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**MOLECULAR DIAGNOSIS OF HEPATITIS C VIRUS IN  
GHANAIAN BLOOD DONORS**

**By**

**NICHOLAS ISRAEL NII-TREBI: 10074739**

**BSc (Ghana), 1998**



**September, 2002**

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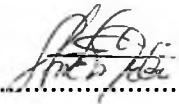
This thesis is submitted to the University of Ghana, Legon in partial fulfillment of the requirement for the award of M.Phil degree in **Biochemistry**

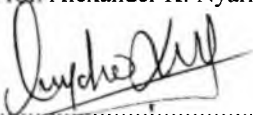


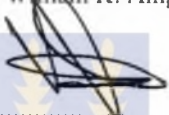
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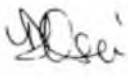
**DECLARATION**

I certify that the work described in this report was carried out by me at the Virology Unit, Noguchi Memorial Institute for Medical Research and the Biochemistry Department of the University of Ghana, Legon under the supervision of Prof. Alexander K. Nyarko, Dr. Yaa D. Osei and Dr. William K. Ampofo.

Nicholas Israel Nii-Trebi  .....Date 30-05-03.....  
Student

Prof. Alexander K. Nyarko  
  
.....  
Supervisor

Dr. William K. Ampofo  
  
.....  
Supervisor

Dr. Yaa D. Osei  
  
.....  
Supervisor



## **DEDICATION**

To my parents, Mr. & Mrs. Ebenezer Trebi .

To my siblings, Caro, Ruth, Pat, Enock, Esther and to all members of the Great Family.



### ACKNOWLEDGEMENTS

I am sincerely grateful to the Lord God Almighty for His protection, provision and guidance for me throughout my graduate program.

I acknowledge my indebtedness to my supervisors, Prof. Alexander K. Nyarko, Dr. William K. Ampofo, both of Noguchi Memorial Institute for Medical Research (N. M. I. M. R), and Dr. Yaa D. Osei of the Department of Biochemistry, University of Ghana, Legon, whose keen interest, selfless devotion, priceless contributions, very helpful criticisms and suggestions helped me to complete this thesis. My heartfelt appreciation goes to Dr. Koichi Ishikawa, Dr. Fukasawa, Dr. Kenzo Tokunaga, and Dr. Soturo Takeo, Japanese visiting scientists for the technical expertise and material support they placed at my disposal that made this work possible.

I wish to thank all the lecturers of the Biochemistry Department, especially Dr. W. S. K. Gbewonyo, and the technical staff for their special assistance. I say a big thank you to my only colleague, Evelyn Y. Ugly-Kwame for her encouragement and support especially during the tough times. My warmest appreciation goes to Messrs Harry Asmah, Charles Brown, David Mensah, Victor Nuvor, Arthur Quarm, Mrs. Regina Apiah-Oppong, Mrs Anita Ghansa, all of the N. M. I. M. R, Mr. Emmanuel Lamptey of Oceanography Department of the University of Ghana, Legon and Mrs. Nancy Commodore for the co-operation, love and essential contributions they offered during the period. Special thanks go to the Administration of the N. M. I. M. R for the permission granted me for the use of their facilities. To the Head of the Virology Unit of the N. M. I. M. R, Dr. Mubarak Osei-Kwasi, and all the members of staff of the Unit, I am very much grateful for their invaluable suggestions and contributions.

Special appreciation is also due to the Head and staff of the National Blood Transfusion Service (N. B. T. S), Korle Bu Teaching Hospital especially Mr. Emmanuel Tetteh and Ms. Margaret Kumaka for their support during sample collection. To my friends, and those unnamed but who all helped in diverse ways to make the completion of this work possible, I say thank you and may God reward all your efforts.

Finally, my warmest appreciation goes to all my dear family members who, due to concentration on this work, were denied my attention and services. May the Good Lord richly bless you all for your love, understanding and care.

