothiocyanate method (RNaid Kit, BIO 101, Vista CA, USA). To 100 μl of serum was added an equal volume of guanidine isothiocyante, 11.2 μl 5% N-laurosarcosine, 1μl β-mercarptoethanol, 2 μl yeast RNA and 20 μl 3M sodium acetate (pH 5.2). The mixture was vortexed and 200 μl phenol/chloroform added, vortexed and incubated on ice for 15 min. It was centrifuged in cold for 15 min, and the aqueous phase was collected into a new Eppendorf tube. An equal volume of RNA binding salt was added, vortexed, then 5 μl RNA binding matrix was added and incubated at room temperature for 5 min. It was centrifuged at 4°C for 1 min at 13,000 x g, the supernatant removed and discarded, pellet was washed twice with 400 μl RNA wash solution. The pellet was resuspended in 30 μl DEPC treated water and incubated at 55°C for 5 min, it was finally centrifuged for 1 min at 13,000 x g and the supernatant collected into a clean Eppendorf tube.

(7.3.1)   **Reverse Transcriptase PCR (RT-PCR)**

(7.3.2)   **5' Untranslated Region (5' UTR)**

10 μl of RNA extracted from 100 μl serum was used for the first round RT-PCR in a final volume of 100 μl containing 200 μM of each dNTP (dGTP, dATP, dCTP and dTTP) with 50 picomoles of two external primers 209 (Garson *et al.*, 1991), and 939 (Okamoto *et al.*, 1990; primer sequence, polarity and binding sites shown in Table 7.3.3), 2 units of Taq polymerase (Pekin-Elmer Cetus, Norwalk,

CT), 1 μl reverse transcriptase enzyme (Gibco superscript T^M). The reaction mixture was overlaid with 100 μl of white mineral oil and incubated for 1 hr at 42°C. The reaction was transferred into a thermal cycler (Pekin-Elmer Cetus), programmed for 50 cycles consisting of denaturation (1 min at 94°C), annealing (1 min at 50°C) and extension (2.5 min at 72°C), The last cycle reaction at 72°C was allowed to continue for 9.9 minutes to ensure complete DNA extension. Five μl of the reaction product was used in the second round nested PCR, with two internal primers (Table 7.3.3). The same conditions and temperature were used in the second round PCR with the exception that, the reaction was for 30 cycles. Positive and negative sera were included in the extraction step, and were included along with double distilled water as controls in both rounds of amplification. 10μl from the PCR product was analyzed by 2% agarose gel electrophoresis at 150 volts for 2 hr 30 min, stained with 0.5μg/ml ethidium bromide, and visualized on a u.v. light box.

(7.3.3)   NS 5 Region

Ten μl of RNA extracted from 100 μl of anti-HCV positive serum was used for RT-PCR. The same method used for the reverse transcription of the 5' UTR in section 7.2.4, was followed. The first round PCR was carried out using two external primers 1203 (+ve sense) and 1204 (-ve sense; see table 7.3.3) thought to be highly conserved amongst different isolates of HCV sequenced, primer 1203(Mellor *et al.*, 1995), binds the HCV RNA at position -7903, and primer 1204 binds at the position 8309 (Mellor *et al.*, 1995). The thermocyler (Perkin Elmer Cetus) was programmed for 60 cycles each consisting of a denaturation step (1

Table 7.3.3 Sequence and sources of primers used for PCR amplifications.

Primer	Sequence (5' to 3')	Position[#]	Sources
5UTR			
209 (-)*	ATACTCGAGGTGCACGGTCTACGAGACCT	8	Garson *et al.*, 1990
939 (+)	CTGTGAGGAACTACTGTCTT	297	Okamoto *et al.*, 1990
211 (-)	CACTCTCGAGCACCCTATCAGGCAGT	29	Garson *et al.*, 1990
940 (+)	TTCACGCAGAAAGCGTCTAG	278	Okamoto *et al.*, 1990
NS5 Region			
1204 (-)	GGAGGGGCGGAATACCTGGTCATAGCCTCCGTGAA	8309	Mellor *et al.*, 1995
1203 (+)	ATGGGGTTCTCGTATGATACCCGCTGCTTTGACTC	7903	Mellor *et al.*, 1995
123 (-)	GCTCTCAGGTTCCGCTCGTCCTCC	8250	Mellor *et al.*, 1995
122 (+)	CTCMACMGTCACKGAGARYGAYAT	7935	Mellor *et al.*, 1995
518 (+)	CTCAACSGTCACSGAGARGGCAT	7935	Mellor *et al.*, 1995
Core Region			
CPS14 (+)	GCGGAATTCGTGGTACTGCCTGATAGGGTG	67	Xu *et al.*, 1994
CPA14 (-)	GGCGGATCCGGAAGATAGAGAAAGAGCAACC	511	Xu *et al.*, 1994
CPS15(+)	GGCGAATTCTCTCGTAGACCGTGCACCATG	26	Xu *et al.*, 1994
CPA15 (-)	GCTAGATCTTCCTGTTGCATAGTTCACGCCG	480	Xu *et al.*, 1994

*(-) negative or (+) positive polarity   [#] According to Choo *et.al.*, (1991)

min at 94°C), annealing step (1 min 50°C), and extension step (2.5 min 72°C). Other conditions remained same as in the section 7.2.4. Various combinations of internal primers were used to optimized the PCR reaction, the first combination was primers 122 and 123 (Mellor *et al.*, 1995). Primer 122 binds HCV RNA at position 7935 and 123 binds at position 8250. The second combination was 518 and 123 (Mellor *et al.*, 1995). Primer 518 was a modified form of primer 122, hence it binds at the same position as 122. The third primer combination was, 517 (Mellor *et al.*, 1995) and 123. Primer 517 was also a modified form of primer 122, and binds at the same position, 7935. The fourth primer combination was 518 and 1204. Primer 1204 is a modified form of primer 123. Primer sequences, polarity and binding position are shown in table 7.3.3.

5 μl of the first PCR product was used in a 50 μl reaction. Other conditions are as in the section 7.2.4 with the exception that the amplification was for 30 cycles, consisting of a denaturation step (95°C 36 sec), annealing step (45°C 1.5 min) and an extension step (68°C 3 min). 10μl of the PCR product was resolved in 2% agarose gel electrophoresis, 1 x TBE at 150 volts for 2 hr 30 min, stained with 0.5μg/ml ethidium bromide, and visualized on a u.v. light box.

(7.3.4) **The Core Region**

Ten μl of the HCV RNA extracted from anti-HCV positive sera was used for RT-PCR of the core region (C). Four primers were selected to amplify a fragment of the C region conserved in published HCV sequences. The two external primers were CPS14 (+ve sense polarity) which binds at nucleotide position -67 and CPA14 (-ve sense polarity) which binds at nucleotide position 511. The two

internal primers were CPS15 (+ve sense polarity) which binds at nucleotide position -26 and CPA15 (-ve sense polarity) which binds at position 480 (Xu *et al.*, 1994). Reverse transcription was carried out as in the section 7.2.4. First round PCR was carried out in a thermocycler programmed for 60 cycles consisting of denaturation step (95°C 1 min), annealing step (42°C 1 min), and extension step (72°C 2.5 min). Other procedures were as those in section 7.2.4 and 7.2.3. Products were analyzed on 1.5% agarose gel electrophoresis in 1% TBE, at 150 volts for 2hr 30 min. Stained and visualized as above.

(7.3.5)　　　　　　　　**Purification Of The PCR Product**

PCR product were purified using a QIAEX DNA extraction kit (QIAGEN GMBH, Hilden, Germany). The mineral oil on top of the reation was completely removed. Products with a single visible band, were purified directly by adding 150 μl buffer QX1 (3:1), and 20 μl of QIAEX II and incubated at room temperature for 10 min, with intermittent vortexing. The mixture was centrifuged for 30 sec, and the supernatant removed, and discarded. The pellet was washed twice with 500 μl of buffer PE, and pelleted by centrifugation for 30 sec. It was air-dried for 15-20 min, suspended by vortexing in 20 μl of 10mM Tris-HCl, pH8.5, and kept at room temperature for 5 min, then was centrifuged for 30 sec, and the supernatant transferred into a clean Eppendorf tube.

Those products with two or more bands were fragmented on 2% low melting point agarors. The band of interest was excised from the gel with a clean sharp scalpel, into a clean Eppendorf tube. The tube was weighed and buffer QX1 three times weight of the gel was added, then 30 μl of QIAEX II was added. It

was incubated at 50°C for 10 min room. All the remaining procedures were as above.

(7.4.1)   **Cloning of the P.C.R. Product**

The TA cloning kit, version 2.3 (Invitrogen, San Diago, CA USA) was used for cloning the purified PCR product. 2 μl of the purified PCR product was ligated into PCR™II (vector) in a 10μl reaction, containing 2 μl of the vector (25 ng/μl pCR™ II), 1 μl 10 x ligation buffer, 4 μl sterile double distilled water, and 1 μl T4 DNA Ligase. The reaction was incubated at 14°C overnight. The next day, it was briefly spun down and kept on ice. 2μl of the ligated cell/vector was used to transform 50 μl competent cells (INV α F'). 2 μl of ice cold 0.5M β-mercaptoethanol was added into a vial of competent cell on ice, mixed properly, and 2 μl of each ligation reaction added. This was mixed gently, without pipetting. The reaction was incubated on ice for 30 min, and then heat shocked at 42°C for 30 sec. It was placed on ice for 2 min, and 450 μl of prewarmed SOC medium added, it was incubated at 37°C for exactly 1 hr at 225 rpm in a shaker. The vials were returned onto ice, and different volumes (50 μl, 100 μl, 150 μl) of the transformed cells were added into 4 ml of top agar containing ampicillin (50μg/ml) and X-gal (40mg/ml). This was spread on LB agar plate containing ampicillin (50μg/ml). The plates were incubated at 37°C overnight.

(7.5.1)   **Plasmid Mini Preparation (QIAGEN)**

The qiagen kit (QIAGEN GMBH, Hilden, Germany) plasmid miniprep kit was used. A single white colony was picked with a sterile Eppendorf tip and

grown in LB medium containing 50µg/ml ampicillin overnight. 150 µl of the overnight culture was spin for 5 min in a microcentrifuge. The bacterial cells pellet were re-suspended in 250 µl of buffer P1, vortexed vigorously and 250 µl buffer P2 added. Each was inverted to mix and incubated at room temperature for 5 min. Then 350 µl ice cold buffer N3 was added, gently mixed by inversion, then incubated on ice for 5 min. It was centrifuged for 10 min, the supernatant was applied into a QIAprep-spin column and centrifuged for 1 min. The spin column was washed with 0.5 ml buffer PB, centrifuged for 1 min, and washed with 0.75 ml of buffer PE. The column was drained by centrifugation twice, each time for 1 min, and the Plasmid DNA was eluted with 80 µl 10 mM TE, pH 8.5 by spinning for 1 min at 13,000 x g. 5 µl of the plasmid minipreparation was digested in a 10 µl reaction containg 0.5µl (6 units) *EcoRI*, 1 µl react 3 [50mM Tris (pH 8.0), 10mM $MgCl_2$, 100mM NaCl] and 3.5 µl of sterile double distilled water with incubation at 37°C for 2hr 30 min. The digested DNA was analyzed by 1.5% agarose gel electrophoresis at 150 volts for 2hr, stained with 0.5µg/ml and visualised on a u.v. light box.

(*7.6.1*)   **PLASMID DNA SEQUENCING**

20 µl of plasmid DNA was relaxed in 1M NaOH and 10 mM EDTA (pH 8.0), kept at room temperature for 30 min. The relaxed plasmid DNA was separated by passing through sepharose CL6B column and kept on ice.

Annealing Reaction

8.5 µl of the relaxed plasmid DNA was annealed to 0.5pg of either reverse or forward sense universal primer. Incubated at 37°C for 30 min. The remaining sequencing procedure and analysis were same as in the section 3.5.1.

Reading of the Sequence

At least six clones were sequenced from each donor in each direction. The sequence of clones from each donors were compared sequence each another.A single consensus file representing the sequence of HCV from each donor were obtained and compared with the HCV sequences in the data base using the Blast programme(Altschul *et al.*, 1990).

CHAPTER 8

(8.1)   **RESULTS**

(8.1.1)   **Survey of Adult Blood Donors for anti-HCV**

200 adult blood donors from 5 transfusion centres (part of serum samples

used in HBV section 3.1.1) in Nigeria, were screened for antibody against hepatitis

C virus (anti-HCV), with a second generation ELISA (Murex U.K). **16** (8 %) were

reactive.