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**LIST OF ABBREVIATIONS**

<b>AMV</b>	Avian myeloblastosis virus
<b>E</b>	<b>as in E1, E2</b> denotes <i>envelope</i> glycoprotein
<b>HAV</b>	Hepatitis A virus
<b>HBV</b>	Hepatitis B virus
<b>HCV</b>	Hepatitis C virus
<b>α-IFN</b>	Alpha interferon
<b>IRES</b>	Internal ribosomal entry site
<b>M-MuLV</b>	Moloney murine leukemia virus
<b>NS</b>	<b>as in NS3, NS4</b> , etc denotes <i>Non-structural</i> protein
<b>PBMC</b>	Peripheral blood mononuclear cells
<b>RdRp</b>	RNA-dependent RNA polymerase
<b>RIBA-3</b>	Third generation <i>recombinant immunoblot assay</i>
<b>RT-PCR</b>	Reverse-transcriptase polymerase chain reaction
<b>5'UTR / 5'NCR</b>	5'-Untranslated region / 5'-Noncoding region

## ABSTRACT

The geographical distribution of different types and subtypes and the prevalence of hepatitis C virus (HCV) vary significantly. Identification of HCV genotypes has become increasingly important for the clinical management and prognosis of HCV infections.

Available evidence shows that HCV is endemic in West Africa, but there is little data on HCV genotypes present in Ghana. This study therefore set out to identify circulating HCV genotypes in healthy Ghanaians and determine the relationship to genotypes from other geographical regions.

Plasma were obtained from 200 blood donors in the year 2002 and tested for antibodies against HCV. RNA was extracted from anti-HCV-positive sera and reverse-transcribed into cDNA. The cDNA preparations were subjected to nested and semi-nested PCR to amplify 5'-untranslated region (5'UTR) and the core sequences, respectively. Nested PCR was then used to identify HCV genotypes using type-specific primers. Semi-nested PCR amplified core sequences were cloned into a TOPO vector (Appendix I). The clones were transformed into *E. coli* cells and cultured; DNA was purified from the cells and sequenced. HCV cDNA sequences were analysed and compared for intra- and inter-donor differences and with reported sequences for similarity with those from other regions.

HCV RNA was detected in only 2 of 13 (15.4%) HCV seropositive donors. Genotype 2 was the only genotype found and subtypes 2a and 2b were detected in one subject while the other had 2a only.

Comparison of nucleotide sequences from position 329 to 658 of the HCV genome showed the following characteristics: (i) the nucleotide sequence similarity was 95 to 96%; (ii) intra- / inter-donor difference in nucleotide sequences observed was 30% and an intra- / inter-donor similarity was at least 94%.

The phylogenetic analyses of the core region indicated that collectively, the Ghanaian HCV RNA extracts formed a diverse single phylogenetic group. The existence of rapid genotype variation within a single individual was observed. This study also shows that HCV genotype 2a is prevalent in Ghana.

## CHAPTER ONE

### INTRODUCTION AND LITERATURE REVIEW

#### 1.1 INTRODUCTION

Viral hepatitis is a systemic disease primarily involving the liver. It presents a major health problem throughout the world (Prince *et al.*, 1993; Alter *et al.*, 1992). Most cases of acute viral hepatitis in children and adults are caused by hepatitis A virus (HAV) and hepatitis B virus (HBV). Hepatitis C virus is the most important agent of chronic or acute parentally transmitted viral hepatitis not attributable to HAV or HBV and is known as non-A, non-B or NANB hepatitis (Dana *et al.*, 1994; Choo *et al.*, 1990; 1989).

Hepatitis C virus causes persistent infections in more than 90% of infected people. An estimated 70 to 90% of infections become chronic with progression to cirrhosis in at least 20 % of infected individuals after 10 to 20 years (Barera *et al.*, 1995; Thomas *et al.*, 1995; Alter *et al.*, 1992). It is also associated with hepatocellular carcinoma (Alter, 1995). Persons with HCV infection may have no symptoms for several years and many infected people do not know they have the virus. No vaccine for HCV is available, and antiviral treatments, generally alpha interferon ( $\alpha$ -IFN), are marginally effective (NIH, 1997). Studies have demonstrated that the subtype of the infecting HCV strain seems to influence the clinical course of the infection as well as the outcome of therapy with alpha interferon (Prati *et al.*, 1996; Takada *et al.*, 1996; Lau *et al.*, 1995).