The anti-HCV positive sera were tested further with the second generation

Recombinant Immunosorbent Assay (RIBA; Chiron, CA, USA), **4** (25%) were

positive and **2** (12.5%) were indeterminate. RIBA results are presented in figure

8.1. 1. The summary of the results is presented in table 8.1.1

(Table *8.1.1*) Results of the RIBA test for the 16 anti-HCV Positive Adult Donors

		RIBA-2				
Strip	Sample	5-1-1	c100-3	c33c	c22-3	Interpretation
01	0020	-	-	-	-	-
02	145	-	-	-	-	-
03	041	-	-	-	-	-
04	230	-	-	-	-	-
05	280A	-	-	-	-	-
06	091	-	-	-	-	-
07	274	-	-	-	-	-
08	089	-	-	+	+	+
11	323	-	-	+	-	Indeterminate
12	155	-	-	-	-	-
13	0202	+	+	+	+	+
14	329	-	-	+	-	Indeterminate
17	281	+	+	+	+	+
18	231	-	-	-	-	-
19	229	-	-	-	-	-
30	116	+	+	+	+	+

Four donors, samples 089, 116, 0202, and 281 (strip nos 08, 13, 17, & 30) were

RIBA positive and RT-PCR positive, two samples 323 and 329 (strip nos 11 & 14)

were indeterminate, sample 230 (strip no 04) was RIBA negative but RT-PCR

positive.

Fig. 8.1.1: RIBA-2 test of 16 ELISA positive, 3 ELISA negative donors and 5 children that were positive in the first round ELISA*. Lanes 01 to 08, 11 to 14 and 17 to 19 and 30 wereanti-HCV positive samples from adult donors, lanes 15, 16 and 25 were anti-HCV negative donors while lanes 20 to 24 were samples from children of pre-school age that were positive in the first round ELISA. Lane 09 = positive control, Lane 10 = negative control. 4 were samples positive (Lane 08, 13, 17, 30, samples 089, 202, 281 and 116), Two were indeterminate (Lane 11 and 14, samples 323 and 329). Others including those of the children were all negative. * These samples were only positive in the first round ELISA, but were negative in the second round ELISA

Recombinant antigen band pattern (RIBA-2)
Band 1 = Strip high control level, Band 2 = 5-1-1, Band 3 = c100-3, Band 4 = c33c, Band 5 = c22-3, Band 6 = SOD, Band 7 = Strip low control level I .

(8.1.2)   **Survey of Pre-School Age Children for anti-HCV**

One hundred pre-school children, attending the paediatric unit of the University College Hospital (U.C.H.), Ibadan (same children as in section 3.1.1.B), were screened for anti-HCV (with the same kit used in section 8.1.2). Five (5%) were positive in the first round ELISA, but none was in the second round ELISA. RIBA test was carried out to test further the 5 sera that were positive in the first round ELISA, however, none was RIBA positive.

(8.1.3)   **SEQUENCING ANALYSIS**

(8.1.4)   **5 ' Untranslated Region (UTR)**

Five sera (**089, 281, 230, 116** and **0202**) of the 16 anti-HCV positive sera, were positive by nested reverse transcriptase polymerase chain reaction (RT-PCR) in the 5' UTR region. The results of the PCR is shown in fig 8.1.4a. One of the these positive sera (donor 230) was RIBA negative. The PCR products were purified and cloned into a bacterial vector, and at least 6 clones from each donor were sequenced. The sequence of clones from each donor were compared with each another and a single file representing the consensus sequences, of all the sequenced clones from each donor were obtained, and were independently compared with the HCV sequence in the EMBL data base using Blast.

Database searching showed that the HCV sequence from donor 089 has 91% nucleotide sequence homology with HC4N3 and HP5AAG both were HCV genotype 4 (data base accession nos X78865 and X58952) and it has 90% nucleotide sequence homology with HPC5AAQ (data base accession no. X58953), which was also a genotype 4. The clones from donor 281 have 96% nucleotide sequence homology with HPC4RNAA1, isolated from the USA, a 95% homology with HPCBCS5, isolated from Britain, 92% with HPCRNAD3 and 91.5% with HPCRND6, both German isolates, 90% homology with HPCRNADK7 isolated from Denmark and 90% similarity with HPCRNADR4 isolated from Dominican Republic; all these isolates are classified as genotype 1, based on the nucleotide sequence of the 5' UTR. (Data base accession nos. for these isolates are M58406, L34386, M84842, M84241, M84839, and M84859). The clones from donor 230 also showed sequence homology with HCV genotype 1. The EMBL blast search

of the sequence showed a 98% homology with HPC4NRAA1, a genotype 1 isolated from the USA, and 94% homology with HPCBCS5, a predicted genotype 1a isolate from the UK, 94% homology with QC14 a genotype 1a isolated from Canada, 93% sequence homology with HPCRND6, a genotype 1a isolate from Germany. This isolate can be classified as genotype 1a, based on the sequence from this region. The clones from donor 116, showed 98% nucleotide sequence homology with HPCRNAZ8 a predicted genotype 4f HCV isolate, 97% nucleotide sequence homology with HPCRNAZ5 a predicted 4e genotype, both isolates were from Zambia. It has 97% nucleotide sequence homology with HPC4GB541, a 4d predicted HCV genotype, (Data base accession nos, M84829, M84828, L29600). It can then be predicted, based on the high degree of nucleotide sequence homology with genotype 4 observed in the 5' UTR that donor 116 was infected with genotype 4. The clones from donor 202 showed a 98% sequence homology with HPCGB116U and GHPC4GB487, both were predicted HCV genotype 4c, isolated from Gabon. It has 98% nucleotide sequence homology with HPCC736UT a 4e predicted HCV isolate from Cameroon, it shared also 96% nucleotide sequence homology with HPCRNAZ1, a genotype 4a isolate from Zambia (data base accession nos L29603, L29616, L29591 and M84845). The data base nucleotide sequence comparison of all these isolates in the 5' UTR predicted three donors (089, 116, 202) were likely infected with HCV genotype 4, two donors (281 and 230) were infected with a genotype 1, since both isolates showed a very high nucleotide sequence homology (above 90%) with genotype 1. Alignment of 5' UTR consensus nucleotide sequence from each donor with HCV prototype 1 is shown in fig 8.1.4b. An existing HCV genotype can readily be identified with sequence analysis of 5' UTR, but this region alone is, however, not sufficient for

classification or identification of a new HCV genotype. In order to confirm

whether these present HCV isolates from Nigeria might represent a new subtypes,

further analysis of two putative coding regions (NS5 and core regions) were

carried out. These two regions were amplified and sequenced. Comparatively,

nucleotide sequence analysis of the NS5 and core regions have proved to be more

reliable in the classification of HCV into various genotypes compared with the 5'

UTR, because of the great diversity showed by these two regions (Okamoto *et al.*,

1992, Simmonds *et al* ., 1993a)
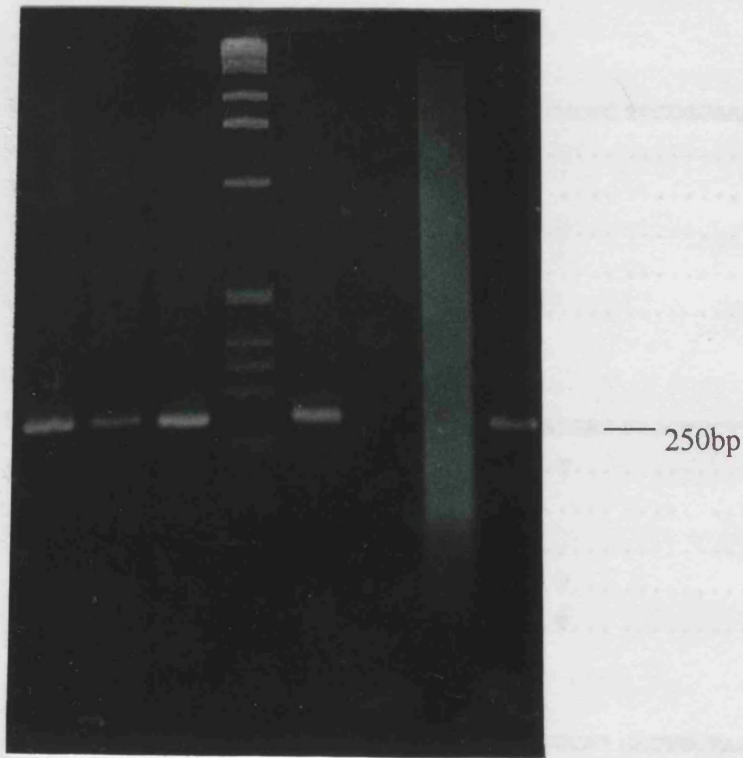
Figure 8.1.4a: Results of Polymerase Chain Reaction of HCV 5' Untranslated Region. Lanes 1, 2, 3 & 5 are samples under test (089, 281, 116, and 0202), Lane 4 = 1Kb Ladder marker*; Lane 6 = sterile double distilled water, 7 = Negative control, 8 = Positive Control. *1Kb ladder size in the appendix.

(Fig.*8.1.4b*) Aligment of 5'UTR consensus nucleotide sequence from 5 donors

(089, 202, 230, 116 & 281) with HCV genotype 1

```
            -250                                                          -190
HCV-1       GGCGTTAGTA TGAGTGTCGT GCAGCCTCCA GGACCCCCCC TCCCGGGAGA GCCATAGTGG
HCV 089     .......... .......T.. A......... .......... .......... ..........
HCV 202     .......... .......T.. A......... .......... .......... ..........
HCV 230     .......... .......... .......... .......... .......... ..........
HCV116      .......... .......T.. A......... .......... .......... ..........
HCV281      .......... ........A. .......... .......... .......... ..........


            -189                                                          -130
HCV-1       TCTGCGGAAC CGGTGAGTAC ACCGGAATTG CCAGGACGAC CGGGTCCTTT CTTGG*ATCA
HCV 089     .......... .........T. ...*....C. ..G...T... .......... ....A..A.
HCV 202     .......... .......... .......C. ..G...... .......... .....*..T.
HCV 230     .......... .......... .......... .......... .......... .....*.....
HCV 116     .......... .......T. .........C. ..G...T... .......... ....A..A.
HCV 281     .......... .......... ...*...... ..G...T... .......... ....A..A.


            -129                                                          -70
HCV-1       ACCCGCTCAA TGCCTGGAGA TTT*GGGCGT GCCCCCGCAA GACTGCTAGC CGAGTAGTGT
HCV 089     .......... ....C...A. ...G..... .......... .......... ..........
HCV 202     .......... ....C...A. ...*..... .......... .......... ..........
HCV 230     .......... .......... ...*..... .......... .......... ..........
HCV 116     .......... ....C..... ...G..... .......... .......... ..........
HCV 281     .......... .......... ...*..... .......... .......... ..........


            -69        -44
HCV-1       TGGGTCGCGA AAGGCC
HCV 089     .......... ......
HCV 202     .......... ......
HCV 230     .......... ......
HCV 116     .......... ......
HCV 281     .......... ......
```

* There is either a nucleotide deletion or insertion at the asterisk position compared to HCV genotype 1.

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(*8.1.5*)   The 5'non structural Region (NS5)

Only three of the anti-HCV positive donors (**089, 230 and 281**), were NS5

positive by RT-PCR of the NS5 region, there were not sufficient sera to carry out

furter tests for donors 116 and 202, after several attempts at NS5 amplification.

None of the samples could be amplified using the method of Valliammai *et al.*,

(1995) with the primers derived from the NS5 region described by Enamoto *et al.*

(1990). The results of PCR and the purified products are shown on fig 8.1.5a and

fig. 8.1.5b.

Comparative nucleotide sequence analysis of this region using Blast against

the EMBL database clearly predicted that donor 089 is infected with HCV type 4.

The virus has 82% sequence homology with CAM600 (data base accession no.

L29590) a predicted HCV genotype 4e isolate from Cameroon, 82% sequence

homology with GB809.3.1 (data base accession no. L29626), also predicted to be

genotype 4e and from Gabon, and 81% sequence homology with GB809.3.2 (data

base accession no. L29627), predicted as genotype 4g from Gabon, and 81%

sequence homology with HPCN5BA (data base accession no. L36438) genotype 4a

from the Central Africa Republic. Pairwise comparison of this sequence with the

representatives of genotypes 4a, 4c, 4d, 4e, 4f, and 4h (table 8.1.5a), confirmed that

this isolate cannot be assigned to those subtypes. The phylogenetic tree analysis

(fig.8.1.5d) predicted that it is genetically more related to the genotype 4e than any

other subtype, but could be a new subtype within the type 4. However, NS5

sequences derived from genotype 4b are not available in the databases at present.

Considering the NS5 nucleotide sequence of the 089 with the representatives of

HCV type 4, based on Simmonds *et al.*, (1994) classification which proposed

sequence similarities of 75% to 86% as a new subtype, this isolate seems to represent a new subtype within the type 4 in addition to the eight subtypes described previously (Stuyver *et al.*, 1994, Mellor *et al.*, 1995).

The donor 230, could be said to have been infected with HCV type 1. The nucleotide sequence analysis from the NS5 region in the blast EMBL showed a 95% sequence homology with HPCNS77 (data base accession no. M62383), a predicted genotype 1a from the USA, 94% sequence homology with HCV-PT-1 (data base accession no. D10646) also genotype 1a, but from Japan. It has a 94% homology with HPHCJ1 an HCV isolate from Japan (data base accession no. D10749), it also has a 94% homology with HPCNS90 (data base accession no. M67463) a predicted 1a HCV isolate from the U.S.A. Pairwise comparison of the nucleotide sequence of this isolate with 12 various HCV genotypes 1a, 1b, and 1c (table 8.1.5b) clearly showed that this isolate (230) can be classified as genotype 1a. The phylogenetic tree analysis predicted genotype 1a. It was found to be within the genotype 1a in the phylogenetic tree (figure 8.1.5e). The nucleotide sequence analysis of the HCV isolate from donor 281 using the Blast agaist the EMBL database, showed nucleotide sequence homology of 85% with HCU14299 (data base accession no.U14299), a predicted HCV genotype 1a isolate from USA, 84% nucleotide sequence homology with HCV-PT-1 (data base accession no D10646) also a genotype 1a HCV isolate from USA and, HPCNS5A14 (data base accession no. D14196) genotype 1a isolate from Japan (Okamoto, unpublished sequence). It has 85% sequence homology with HPCPOLYP a predicted 1a genotype (an Okamoto unpublished sequence, data base accession no. M32084). The pairwise comparison with 12 different isolates is shown in table 5.1.5c. The phylogenetic tree analysis (fig.8.1.5e), predicted it to be a new genotype of HCV within the type 1.

Figure 8.1.5a: Results of the Polymerase Chain Reaction of HCV NS5 Region.

Gel A; Lane 1 empty, Lanes 2 and 13 = 1Kb Ladder Marker*; Lanes 3-6 = samples,

Lane 7 = Positive Control; Lane 8 = Negative Control ; Lane 10 = Sterile Distilled

Water.

Gel B; Lanes 1 and 12 = 1 Kb Ladder Marker; Lane 2 = Negative Control; Lane 3

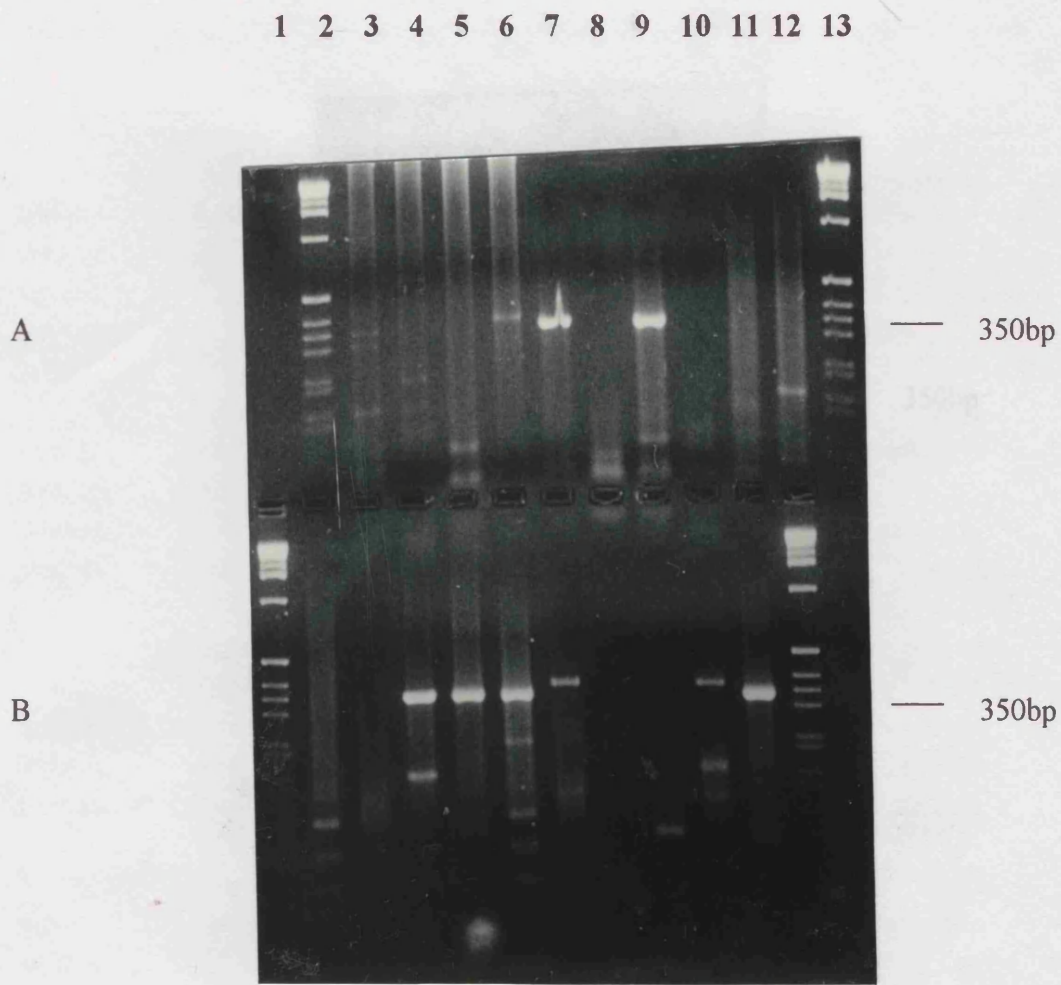= Sterile Distilled Water; Lanes 4-8 = samples, Lane 11 = Positivve Control.

—— 350bp

Figure 8.1.5b: Purified Polymerase Chain Reaction Product of the NS5 Region. Lanes 1 to 3 are purified PCR products, Lane 4 = Empty, Lane 5 = 1kb Ladder marker* (1kb marker sizes in appendix).

(Fig.*8.1.5*c) Alignment of the consensus nucleotide sequences of part of NS-5

region of the 3 HCV isolates (230, 281, 089) with HCV-1

```
          7955                                                8004
HCV-1     ACATCCGTAC GGAGGAGGC AATTTACCAA TGTTGTGACC TGGACCCCCA
HCV230    .......... ......... ...CA..... .......... ..........
HCV281    .......... ...A..... .......... A......... ...C....TG
HCV089    C..A....CA C.GA....G .CATCAGG.CC .AG.GAGAG GTCTAT.AGT


          8005                                                8059
HCV-1     AGCCCGCGTG GCCATCAAGT CCCTCACTGA GAGGCTTTAT GTTGGGGGCC
HCV230    G.....T... .......... .......... .......... ..........
HCV281    A.G...A... .....A.... .......... .......... ...C....G.
HCV089    .TTGT.ACCT CATGCAC.A. AG.AAGGG.G ACCT..GCGG TG.CC.TAGG


          8060                                                8109
HCV-1     CTCTTACCAA TTCAAGGGGG GAAAACTGCG GCTACCGCAG GTGCCGCGCG
HCV230    .......... .......... ..G.....T. .......... ..........
HCV281    A.......C ..C.AA..T. ........C. .......TC. ......T..A
HCV089    TG.CGCG.T. GCGGC.TATT C.CC..CAGC TTCGGGAACA CACTGA..T.


          8110                                                8159
HCV-1     AGCGGCGTAC TGACAACTAG CTGTGGTAAC ACCCTCACTT GCTACATCAA
HCV230    .......... .......... .......... .......... ..........
HCV281    ...C.G..G. ....C..... ......C... .....T..C. .T...C....
HCV089    CTACCTTA.G GCCAC.GCC. .ACA.AGGG. GG.GGG.... CT.CGGGA.C


          8160                                                8209
HCV-1     GGCCCGGGCA GCCTGTCGAG CCGCAGGGCT CCAGGACTGC ACCATGCTCG
HCV230    .......... .......... .......... ..G....... ..........
HCV281    A...A.C..G .......... .....CA... ..A....... ..........
HCV089    ACA.T.ACGT ..TACCTT.A GGC..CA..C G.ACAGAG.G CGGCG.GA.T


          8210                                                8259
HCV-1     TGTGTGGCGA CGACTTAGTC GTTATCTGTG AAAGTGCGGG GGTCCAGGAG
HCV230    .......... .......... ........C. .G........ ..........
HCV281    ....C..... ....C.T..T ..C....... ....C.GC.. ..........
HCV089    .AAAGATTGC ACCA.GCTGG TC.GCGGCGA CT.CCTG.TC .TCATGCCT.


          8260          8270
HCV-1     GACGCGGCGA GCCTGAGAGCC
HCV230    ....A.CG.. A..........
HCV281    ........A. ...........
HCV089    A.A.T.A..G CGTC..CGAGG
```

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(Table *8.1.5a*) Pairwise comparison of nine partial nucleotide sequences derived from the NS5 region of isolates of HCV genotype 1

	1a			1b			1c		
	M62321	M67463	HCV230[+]	M58335	D90208	M84754	L23446	L23447	HCV281[+]
M62321*	100	96	95	82	80	81	84	82	85
M67463*		100	94	81	80	80	83	81	84
HCV230[+]			100	78	78	77	84	82	84
M58335*			·	100	92	95	83	81	78
D90208*					100	91	76	76	80
M84754*						100	75	76	79
L23446*							100	95	77
L23447*								100	76
HCV281[+]									100

The values represent percentage nucleotide identity between representatives of HCV genotypes 1a, 1b and 1c

* Database accession number

[+] Nigerian HCV isolates

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(Table *8.1.5b*) Pairwise comparison of 14 partial nucleotide sequences derived from the NS5 region of isolates of HCV genotype 4

	L23470	L29602	L29605	EUYEM2	L29590	L29626	L29596	L29618	L29621	L29613	L29611	HCV089	L36438	L36437
L23470*	100	83	83	79	86	84	80	78	77	80	79	80	79	82
L29602*		100	93	76	85	85	82	78	77	81	80	79	80	81
L29605*			100	81	86	82	82	79	79	82	82	79	81	81
EUYEM2*				100	84	83	82	77	76	81	80	76	81	79
L29590*					100	93	84	80	80	86	86	82	83	83
L29626*						100	85	80	80	86	85	82	81	83
L29596*							100	81	80	84	83	79	81	81
L29618*								100	99	84	84	76	81	76
L29621*									100	84	84	76	80	76
L29613*										100	99	80	81	81
L29611*											100	80	80	81
HCV 089[+]												100	81	80
L36438*													100	79
L36437*														100

The values represent percentage nucleotide identity with genotypes 4a (L23470), 4c (L29602, L29605), 4d (EUYEM2), 4e (L29590, L29626), 4f (L29596), 4g (L29618, L29621), 4h (L29613, L29611) and two unassigned sequences CAR1 (L36438) and CAR4 (L36437).

Pairwise comparisons are made over 329 nucleotides (nt 8276-8605 according to Choo *et al.*, 1991), except for L23470 where only 222 nucleotides are available in the database.

* Database accession number, EUYEM2 is from Mellor *et al.* (1995)

[+]Nigerian HCV isolate.