HCV has a single-stranded RNA genome of positive (protein-coding) polarity. It has a genetic organization similar to that of the *Flaviviridae* family, which includes the pesti-

and the flaviviruses to which the yellow fever virus belongs (Alter, 1995; Choo *et al.*, 1991; Miller *et al.*, 1990). Around 96% of the genome codes for 9 viral proteins, of which 3 are structural and 6 non-structural, with non-coding regions at both the 5' and 3' ends (Choo *et al.*, 1991). These structural and non-structural proteins form the basis of diagnostic tests to detect the presence of anti-HCV antibodies and the viral genome.

HCV has a high rate of genetic mutation (Ogata *et al.*, 1991), and as a result, extensive genetic heterogeneity of HCV occurs in infected individuals. HCV isolates are found as either a group of isolates with very closely related genomes, referred to as quasispecies, or genetically distinct groups called 'genotypes' (Bukh *et al.*, 1995). Since the original classification of HCV into 6 major genotypes, sequence comparisons of HCV derived from a much wider range of geographic locations have provided evidence for the existence of a large number of variants (Simmonds *et al.*, 1994). Presently at least 50 subtypes of HCV have been described on the basis of phylogenetic analysis (Smith *et al.*, 1997; Simmonds *et al.*, 1995; Stuyver *et al.*, 1994).

HCV typing has been established by methods mainly based on polymerase chain reaction (PCR) and / or serology (Cerino *et al.*, 1996). All HCV genotyping assays operate on the crucial assumption that the region analysed – the 5' untranslated region (5' UTR), core, NS4 or NS5b (see Fig. 1) is representative of the whole genome. This assumption would break down if recombination between HCV genotypes occurred during virus replication. Comparisons of genotyping assays based on sequence analysis of different regions of the genome, and between PCR-based and serological typing assays have given remarkably

consistent results (Fields *et al.*, 1996). This suggests that genotyping is a valid procedure despite the theoretical possibility of recombination (Bhattacharjee *et al.*, 1995; Lau *et al.*, 1995; Tanaka *et al.*, 1994).

The availability of the nucleotide sequence of HCV makes use of reverse transcriptase (RT) PCR, which targets the highly conserved 5' noncoding regions (5' NCR) as a direct and reliable assay. Many of the current methods for genotyping rely on the following (i) PCR amplification of virus sequences in clinical specimens, using type-specific primers that selectively amplify different genotypes (Okamoto *et al.*, 1993) (ii) Analysis of the PCR product by hybridization with genotype-specific probes (Stuyver *et al.*, 1994), (iii) Restriction fragment length polymorphisms (RFLP) (Gish *et al.*, 1999; Davidson *et al.*, 1995; McOmish *et al.*, 1993) (iv) Reverse dot blot (Stuyver *et al.*, 1996), and (iv) Heteroduplex mobility analysis (White *et al.*, 2000).

First, second and third generation HCV assays target antigens to the core region (c22c) and one or more non-structural regions - NS3 (c33), NS4 (c100-3) or NS5. These assays proved sensitive and specific for screening blood donations to eliminate post-transfusion HCV infections (Aach *et al.*, 1991). However, low incidence, sequence diversity or impairment of immune response has rendered such HCV assays, especially the first and second-generation assays, prone to non-specific cross-reactivity. Besides, non-specific reactions occasionally originate from the bacterial fragment of the fusion protein, during expression of HCV recombinant proteins in bacterial cells for anti-HCV tests.

In West Africa, although HCV is recognized as endemic (Jeannel *et al.*, 1998), information on HCV infection in the Ghanaian population is limited. Recent information on HCV seroprevalence was generated by investigations on seroprevalence of blood-borne infectious diseases in Ghana. Initial screening using a particle agglutination assay showed that 8.4% of the healthy donors were HCV positive. However, supplementary analysis using third-generation recombinant immunoblot assay (RIBA 3) and PCR confirmed HCV seroprevalence as only 0.9% (Ampofo *et al.*, 2002).