Fig.8.1.5d The rootless phylogenetic tree analysis of the NS5 region of HCV isolates (089, 230, & 281) using a maximum likelihood algorithm (courtesy Dr. Janet Mellor)

***** NIGERIAN HCV ISOLATE**

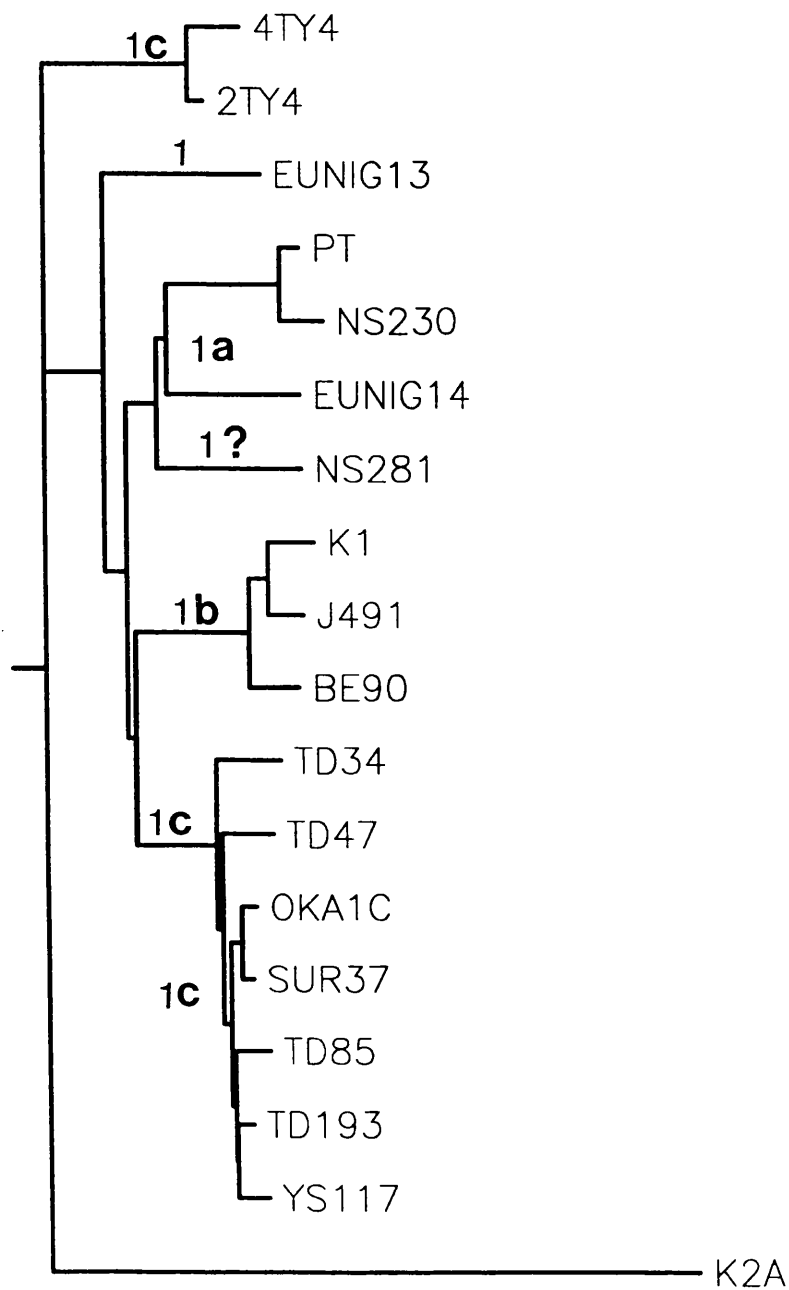**Fig. 8.1.5e.** The rootless phylogenetic tree analysis of the NS5 nucleotide sequence of the two predicted HCV genotype 1 isolates (230 & 281), using a maximum likelihood logrithm analysis (Courtesy Dr. Janet Mellor).

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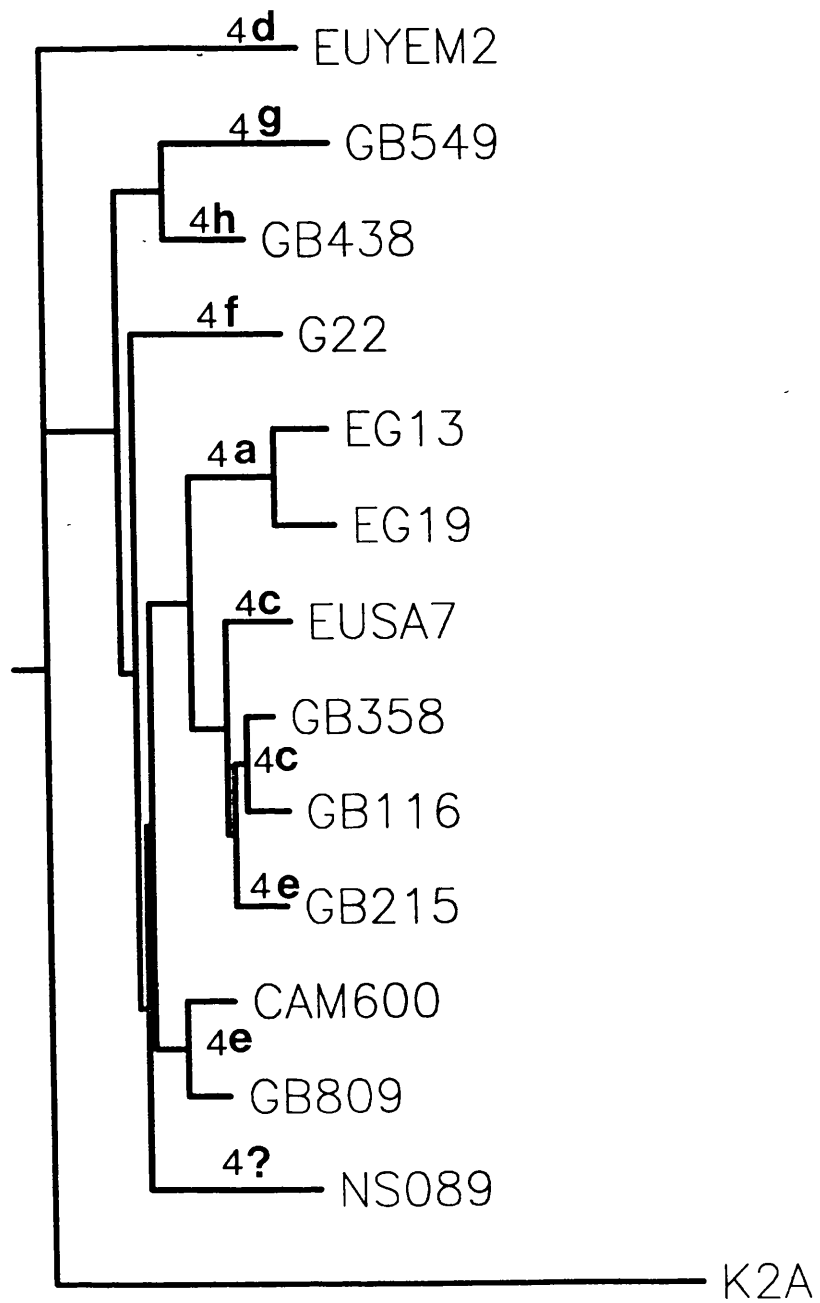
Fig 8.1.5f. The phylogenetic tree ananlysis of the NS5 nucleotide sequence of the predicted HCV genotype 4 isolate (089), using maximum likelihood logrithm analysis (courtesy of Dr. Janet Melor).

(Table *8.1.*6) Pairwise comparison of 8 partial nucleotide sequences derived from the nucleocapsid region of isolates of HCV genotype 4.

	u10236	u10235	u10239	u10238	l29587	l29624	u10240	HCV089
u10236*	100	91	89	89	92	93	92	91
u10235*		100	89	88	90	91	92	88
u10239*			100	95	90	90	92	89
u10238*				100	91	92	91	90
l29587*					100	96	91	91
l29624*						100	91	92
u10240*							100	89
HCV 089[+]								100

The values represent percentage nucleotide identity over 463 nucleotides (nt 342-811 accorging to Choo *et al.*, 1991) and between various representatives of genotype 4a (U10236), 4b (U10235), 4c (U10239, U10238), 4e (L29587, L29624), 4f (U10240).
* Database accession number
[+] Nigerian HCV isolate

(8.1.6) **Core Region**

Only two donors (**089** and **281**) were RT-PCR positive using the primers for core region. All attempts to get amplify sample 230 were futile, although, this donor (230) was ELISA positive, RIBA negative, but was positive for both 5'UTR and NS5 regions. The PCR result and *EcoRI* digestion of the cloned products are shown in fig.8.1.6a and fig. 8.1.6b. The sequence analysis using Blast against the EMBL databse showed donor 089 was infected with HCV with a close nucleotide sequence homology with HCV of genotype 4. It has 93% sequence homology with HCV GB809 (accession no. L29624), predicted as genotype 4e from Gabon, 92% sequence homology with DK13 (accession no. U10192), genotype 4d from Denmark, 92% sequence homology with CAM600 (accession no. L29587) predicted genotype 4e from Cameroon, 92% sequence homology with Z4 (data base accession no. U10236) predicted genotype 4a from Zambia. From the pairwise comparison of 089 nucleocapsid sequence with representatives of genotypes 4a, 4b, 4c, 4e and 4f, it is clear that the 089 sequence is distant from that of the genotype 4b isolate. Considering the NS5 and nucleocapsid sequence data together, it seems that isolate 089 represents a new subtype within genotype 4 in addition to the eight subtypes described previously (Stuyver *et al.*, 1994, Mellor *et al.*,1995). It has therefore been designated provisionally genotype 4i.

Nucleotide sequence analysis of the core region of HCV 281 showed 93% homology with HC-G9 and YS117 (data base accession nos. D14853, D16189) both predicted as genotype 1c from Indonesia. It showed 91% nucleotide sequence homology with F2 (accession no. X76408) predicted 1b HCV isolate from France. The alignment of the consensus core region sequence from two donors (089 & 281) with HCV-1 and Gabon isolate HC-G6 is shown in fig. 8.1.6c.
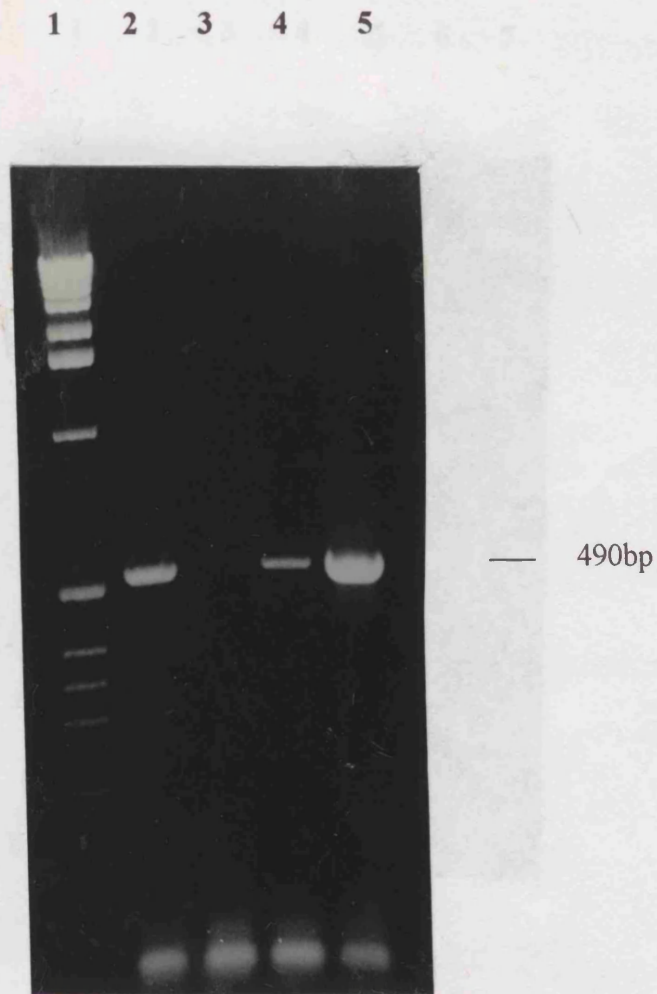
490bp

Figure 8.1.6a: Results of the Polymerase Chain Reaction of the HCV Core

Region. Lane 1 = 1Kb Ladder Marker, Lane 2 = Positive Control, Lane 3 =
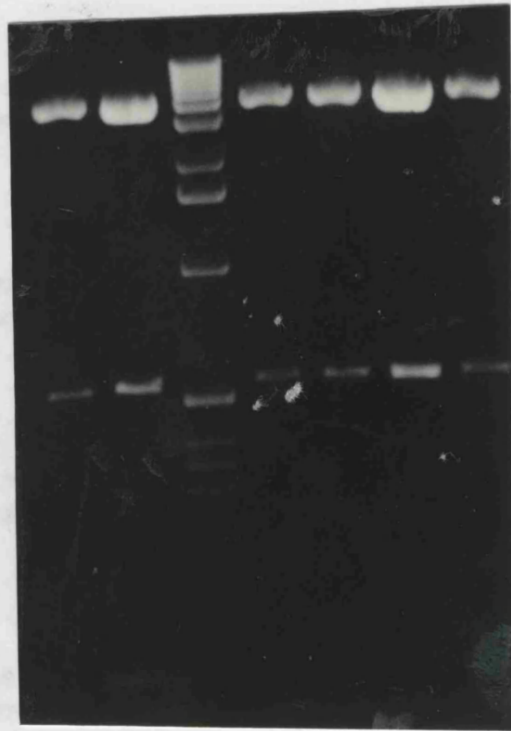
Negative Control, Lane 4 & 5 sample.

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Figure 8.1.6b: EcoRI Digested Clones of the HCV Core Region.

Lanes 1 & 2 and 4 to 7 are Digested Clones, Lane 3 = 1Kb Ladder Marker.

(Fig 8.1.6c)   Aligment of the 2 HCV Isolates (089 and 281) core region sequences, with

HCV type1 prototype (HCV-1) and Gabonese (HC-G6) isolate

```
         -20
HCV-1    TCTCGTAGAC CGTGCACCAT GAGCACGAAT CCTAAACCTC AAAAAAAAAA CAAACGTAAC
HC-G6    .......... .......... .......... .......... ...G.....C ..........
HCV89    .......... .......... .......... .......... ...G.....C ..........
HCV281   .......... .......... .......... .......... ...G.....C ..........

         31
HCV-1    ACCAACCGTC GCCCACAGGA CGTCAAGTTC CCGGGTGGCG GTCAGATCGT TGGTGGAGTT
HC-G6    ........C. ....CAT... ...T...... ....C.T.. .C........ ..........
HCV089   ........C. ....CAT... ...T...... .....T... .C........ ...C......
HCV281   ........C. ....CAT... ...T...... ....C.T.. .......... ...C......

         131
HCV-1    TACTTGTTGC CGGCAGGGG CCTAGATTGGG TGTGCGCGCG ACGAGAAAGA CTTCCGAGCG
HC-G6    .......... .......... ..C..G.... .......... ...T.G.... ....G.....
HCV089   .......... .......... ...C...... .......... ...TC.G... ..........
HCV281   .......... .......... ..C..G.... .......... ......G... ..........

         181
HCV-1    GTCGCAACCT CGAGGTAGAC GTCAGCCTAT ATCCCCAAGG CTCGTCGGCC CGAGGGCAGG
HC-G6    .......... ..T..G.... .......... .......... ...A....AT .T........
HCV89    .......... .......... T..T...... ....AG.C.. .C..A..... ..........
HC281    .......... ..T..A...G .G........ .......... ..A..C.... ..........

         231
HCV-1    ACCTGGGCTC GGGATGGCTC CTGTCTCCCC GTGGCTCGGC CTAGCTGGGG CCCCACAGAC
HC-G6    T......... .......... .....A.... .T...T..C. T........T ..........
HCV089   ..G.T.G... .......... T........G .......... .......... ..........
HCV281   T......... ..C....... ..C....... ....A.... .A........ .......C..

         291
HCV-1    TACCTCATGG GCCCCGGCG TAGGTCGCGC AATTTGGGTA AGGTCATCGA TACCCTTACG
HC-G6    ..A....... ......A... .........A ..G.CT.... ....A.AT.. .........A
HCV089   ..A....... ....AG.G. ..C....... ....CT.... ....G.AT.T ..........
HCV281   .....T.... .....T...T .........C .....A.... ..C....... ..........

         361
HCV-1    TGGGCTTCGC CGACCCTTGG CCCCTCTATG GCAGCCCCGG GTAATGAGGG CTGCGGGTGG
HCV-G6   .....C.... .C........ .......... .....A. T.......T. .........T
HCV089   .......... .......... .........T .......... .....C.... .C....A...
HCV281   .....G.... .T..C...GC .......... ..A....T.. .......T.. ..........

         421
HCV-1    GGTACATACC GCTCGTCGGC GCCCCTCTTG GAGGCGCTGC CAGGGCCCTG GCGCAT
HC-G6    .......... ...A....A. .....CG.G. .C....TC.. .......... ..A...
HCV089   ......A... .......... ..T.T..... ....C..G.. ......G.G. ..C.T.
HCV281   ....T..A.. ...G...... .......... ..C..A.... ..A.T..A.. ..A...
```

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CHAPTER 9

(9.1)   **Discussion**

Various reports have shown that the prevalence of HCV infection is generally

high in sub-Saharan Africa (Coursaget *et al.*, 1990, Delaporte *et al.*, 1993). In this

work, a pilot survey of HCV infection amongst healthy adult blood donors and

children of pre-school age in Nigeria was carried out with the aim of determining the

prevalence of HCV infection in these two groups. This study is one of the first surveys

of HCV infection in Nigeria. An 8% anti-HCV positivity rate was found among the

adult donors by ELISA, but none of the children was positive. The high prevalence

of anti-HCV among adults donors and not among the children suggests transmission

later in life possibly as a result of infection during blood transfusion, and (or)

community acquired. It is also possible that some children might have been infected,

but, sera were collected during the HCV incubation period (window period). This is

also an indication that vertical transmission of HCV infection from mother to infant,

does not occur as frequently seen with HBV or HIV-infected mothers. Although HCV

infection has been demonstrated in infants, these infants came from both anti-HCV

negative and anti-HCV positive mothers (Reesink *et al.*, 1990). The 16 or 8% anti-

HCV found among the adult donors is very high, especially when confirmatory tests

could only confirmed between 4 (RIBA) and 5 (RT-PCR) of the 16 anti-HCV donors

as positive. Although some reported cases from other parts of sub Saharan Africa

based on anti-HCV positive (ELISA) were comparable to what was found in this

work, for example, 12.5% prevalence of anti-HCV was reported from Cameroon

(Louis *et al.*, 1994), 7.9% from Libya (Saleh *et al.*, 1994), and Gabon 6.5% out of

which 46% of the anti-HCV positive were HCV RNA positive by RT-PCR (Delaporte

et al., 1993). With the exception of the reported case from Gabon, no confirmatory tests were done for other reports. The discrepancy observed in the ELISA result and the confirmatory tests (RIBA & RT-PCR) is as a result of false positivity associated with the ELISA method. This further demonstrates the reliability of the RIBA and RT-PCR as a confirmatory test for HCV infection. It is doubtful if the third generation ELISA could eliminate the false positivity associated with the second generation, since the third generation seems to be more sensitive than the second generation, hence there is every possibility that more false positive would be picked using the third second generation ELISA.

For the first time, genotypes of HCV isolates from Nigeria were determined. Numerous criteria to define HCV genotypes have been proposed; PCR with genotyping specific primers in the C region (Okamoto *et al.*, 1992), analysis of amplification products of the NS5 region by slot blotting (Enamoto *et al.*, 1990), or of the 5'UTR (McOmish *et al.*, 1993), by restriction fragment length polymorphism, sequence analysis of the 5'UTR, E1 or NS5 gene (Bukh *et al.*, 1992, 1993, Okamoto *et al.*, 1993, Simmonds *et al.*, 1993a), phylogenetic analysis (Simmonds *et al.*, 1993b, Stuyver *et al.*, 1994), but the genetic classification of HCV through sequencing has, however, evolved as the most extensively used method. This system allowed for partial sequences of different segments of the genome such as the 5' untranslated region (5' UTR), and putative coding regions (NS5, core and envelope), to be enough for the classification of HCV isolates into various genotypes throughout the world (Simmonds *et al.*, 1994). Many HCV genotypes have been reported world wide, but none has been reported from Nigeria. In the present work, healthy adult blood donors from five of the blood transfusion centres across the country were screened for anti-

HCV, confirmed by RIBA-2 and RT-PCR. Nucleotide sequence comparison of the 5' UTR of the HCV isolated from five donors that were positive in this region, with sequences of various HCV isolates in the EMBL data base, showed the majority (3/5) of the donors were infected with HCV genotype 4, and the remaining (2/5) were infected with genotype 1. However, the analysis of this region alone is not sufficient to predict accurately all the subtypes.

Only three of the PCR-positive sera could be amplified in the NS5 region. The nucleotide sequence analysis of this region, and the pairwise comparison of the representatives of genotype 1, showed donor 230 to be infected with HCV genotype 1a, and the phylogenetic analysis further confirmed this isolate to be within the cluster of the genotype 1a. The second donor (281) seems to be infected with a novel virus, which, the nucleotide sequence was closely related to various genotype 1, but based on the criteria of Simmonds *et al.*, (1994) for HCV classification, and the phylogenetic analysis of the nucleotide sequence, both at the NS5 region and the nucleocapsid region, it represents a novel genotype within genotype 1. The third donor (089) was infected with HCV which has sequence homology closely related to genotype 4, the results of the nucleotide sequence at NS5 region, nucleocapsid region and phylogenetic tree analysis confirmed this virus to be a new subtype within the genotype 4.

The nucleotide sequence analysis of the core region further confirmed the observation made in the NS5 region, although only two of the samples were sequenced in this region (089 & 281). The HCV isolated from donor 089 has a nucleotide sequence closely related to various HCV genotype 4 of African origin 4e (Gabon and Cameroon), and 4a (Zambia), but the nucleotide sequence homology was closer to genotype 4e. The core region sequence analysis of HCV isolate from donor 281 showed a high homology to genotype 1c (Indonesia isolate) and a genotype 1b

(France), but the homology is more towards the genotype 1c. There is at present, no percentage homology cutoff guideline proposal for core region sequence analysis, as there was for NS5 region (Simmonds *et al.*, (1994). The application of the core region sequence to the classification of HCV into genotypes then depends on the percentage nucleotide sequence homology observed within this region in the data base sequence analysis. The result obtained from this method suggested HCV 281 may be a genotype close to 1c, but cannot be classified as 1c, according to the pairwise comparison with representatives of 1a, 1b, and 1c, and the phylogenetic tree analysis of the NS5 region, this virus appeared to be novel and it is proposed as a new genotype 1d.

The HCV sample 089, could not be classified among any existing genotype, on the basis of the core sequence, although it is very close to 4e. The pairwise comparison of the nucleocapsid sequence with the representatives of genotypes 4a,4b, 4c, 4e, and 4f and the phylogenetic tree analysis of the NS5, confirmed the isolate (089) as a new subtype within genotype 4. Considering the NS5, and nucleocapsid sequences and the phylogenetic tree analysis, it seems isolate 089 represents a new subtype within genotype 4 in addition to the eight subtypes described previously (Stuyver *et al.*, 1994, Mellor *et al.*, 1995). It has been designated provisionally as a new genotype 4i. Various studies in African countries, such as Gabon, Zaire, Egypt and Burundi suggested HCV genotype 4 to be predominant in this continent (Bukh *et al.*, 1993, Simmonds *et al.*, 1993, Stuyver *et al.*, 1993, Xu *et al.*, 1994), but in the present study, genotypes 1 and 4 were found in Nigeria. Of the five that were sequenced in 5'UTR, three (089, 116, & 0202) were predicted to be genotype 4 and the remaining two (230 & 281) to be genotype 1. However, of the three that were sequenced in the NS5 region, two were predicted to be genotype 1 (230 & 281) and

one (089) predicted to be genotype 4. Two (089 & 281) were predicted as novel isolates by phylogenetic tree analysis, and each belongs to new subtypes within genotype 1 and 4. Nkengasong *et al.*, (1995) reported predominantly genotypes 1 and 2 from Cameroon, although non was sequenced. Since this work is the first report on the HCV genotypes from Nigeria, it is not possible to confirm which of these genotypes is predominant. Although, three regions from these HCV isolates were sequenced, the number of isolates sequenced thus far was not large enough to draw a conclusion as to whether other HCV genotypes may be present in Nigeria. However, the present studies confirmed the existence of at least two HCV genotypes in Nigeria.

The implications of the presence of various genotypes of HCV in one country, will be in the treatment of HCV infection and in the vaccine production, since there are indications that different subtypes of HCV may have different clinical relevance, mostly in their response to interferon treatment (Kanai *et al.*, 1992, Dusheiko *et al.*, 1994). It may also have a role to play in the development of hepatocellular carcinoma, and different genotypes may evoke different serological responses.