The demonstration of HCV seroprevalence in Ghana illustrated the need for HCV screening for would-be blood donors (Ampofo *et al.*, 2002; Acquaye *et al.*, 2000; Wansborough-Jones *et al.*, 1998). Currently, anti-HCV screening among blood donors is under implementation in the country. Although present HCV screening assays may be useful at preventing transmission of HCV infection, employing molecular technology to confirm HCV positivity may prove useful especially in reducing significant loss of blood donations caused by lack of specificity of most screening assays (Courouce *et al.*, 1998). In addition to ensuring the maximum possible safety of transfused blood and components, the proper management of HCV infection is very important. It has been documented that certain genotypes of HCV are associated with rapid disease progression (Hotta *et al.*, 1997). Besides, antigenic differences between genotypes have implications for development of vaccines for HCV and for the optimal design of serological screening and confirmatory assays for HCV. Molecular diagnosis of HCV is therefore very essential for the clinical management and public health control of HCV infection.

## AIMS AND OBJECTIVES

Genotypic differentiation of HCV has served as an important epidemiological tool for the study of the geographic distribution of HCV genotypes, their routes of transmission, and their association with certain risk factors. Heterogeneity in sequence seen among HCV genotypes may be associated with various antigenic and biological properties (Zein *et al.*, 1995). Besides, outcome of liver disease and rates of response to interferon therapy may vary according to HCV genotype (Yoshioka *et al.*, 1992). Therefore, identification of HCV genotypes may provide for prognosis and therapy. In Ghana, although currently blood for transfusion is routinely screened for HCV in addition to human immunodeficiency virus (HIV) and hepatitis B virus (HBV), there is little detail on occurrences of HCV subtypes in the country. This study was therefore conducted to identify HCV genotypes in healthy Ghanaians. This would generate data on HCV genotype distribution, to inform policy making on the management of HCV infections in the country. The study was conducted to specifically:

- 1) Screen blood donors in Accra for hepatitis C virus infection,
- 2) Extract and genotype hepatitis C virus from infected blood specimens and
- 3) Determine intra- and inter-donor HCV subtype differences by sequence analyses.

## 1.2 LITERATURE REVIEW

Hepatitis C virus infection is an important public health problem, affecting the well being of an estimated 75-150 million people worldwide. Prevalence of HCV infection in blood donors worldwide is summarised in Fig 1. Prevalences generally vary between 0.04% and 3.5% in most parts of the world (Shakil *et al.*, 1995; Dow *et al.*, 1993, Tretskaia *et al.*, 1993, Galban *et al.*, 1992). Relatively higher prevalences (4 -14%) have been recorded in Africa (Iliako *et al.*, 1995; Ndume and Skalsky, 1993). The highest prevalence (26%) thus far reported was found in Cairo, Egypt (Bassily *et al.*, 1995).

The precise mode of acquisition HCV is often uncertain. However, HCV is known to be transmitted by parenteral, or inapparent parenteral routes such as blood transfusion, exposure to blood or blood components, intravenous drug abuse, sex (though inefficient and infrequent means) and maternal-infant transmission (relatively uncommon). Hepatitis C virus infection may remain clinically silent or progress to cirrhosis. The illness caused by HCV infection has a complex natural history, and the ultimate, long-term prognosis for patients with chronic hepatitis is difficult to predict. However, the rate of disease progression and the morbidity associated with HCV are known to be affected by many interactive factors. They include age of acquisition, concomitant alcohol abuse, gender, coexisting viral disease, host immune response, level of viraemia, HCV genotype and degree of viral diversity (Zein, 2000; Dusheiko *et al.*, 1993; Gordon *et al.*, 1993; Pozzato *et al.*, 1991).



Fig. 1. Worldwide prevalence of anti-HCV among volunteer blood donors (Decker and Troonen, 2001).

The structure and assembly of HCV as well as the function of various HCV genome-coded proteins play a significant role in production of genetic variants. This variation results in differences in infectivity, severity or spectrum of disease and response to treatment (Dusheikko *et al.*, 1994).