Chapter 10

(*10.1*)   General Discussion

Through the first part of this study, it has been possible to confirm that hepatitis B virus infection is highly prevalent in Nigeria. From the current rate of HBsAg carriage among the healthy adult donors and children of pre-school age, it can be concluded that the infection is high in the general population notwithstanding, the fact that the majority of blood donors in Nigeria are male. Comparison of adult blood donors and pre-school age children showed an early infection. A strategy for the control of the disease through neonate vaccination is hence recommended, and should be introduced as early as possible. Among the major routes of transmission that have been identified are vertical transmission, mother to child (Stevens *et al.*, 1975), and horizontal transmission scarification, circumcision and tribal marks with unsterile materials (Botha *et al.*, 1984), these routes can be efficiently controlled through vaccination of neonates. Scarification in any form should be discouraged. Also, one other major source of infection is believed to be through contaminated syringes and needles, therefore it is of tremendous importance that needles and syringes should be properly sterilized if they are to be reused or should be discarded if disposable can be afforded.

The results of nucleotide sequence analysis of the pre-core region indicated that, pre-core mutations are not very common in the HBV from Nigeria. Although, HBV DNA positivity in the absence of HBeAg was common in the donor samples, the most common pre-core mutation that aborts HBeAg synthesis was rare. This contrasts with various reports from some other parts of the world, where absence of HBeAg in viraemic patients frequently is associated with the pre-core

mutation (see section 2.3.9). The absence of HBeAg in many of the viraemic blood donors that were PCR positive, but very few had any mutation within the pre-core sequence, may be an indication that these HBV isolates (from Nigeria) may be associated with other mutations, such as mutations in the core promoter which also abort HBeAg synthesis (Okamoto *et al.*, 1994). There is need to sequence more Nigerian HBV isolates in the core promoter region to reach a conclusion.

In the second part of this study, 8 % of donors were reactive in the second generation ELISA, but none of the children was positive. Although, very few out of this number were HCV RNA positive, this may be an indication that most of these donors have resolved of the disease or are currently infected with parasites such as malaria or yellow fever which may also be ELISA reactive or in association with GB virus which is believed to suppress HCV replication and could still be ELISA positive. The situation of HCV infection in Nigeria may be a more representation of HCV infection in males, rather than in the general adult population, since majority of the donors in Nigeria are usually men. However, the present study implies that perinatal transmission is not a major route of infection, since none of the children screened was positive. Much has to be done to control the spread of the infection, this can be done by making sure that sources of infections are adequately controlled. The age at which the infection occurred is yet to be investigated.

Various genotypes of HCV have been described throughout the world, and it has been suggested that the various genotypes may have different clinical relevance, especially in their severity of liver disease and in response to interferon

treatment (Dusheiko *et al.*, 1994). Genotypes of HCV have been found in Nigeria, genotypes 1a, 1d and 4i. The clinical significance of the existence in Nigeria of genotype 1, which is believed to have a world wide distribution but not to be very common in sub-Saharan Africa, is not yet known. However, there are suggestions that genotype 1 are less sensitive to interferon treatment. It must be stated that there was no single co-infection of HBV and HCV observed in the present study.

CHAPTER 11

(11.1) CONCLUSION

From this study it can be concluded that Nigeria is endemic with both hepatitis B and C viruses. There are present two major subtypes of HBV in Nigeria, *ayw* and *adw*. The subtype *ayw* is in the majority. The prevalence of HBV infection is higher than that of the HCV in the general population. HCV infection is higher among the donors, than in the children, in contrast to what was found in HBV, where infection among the children was higher than that of the adult donors. At least two major genotypes of HCV are present in Nigeria. Genotypes 1 and 4. The spread of HBV should be contained as soon as possible because it seems to be more of public health problem of the two viruses.

Appendix

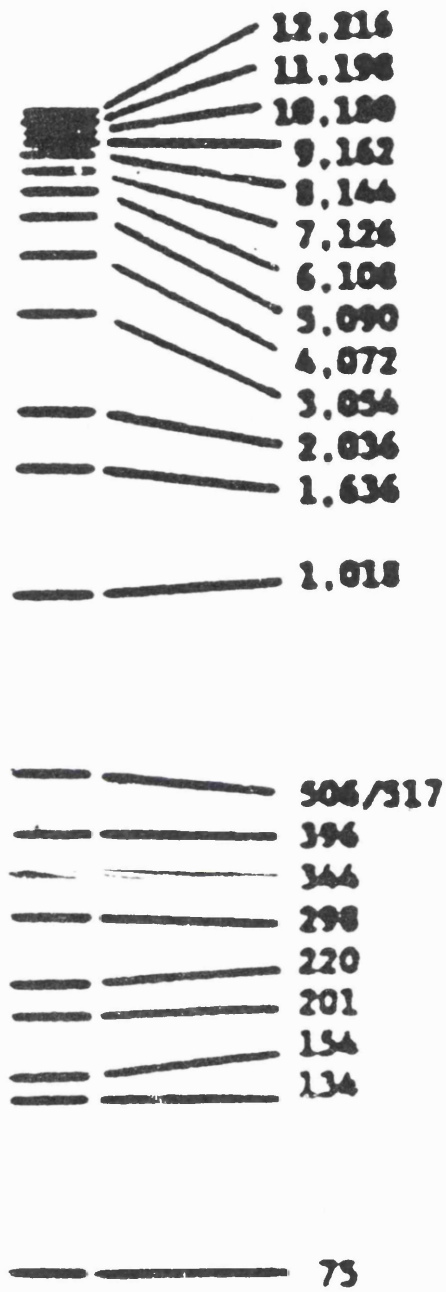|		
HBsAg ELISA wash fluid	=	0.1M Piperazine, 0.1mM Magnesium ions, 0.1mM Zinc ions, and 0.1% Protein
ELISA stop solution	=	2 M Sulphuric Acid
Anti-HBs washing soln	=	1 x Phosphate buffer, 0.1% Tween, and 0.001% Thimerosal
D.T.M	=	100μM each of dATP, dGTP, dTTP in 250 Tris-HCl (pH8), 25mM $MgCl_2$, 50mM β-mercaptoethanol.
OL/2	=	45U/ml P(dN)^6 (Pha rmacia 27-2166-01) in Tris-HCl (pH8)
EDTA	=	Ethylene Diamine Tetra Acetic acid
TCA	=	Trichloroacetic Acid
TNE	=	0.1M Tris.Cl (pH8), 0.01M EDTA (pH8), 1M NaCl
TE	=	10mM Tris.Cl (pH8) , 1mM EDTA (pH8)
1 X SSC	=	0.15M NaCl + 0.015M Na-Acetate
10 x Taq buffer	=	10mM Tris HCl (pH8.3), 50mM KCl, 1.5mM $MgCl_2$
Enzyme react 2	=	50mM Tris HCl (pH8), 10mM $MgCl_2$, 50mM NaCl
Enzyme react 3	=	50mM Tris (pH8), 10mM $MgCl_2$, 100mM NaCl
Ligation buffer	=	0.25M Tris-HCl (pH7.6), 50mM $MgCl_2$, 5mM ATP, 5mM DTT, 25% (w/v) Polyethylene glycol-800
YT broth	=	1 % Bacto Tryptone, 1 % Bacto Yeast, 0.5 % NaCl
YT Agar	=	1 % Bacto Tryptone, 0.8 % Bacto Agar, 0.8 % NaCl
X-gal	=	5-Bromo-4-choloro-3-indoly-β-D-galactoside
IPTG	=	100mM Isopropylthio-β-D-galactoside

KEY TO AMBIGOUS OR INCOMPLETLY SPECIFIED BASES

R   =   A or G

Y   =   C or T

M   =   A or C

K   =   G or T

S   =   G or T

W   =   A or T

H   =   A or C or T

B   =   C or G or T

V   =   A or C or G

D   =   A or G or T

N   =   A or C or T

Key to Amino acid symbols

Amino acid	Symbol	Amino acid	Symbol
Alanine	A	Arginine	R
Asparagine	N	Aspartic acid	D
Cysteine	C	Glutamine	Q
Glutamic acid	E	Glycine	G
Histidine	H	Isoleucine	I
Leucine	L	Lysine	K
Methionine	M	Phenylalanine	F
Proline	P	Serine	S
Threonine	T	Tryptophan	W
Tyrosine	Y	Valine	V

12,216
11,198
10,180
9,162
8,144
7,126
6,108
5,090
4,072
3,054
2,036
1,636
1,018

506/317
396
344
298
220
201
154
134

75

1 Kb Ladder Fragment size (base pairs)

CHAPTER 13

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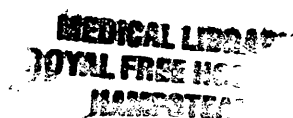
Genotypes of Hepatitis C Virus in Nigeria

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A pilot survey of hepatitis C virus (HCV) infection in Nigeria was carried out on healthy adult blood donors and children of preschool age. Sixteen of 200 (8%) donors were positive for antibodies using a second generation enzyme-linked immunosorbent assay (ELISA) but all of the children were negative. Supplementary testing of the ELISA-positives using a recombinant immunoblot assay (RIBA-2) confirmed the presence of antibody in four and two others were indeterminate. Four of the anti-HCV-positive sera and one found positive by ELISA but which was negative by RIBA-2 were found to be positive for HCV RNA using reverse transcriptase-polymerase chain reaction (RT-PCR) and primers specific for the 5' untranslated region (5'UTR) of the HCV genome. The NS5 and core regions also were amplified and the PCR products from all three regions were sequenced. Sequences from the 5'UTR could be divided into two groups: one group comprised three isolates with greater than 95% sequence identity with published sequences of genotype 1 and the other comprised two isolates with greater than 93% sequence identity with genotype 4. Analysis of three sequences amplified from the NS5 region confirmed this assignment to genotypes 1 and 4. Pairwise comparisons of the NS5 region sequences with representatives of 1a, 1b, 1c (for the first group) and 4a–4h (for the second group) show the first group to include subtypes classifiable as 1a and a novel sequence and the second group to include a novel sequence within genotype 4. Sequence analysis of the core region was consistent with this interpretation. These data confirm the presence of at least two major HCV genotypes in Nigeria (genotypes 1 and 4) and we report two novel sequences which have been designated provisionally as genotypes 1d and 4i. © 1996 Wiley-Liss, Inc.

INTRODUCTION

Hepatitis C virus (HCV) has been identified as the etiological agent responsible for most cases of parenter-

ally transmitted non-A, non-B hepatitis [Choo et al., 1990; Kuo et al., 1989]. The HCV genome is a positive polarity, single-stranded RNA of approximately 9.4 kilobases (kb) in length [Choo et al., 1991]. The genome consists of three regions, a 5' untranslated region (5'UTR) which comprises approximately 341 nucleotides [Han et al., 1991], a single continuous open reading frame encoding a polyprotein of 3,010 amino acid residues [Choo et al., 1991], and a short 3'UTR [Takamizawa et al., 1991]. The 5'UTR and region encoding the nucleocapsid protein (C) are relatively conserved [Takeuchi et al., 1990; Han et al., 1991; Bukh et al., 1992, 1994], while regions encoding envelope glycoproteins 1 (E1) and 2 (E2) are highly variable and a hypervariable region (HVR-1) is present in the N-terminus of E2 [Weiner et al., 1991]. The remainder of the open reading frame encodes non-structural polypeptides and is moderately variable.