### **Genome Organization of HCV**

The genome organization of HCV (Fig. 2) is similar to that of the flaviviruses and pestiviruses (Miller *et al.*, 1990; Bradley, 1985). It is an enveloped particle harbouring a plus-strand RNA that has a length of about 9600 nucleotides (Fig 2). The genome carries a single long open reading frame (ORF) that occupies around 96% of the entire genome. The ORF encodes a single large polyprotein precursor of approximately 3,010 amino acids, that is cleaved co- and posttranslationally by host and viral proteases to yield nine individual structural and nonstructural viral proteins: core, envelope 1(E1), E2; non-structural (NS) 2, NS3, NS4a, NS4b, NS5a and NS5b. The polyprotein gene is flanked by non-coding regions at both the 5' and 3' ends (Choo *et al.*, 1991).

The structural proteins are located in the amino terminal one-third region and the nonstructural replicative proteins in the remaining region of the genome (Reed *et al.*, 1998). The first 40 nucleotides of the RNA genome are not required for translation but are involved most likely in replication (Boyer and Haenni, 1994). Translation of the HCV open reading frame (ORF) is directed through a 341-nucleotide long 5' NTR functioning as internal ribosome entry site (Tsukiyama-Kohara *et al.*, 1992). The 3' non-translated region (NTR) has a tripartite structure composed of a variable sequence