The prevalence of HCV infection generally is low in Western countries, but a higher prevalence has been reported in some parts of southern and eastern Europe [Esteban et al., 1989]. The prevalence is quite high in Japan [Hayashi et al., 1995], Egypt, and sub-Saharan Africa [Coursaget et al., 1990; Delaporte et al., 1993]. Numerous HCV isolates from different geographical origins have been sequenced partially or completely; nucleotide sequence comparison and variation of the deduced amino acid sequences reveal that the HCV genome displays a rather high heterogeneity both among infected individuals [Enomoto et al., 1990] and within a single individual [Tanaka et al., 1992]. Several authors have proposed systems of nomenclature of HCV genotypes [Enomoto et al., 1990; Nakao et al., 1991; Chan et al., 1992; Okamoto et al., 1992; Simmonds et al., 1994b]. Recently, efforts have been made to find an acceptable unified system of nomenclature and Simmonds et al. [1994a] have proposed a system based on the nucleotide sequence divergence within the NS5 region, since sequence analysis of the entire genomes of many isolates

TABLE I. Primers Used for PCR Amplifications

Primer	Sequence (5' to 3')	Position[a]	Reference
5'UTR			
939 (+)[b]	CTGTGAGGAACTACTGTCTT	-297	Okamoto et al. [1990]
209 (−)	ATACTCGAGGTGCACGGTCTACGAGACCT	8	Garson et al. [1990]
940 (+)	TTCACGCAGAAAGCGTCTAG	-278	Okamoto et al. [1990]
211 (−)	CACTCTCGAGCACCCTATCAGGCAGT	-29	Garson et al. [1990]
Core			
CPS14 (+)	GCGGAATTCGTGGTACTGCCTGATAGGGTG	-67	Xu et al. [1994]
CPA14 (−)	GGCGGATCCGGAAGATAGAGAAAGAGCAACC	511	Xu et al. [1994]
CPS15 (+)	GGCGAATTCTCTCGTAGACCGTGCACCATG	-26	Xu et al. [1994]
CPA15 (−)	GCTAGATCTTCCCTGTTGCATAGTTCACGCCG	480	Xu et al. [1994]
NS5			
1203 (+)	ATGGGGTTCTCGTATGATACCCGCTGCTTTGACTC	7903	Mellor et al. [1995]
1204 (−)	GGAGGGGCGGAATACCTGGTCATAGCCTCCGTGAA	8275	Mellor et al. [1995]
518 (+)	CTCAACSGTCACSGAGARGGCAT	7935	Mellor et al. [1995]
123 (−)	GCTCTCAGGTTCCGCTCGTCCTCC	8250	Mellor et al. [1995]

[a]According to Choo et al. [1991].
[b](+) positive or (−) negative polarity.

TABLE II. Results of RIBA and RT-PCR of 16 Anti-HCV ELISA-Positive Donors*

Sample	RIBA-2					RT-PCR		
	5-1-1	C100-3	C33c	C22-3	Interpretation	5'UTR	NS5	Core
020	−	−	−	−	−	−	NT	NT
041	−	−	−	−	−	−	NT	NT
089	−	−	+	+	+	+	+	+
091	−	−	−	−	−	−	NT	NT
116	+	+	+	+	+	+	−	−
145	−	−	−	−	−	−	NT	NT
155	−	−	−	−	−	−	NT	NT
202	+	+	+	+	+	+	−	−
220	−	−	−	−	−	−	NT	NT
230	−	−	−	−	−	+	+	−
231	−	−	−	−	−	−	NT	NT
274	−	−	−	−	−	−	NT	NT
280A	−	−	−	−	−	−	NT	NT
281	+	+	+	+	+	+	+	+
323	−	−	+	−	Indeterminate	−	−	−
329	−	−	+	−	Indeterminate	−	−	−

*Four donors were RIBA-positive and two indeterminate. Five were RT-PCR-positive, including a RIBA-negative sample (230). NT = not tested.

in an unrealistic proposition. This system recognizes degrees of divergence among various HCV isolates. Thus, new isolates with less than 72% nucleotide identity with isolates of known sequence potentially may be considered new genotypes; subtypes within a genotype have 75%–86% identity with one another; and isolates within a subtype have greater than 88% identity [Simmonds et al., 1994a].

There is a suggestion that progression and outcome of HCV disease and response to antiviral therapy such as with interferon may vary with genotype [Kanai et al., 1992; Chan et al., 1993; Chemello et al., 1994]. Furthermore, HCV of diverse genotype may elicit serological responses which are undetectable by commercial assays and also may be undetectable by the polymerase chain reaction (PCR) where sequence variation occurs in the primer binding sites. The presence of diverse genotypes within a population may have implications for diagnostic testing and future vaccine development.

Nucleotide sequences have been determined for HCV

isolates from many countries worldwide but relatively few have been reported from sub-Saharan Africa. The majority of these were of genotype 4 [Bukh et al., 1992; Simmonds et al., 1993; Xu et al., 1994; Stuyver et al., 1994]. Moreover, none has been reported from Nigeria. In the present study, a pilot survey of HCV infection was carried out among adult donors and children of preschool age in Nigeria. We aimed to correlate antibody positivity with viremia, as determined by the PCR, and to determine the predominant genotypes by sequence analysis.

MATERIALS AND METHODS

Serum samples from 200 healthy adult blood donors and 100 children of preschool age were screened for anti-HCV using a second generation enzyme-linked immunosorbent assay (ELISA) (Murex Diagnostics, Dartford, UK). The ELISA-positive sera were tested further with second generation recombinant immunoblot assay

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         -250
HCV-1    GGCGTTAGTA TGAGTGTCGT GCAGCCTCCA GGACCCCCCC TCCCGGGAGA GCCATAGTGG

HCV 089  .......... .......T.. A......... .......... .......... ..........

HCV 202  .......... .......T.. A......... .......... .......... ..........

HCV 230  .......... .......... .......... .......... .......... ..........

HCV 116  .......... .......T.. A......... .......... .......... ..........

HCV 281  .......... .......... A......... .......... .......... ..........


         -189
HCV-1    TCTGCGGAAC CGGTGAGTAC ACCGGAATTG CCAGGACGAC CGGGTCCTTT CTTGG*ATCA

HCV 089  .......... ........T. ...*....C. ..G...T... .......... .....A..A.

HCV 202  .......... .......... .........C. ..G....... .......... .....*..T.

HCV 230  .......... .......... .......... .......... .......... .....*....

HCV 116  .......... ........T. .........C. ..G...T... .......... .....A..A.

HCV 281  .......... .......... ...*...... ..G...T... .......... .....A..A.


         -129
HCV-1    ACCCGCTCAA TGCCTGGAGA TTT*GGGCGT GCCCCCGCAA GACTGCTAGC CGAGTAGTGT

HCV 089  .......... ....C...A. ...G...... .......... .......... ..........

HCV 202  .......... ....C...A. ...*...... .......... .......... ..........

HCV 230  .......... .......... ...*...... .......... .......... ..........

HCV 116  .......... ....C..... ...G...... .......... .......... ..........

HCV 281  .......... .......... ...*...... .......... .......... ..........


         -69
HCV-1    TGGGTCGCGA AAGGCC

HCV 089  .......... ......

HCV 202  .......... ......

HCV 230  .......... ......

HCV 116  .......... ......

HCV 281  .......... ......
```

Fig. 1. Sequences of cDNA amplified from the 5'UTR of five HCV isolates from adult blood donors in Nigeria. The sequences are aligned with the HCV prototype, genotype 1a, and numbered according to Choo et al. [1991]. Asterisk (*) represents deletion in the sequence compared to HCV-1.

(RIBA-2) (Chiron Corporation, Berkeley, CA) and for HCV RNA using reverse transcriptase (RT)-PCR.

For RT-PCR, RNA was extracted from 100 μl of serum using the guanidinium isothiocyanate method (RNaid Kit, BIO 101, Vista, CA). The pellets were resuspended in 30 μl of diethylpyrocarbonate (DEPC)-treated water and 10 μl was used for cDNA synthesis with Moloney murine leukemia virus RT (Pharmacia, Uppsala, Sweden) using random hexanucleotides as primers. PCRs were carried out using the primers listed in Table I.

For amplification of the 5'UTR, first round PCR was carried out in a 100 μl reaction using external primers 939 and 209 and 50 cycles of 94°C for 1 minute, 50°C for 1 minute, and 72°C for 2.5 minutes with a final extension time of 9.9 minutes. Ten microliters of the first round PCR product was used for the second round,

nested PCR with internal primers 940 and 211 for 30 cycles with the same conditions. Both rounds of amplification, and extraction steps included appropriate positive and negative controls.

The nucleocapsid region was amplified by nested PCR using primers described by Xu et al. [1994]. First round PCR was carried out using external primers CPS14 and CPA14 and 50 cycles of 95°C for 1.5 minutes, 42°C for 1.5 minutes, and 72°C for 2.5 minutes with a final extension time of 9.9 minutes. Second round PCR was carried out using internal primers CPS15 and CPA15 and 30 cycles with the same conditions.

PCR of the NS5 region was carried with primers described by Mellor et al. [1995] which was highly conserved among different isolates of HCV. First round PCR was carried out using external primers 1203 and 1204

```
                       7955
HCV-1          ACATCCGTAC GGAGGAGGCA ATTTACCAAT GTTGTGACCT GGACCCCCAA GCCCGCGTGG
HCV 230        .......... .......... ...CA..... .......... .......... .G....T...
HCV 281        .......... ...A...... .......... A......... ...C....TG A.G...A...
HCV 089        C..A....CA C.GA....G. CATCAGG.CC .AG.GAGAGG TCTAT.AGT. TTGT.ACCTC


                       8015
HCV-1          CCATCAAGTC CCTCACTGAG AGGCTTTATG TTGGGGGCCC TCTTACCAAT TCAAGGGGGG
HCV 230        .......... .......... .......... .......... .......... ..........
HCV 281        .......A.. .......... .......... ..C....G.A ........C. .C.AA..T..
HCV 089        ATGCAC.A.A G.AAGGG.GA CCT..GCGGT G.CC.TAGGT G.CGCG.T.G CGGC.TATTC


                       8070
HCV-1          AAAACTGCGG CTACCGCAGG TGCCGCGCGA GCGGCGTACT GACAACTAGC TGTGGTAACA
HCV 230        .G.....T.. .......... .......... .......... .......... ..........
HCV 281        .......C.. .........TC .....T..A. ..C.G..G.. ...C...... .....C....
HCV 089        .CC..CAGCT TCGGGAACAC ACTGA..T.C TACCTTA.GG CCAC.GCC.. ACA.AGGG.G


                       8130
HCV-1          CCCTCACTTG CTACATCAAG GCCGGGCAG CCTGTCGAGC CGCAGGGCTC CAGGACTGCTA
HCV 230        .......... .......... .......... .......... .......... .G.........
HCV 281        ....T..C.. T...C....A ..A.C..G.. .......... ....CA..... .A........
HCV 089        G.GGG....C T.CGGGA.CA CA.T.ACGT. .TACCTT.AG GC..CA..CG .ACAGAG.GC


                       8190
HCV-1          CCATGCTCGT GTGTGGCGAC GACTTAGTCG TTATCTGTGA AAGTGCGGGG GTCCAGGAGG
HCV 230        .......... .......... .......... .......C.. G......... ..........
HCV 281        .......... ...C...... ...C.T..T. .C........ ...C.GC... ..........
HCV 089        GGCG.GA.T. AAAGATTGCA CCA.GCTGGT C.GCGGCGAC T.CCTG.TC. TCATGCCT.A


                       8250
HCV-1          ACGCGGCGAG CCTGAGAGCC
HCV 230        ...A.CG..A ..........
HCV 281        .......A.. ..........
HCV 089        .A.T.A..GC GTC..CGAGG
```

Fig. 2. Nucleotide sequence of the NS5 region from isolates HCV230, HCV281, and HCV089. The sequences are aligned with the HCV prototype, genotype 1a, and numbered according to Choo et al. [1991].

with 50 cycles of 95°C for 1 minute, 44°C for 1 minute, and 72°C for 2.5 minutes with a final extension time of 9.9 minutes. The second round PCR was carried out using internal primers 518 and 123 (and, in one case, a seminested reaction with primers 518 and 1204) and 30 cycles of 95°C for 36 seconds, 45°C for 1.5 minutes, and 68°C for 3 minutes with a final extension time of 9.9 minutes.

PCR products were purified using a QIAGEN DNA extraction kit (QIAGEN GMBH, Hilden, Germany). Two microliters (approximately 10 ng) of the purified PCR product was cloned into the TA cloning vector (Invitrogen, San Diego, CA) and used to transform competent cells (INVaF'). Twenty microliters (approximately 10 ng) of the extracted DNA was denatured in 1 M NaOH, 10 mM EDTA (pH 8.0), and kept at room temperature for 30 minutes. The relaxed plasmid DNA was separated by spinning through sepharose CL6B and kept on ice. This was then annealed to the primer and sequencing reactions were carried out using T7 DNA polymerase (Sequenase version 2.0, United States Biochemical Corporation, Cleveland, OH) according to the manufacturer's instructions. Each cloned amplicon was sequenced on both strands; an average of six templates was sequenced in each case. Individual sequences were compared independently to the EMBL data base using the BLAST alignment search program [Karlin and Altschul, 1990; Altschul et al., 1990].

RESULTS

Sixteen (8%) of the 200 adult blood donors, but none of the 100 preschool children, were anti-HCV-positive by ELISA. Four (25%) of the ELISA-positives were positive on supplemental testing using the second generation RIBA and 2 (12.5%) were indeterminate. Table II shows the results of the RIBA testing and RT-PCR (5'UTR,

TABLE III. Pairwise Comparison of Nine Partial Nucleotide Sequences Derived From the NS5 Region of Isolates of HCV Genotype 1 (Nt 8295–8625 According to Choo et al. [1991])*

	1a			1b			1c		
	M62321	M67463	HCV230[a]	M58335	D90208	M84754	L23446	L23447	HCV281[a]
M62321[b]	100	96	95	82	80	81	84	82	85
M67463[b]		100	94	81	80	80	83	81	84
HCV230[a]			100	78	78	77	84	82	84
M58335[b]				100	92	95	83	81	78
D90208[b]					100	91	76	76	80
M84754[b]						100	75	76	79
L23446[b]							100	95	77
L23447[b]								100	76
HCV281[a]									100

*The values represent percentage nucleotide identity between various representatives of HCV genotypes 1a, 1b, and 1c.
[a]Nigerian HCV isolates.
[b]Data base accession number.

NS5, and nucleocapsid regions) for the 16 anti-HCV-positive adult donors. All four RIBA-positive samples were positive for RT-PCR targeted to the 5′UTR. The two RIBA indeterminate samples were negative by RT-PCR. However, 1 of the 10 RIBA-negative samples was positive using RT-PCR.

5′UTR sequences were determined for the five positive samples. Figure 1 is an alignment of these sequences with that of HCV-1. Nucleotide sequence comparison showed that the isolates can be divided into two groups. The first group consists of the HCV isolates from donors 230 and 281 which have greater than 93% identity to various HCV genotype 1 isolates from the United States, Germany, the United Kingdom, and Canada. The second group is from donors 089, 116, and 202 and have greater than 95% homology with published HCV genotype 4 sequences of sub-Saharan African origin [Bukh et al., 1992; Xu et al., 1994]. None of the sequences has 100% identity with any sequence in the data base.

Amplification of the NS5 and nucleocapsid regions was attempted only for the seven samples which were RIBA-positive or indeterminate or positive using RT-PCR of the 5′UTR. None of the samples could be amplified using the method of Valliammai et al. [1995] and primers derived from the NS5 region described by Eno-moto et al. [1990] and Chan et al. [1992]. However, three of these samples (089, 230, and 281), all of which were positive using RT-PCR of the 5′UTR, were amplified following attempts with a variety of primer pairs as described by Mellor et al. [1995]. Nested PCR with primers 518 and 123 (Table I) amplified cDNA from samples 230 and 281, while only seminested PCR with primers 518 and 1204 was successful for sample 089.

The results of the sequence analysis of the NS5 region are shown in Figure 2. Comparison with sequences in the data base confirmed the provisional assignment of genotype made from the 5′UTR sequences. The sequence of isolate 230 has greater than 94% homology with genotype 1a sequences, while that of isolate 281 has only 76–85% homology with the various genotype 1 sequences in the data base. Pairwise comparison of the two sequences with seven representatives of genotypes 1a, 1b, and 1c (Table III) clearly shows that isolate 230 can

be classified as genotype 1a, but isolate 281 cannot be assigned to the existing subtypes of genotype 1 according to the criteria of Simmonds et al. [1994a,b] because it has less than 86% nucleotide identity with members of those subgroups. Isolate 281 may, therefore, be considered a novel subtype within genotype 1.

The sequence derived from isolate 089 has greatest similarity (82%) with an HCV genotype 4 sequence originating from sub-Saharan Africa. Pairwise comparison of this sequence with the representatives of the genotypes 4a, 4c, 4d, 4e, 4f, 4g, and 4h (Table IV) confirmed that this isolate cannot be assigned to those subtypes. However, NS5 sequences derived from genotype 4b are not available in the data bases at present.

Only two of the samples (281 and 089) could be amplified using primers from the nucleocapsid region following a repeated attempt with reduction of the annealing temperature from 45 to 42°C. These and the others shown as negative in Table II could not be amplified using a 45°C annealing temperature. Sample volumes were insufficient for a repeated attempt with the latter set except for sample 230, which could not be amplified.

Sequence analysis of the nucleocapsid region further confirmed isolate 281 as genotype 1 and isolate 089 as genotype 4. The alignment of these sequences with HCV-1 and the Gabonese type 4c [Xu et al., 1994] is shown in Figure 3. Table V is a pairwise comparison of the isolate 089 nucleocapsid sequence with representatives of genotypes 4a, 4b, 4c, 4e, and 4f. While subtype to subtype sequence variation in HCV is less for the nucleocapsid region than NS5, it is clear that the 089 sequence is distant from that of the genotype 4b isolate. Considering the NS5 and nucleocapsid sequence data together, it seems that isolate 089 represents a new subtype within genotype 4 in addition to the eight subtypes described previously [Stuyver et al., 1994; Mellor et al., 1995]. We have designated this provisionally genotype 4i.

DISCUSSION

A small-scale survey of HCV infection in Nigeria was carried out on adult blood donors and children of preschool age. It should be noted that the donor population

TABLE IV. Pairwise Comparison of 14 Partial Nucleotide Sequences Derived From the NS5 Region of Isolates of HCV Genotype 4*

	L23470	L29602	L29605	EUYEM2	L29590	L29626	L29596	L29618	L29621	L29613	L29611	HCV089	L36438	L36437
L23470[a]	100	83	83	79	86	84	80	78	77	80	79	80	79	82
L29602[a]		100	93	76	85	85	82	78	77	81	80	79	80	81
L29605[a]			100	81	86	82	82	79	79	82	82	79	81	81
EUYEM2[a]				100	84	83	82	77	76	81	80	76	81	79
L29590[a]					100	93	84	80	80	86	86	82	83	83
L29626[a]						100	85	80	80	86	85	82	81	83
L29596[a]							100	81	80	84	83	79	81	81
L29618[a]								100	99	84	84	76	81	76
L29621[a]									100	84	84	76	80	76
L29613[a]										100	99	80	81	81
L29611[a]											100	80	80	81
HCV089[b]												100	81	80
L36438[a]													100	79
L36437[a]														100

*The values represent percentage nucleotide identity with genotypes 4a (L23470), 4c (L29602, L29605), 4d (EUYEM2), 4e (L29590, L29626), 4f (L29596), 4g (L29618, L29621), 4h (L29613, L29611), and two unassigned sequences CAR1 (L36438) and CAR4 (L36437). Pairwise comparisons are made over 329 nucleotides (nt 8276–8605 according to Choo et al., 1991) except for L23470, where only 222 nucleotides are available in the data base.
[a]Data base accession number; EUYEM2 is from Mellor et al. [1995].
[b]Nigerian HCV isolate.

```
        -20
HCV-1   TCTCGTAGAC CGTGCACCAT GAGCACGAAT CCTAAACCTC AAAAAAAAAA CAAACGTAAC
HC-G6   .......... .......... .......... .......... ...G.....C ..........
HCV89   .......... .......... .......... .......... ...G.....C ..........
HCV281  .......... .......... .......... .......... ...G.....C ..........

        31
HCV-1   ACCAACCGTC GCCCACAGGA CGTCAAGTTC CCGGGTGGCG GTCAGATCGT TGGTGGAGTT
HC-G6   ........:C. ....CAT... ...T...... ......C.T. .C........ ..........
HCV089  ........C. ....CAT... ...T...... ........T. .C........ ...C......
HCV281  ........C. ....CAT... ...T...... ......C.T. .......... ...C......

        131
HCV-1   TACTTGTTGC CGGCAGGGG CCTAGATTGGG TGTGCGCGCG ACGAGAAAGA CTTCCGAGCG
HC-G6   .......... ......... ..C..G..... .......... ...T.G.... ....G.....
HCV089  .......... .......... ..C....... .......... ...TC.G... .....G....
HCV281  .......... .......... ..C..G.... .......... ......G... ..........

        181
HCV-1   GTCGCAACCT CGAGGTAGAC GTCAGCCTAT ATCCCCAAGG CTCGTCGGCC CGAGGGCAGG
HC-G6   .......... ..T..G.... .......... .......... ...A....AT .T.....A..
HCV89   .......... .......... T..T...... .T..AG.C.. .C..A..... ..........
HC281   .......... ..T..A...G .G........ .....C.... ..A..C.... ..........

        231
HCV-1   ACCTGGGCTC GGGATGGCTC CTGTCTCCCC GTGGCTCGGC CTAGCTGGGG CCCCACAGAC
HC-6    T......... .......... .....A.... .T...T..C. T........T ..........
HCV89   ..G.T.G... .......... T........G .......... .......... ..........
HCV281  T......... ..C....... ..C....... .....A.... .A........ .......C..

        291
HCV-1   TACCTCATGG GCCCCCGGCG TAGGTCGCGC AATTTGGGTA AGGTCATCGA TACCCTTACG
HC-G6   ..A....... .....A... ..........A ..GTCT.... ....A.AT.. .........A
HCV089  ..A....... .....AG.G. ..C....... ....CT.... ....G.AT.T ..........
HCV281  .....T.... .....T...T .........C .....A.... ..C....... ..........

        361
HCV-1   TGGGCTTCGC CGACCCTTGG CCCCTCTATG GCAGCCCCGG GTAATGAGGG CTGCGGGTGG
HCV-G6  .....C.... .C........ .......... ........A. T.......T. .........T
HCV089  .......... .......... .......... T......... .....C.... .C....A..G
HCV281  .....G.... .T..C...GC .......... ..A....T.. .......T.. ..........

        421
HCV-1   GGTACATACC GCTCGTCGGC GCCCCTCTTG GAGGCGCTGC CAGGGCCCTG GCGCAT
HC-G6   ...A...... ...A....A. .....CG.G. .C....TC.. .......... ..A...
HCV089  ......A... .......... ..T.T..... ....C..G.. ......G.G. ..C.T.
HCV281  ....T..A.. ...G...... ........T. ..C..A.... ..A.T..A.. ..A.A.
```

Fig. 3. Sequence of the core region from two Nigerian isolates of HCV (089 and 281) from Nigeria. The sequences are aligned with prototype HCV-1 and Gabonese HCV type 4 (HC-G6) and numbered according to Choo et al. [1991].

in Nigeria predominantly is male. Using a second generation anti-HCV ELISA, a positivity rate of 8% was found for the donors. This is unusually high but the results of supplementary testing and RT-PCR analysis imply a high rate of false positivity. None of the children tested was positive, lending support to the view that mother to infant transmission of HCV is infrequent [Zanetti et al., 1995] and infection probably is acquired later in life through exposure in the community, including percutaneous practices and blood transfusion (although the latter is uncommon in Nigeria).

Through nucleotide sequence analysis from the 5'UTR, NS5, and nucleocapsid regions, combined with pairwise comparisons with HCV sequences in the data bases, we determined that there are at least two major HCV genotypes, 1 and 4, in Nigeria. Genotype 1 is believed to be distributed worldwide and genotype 4 is distributed widely in Africa. Novel subtypes were identifed within both genotypes and these we have designated provisionally 1d and 4i. Genotypes 1 and 4, along with genotype 2, also have been reported from neighboring Cameroon [Ngengasong et al., 1995]. We cannot rule out the possibility of HCV with more divergent sequences and which are not detected by these assays. Failure to amplify the NS5 region of two and the nucleocapsid region of three of the 5'UTR PCR-positive sera may be a further indication of diversity among these viruses.

TABLE V. Pairwise Comparison of Eight Partial Nucleotide Sequences Derived From the Nucleocapsid Region
of Isolates of HCV Genotype 4*

	U10236	U10235	U10239	U10238	L29587	L29624	U10240	HCV089
U10236[a]	100	91	89	89	92	93	92	91
U10235[a]		100	89	88	90	91	92	88
U10239[a]			100	95	90	90	92	89
U10238[a]				100	91	92	91	90
L29587[a]					100	96	91	91
L29624[a]						100	91	92
U10240[a]							100	89
HCV089[b]								100

*The values represent percentage nucleotide identity over 463 nucleotides (nt 342–811 according to Choo et al., 1991) and between various representatives of genotypes 4a (U10236), 4b (U10235), 4c (U10239, U10238), 4e (L29587, L29624), and 4f (U10240).
[a]Data base accession number.
[b]Nigerian HCV isolate.

In conclusion, despite the small size of this survey, HCV infection seems to be highly endemic in Nigeria. Detection of HCV in adults but not children of preschool age implies that perinatal transmission is not a major route of infection. There are at least two major genotypes of HCV in Nigeria, genotypes 1 and 4, and novel subtypes are present within these genotypes.

ACKNOWLEDGMENTS

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