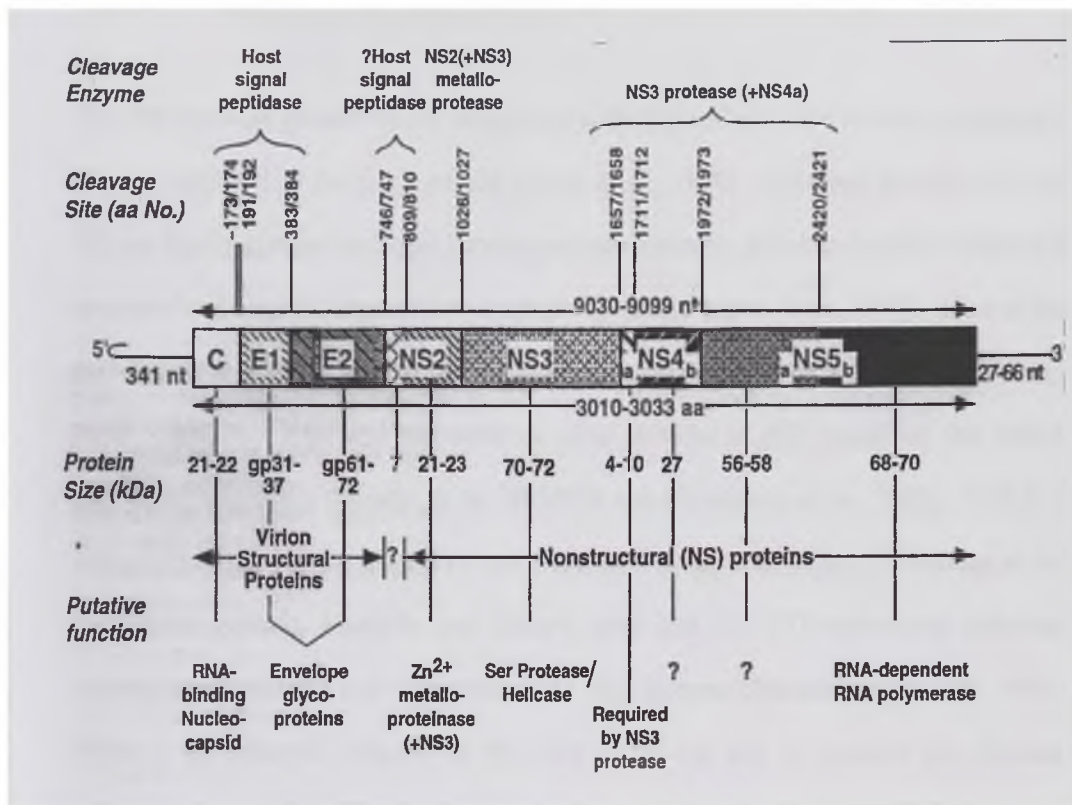


Fig. 2. Organization of the hepatitis C virus genome and polyprotein cleavage products. A schematic representation of the HCV genome indicating the positions of the structural and non-structural proteins within the polyprotein as well as the 5' and 3' NTRs. The polyprotein cleavage products, cleavage sites for host cell signalises, the NS2-3 proteinase, the NS3 proteinase and an unknown cellular proteinase (denoted by ?) are indicated. (nt – nucleotides; aa –amino acids, UTR – untranslated or non-coding region, kDa – kilodalton) (Houghton, 1996).

following the stop codon of the ORF, a poly (U) tract of heterogeneous length and a highly conserved 98 nucleotide sequence essential for replication *in vivo* (Kolykhalov *et al.*, 2000; Yanagi *et al.*, 1999; Tanaka *et al.*, 1996).

The first cleavage product of the polyprotein is the highly basic core protein, forming the major constituent of the nucleocapsid (Yasui *et al.*, 1998). Envelope proteins (E1 and E2) are highly glycosylated type 1 transmembrane proteins, forming disulfide-linked and non-covalently-linked heterodimers, respectively (Deleersnyder *et al.*, 1997). Most of the non-structural proteins 2-5B are required for the replication of the viral RNA (Lohmann *et al.*, 1999 *b*). NS2 and the amino-terminal domain of NS3 constitute the NS2-3 proteinase, catalysing cleavage at the NS3/NS4 site (Hirowatari *et al.*, 1993). NS3 is a bifunctional molecule carrying (1) a serine-type proteinase responsible for cleavage at the NS3/NS4a, NS4a/b, NS4b/5a and NS5a/b sites and (2) NTPase/helicase activities essential for translation and replication of the HCV genome (Bartenschlager *et al.*, 1993). NS4a is an essential cofactor of the NS3 proteinase and is required for efficient polyprotein processing (Tanji *et al.*, 1995a; Bartenschlager *et al.*, 1994). The functions of the hydrophobic NS4b and the highly phosphorylated NS5a in replication are so far unknown. NS5a appears to be involved in resistance of the infected cell to the antiviral effect of IFN. NS5b has been identified as the RNA-dependent RNA polymerase (RdRp) (Al *et al.*, 1998; Yuan *et al.*, 1997).

### **Replication cycle of HCV**

The dynamics of HCV replication can be deduced from the rapid rates of virus production and emergence of mutants (Zeuzem *et al.*, 1998). There is lack of a

convenient animal model and an efficient cell culture system for the study of HCV. As such, current understanding of the molecular mechanisms of HCV replication is based primarily on analogies to the closely related flavi- and pestiviruses. Characterization of recombinant proteins has also provided some information on HCV replication. Using these systems, the HCV replication cycle comprises the following: (1) penetration of the host cell and liberation of the genomic RNA from the virus particle into the cytoplasm; (2) translation of the input RNA, processing of the polyprotein and formation of a replicase complex associated with intracellular membranes; (3) utilization of the input plus-strand for synthesis of a minus-strand RNA intermediate; (4) production of new plus-strand RNA molecules which in turn can be used for the synthesis of new minus strands, for polyprotein expression or packaging into progeny virions; (5) release of virus from infected cell (Bartenschlager *et al.*, 2000). Fig. 3 illustrates the model.

#### *Attachment and entry*

The first step in the life cycle of HCV is the attachment of the infectious particle to the host cell. This requires a specific interaction between a receptor on the cell surface and a viral attachment protein on the surface of the particle. CD81 (CD denotes *cluster of differentiation*, a family of molecular structures on a cell surface) was identified as a putative receptor based on its strong interaction with E2 as well as with virus particles *in vitro* (Pileri *et al.*, 1998). HCV also enters cells by binding to low-density lipoprotein (LDL) receptors (Thomssen *et al.*, 1992). While the nature of the HCV receptor is not known currently, the major envelope glycoprotein E2 is thought to be used for initiating

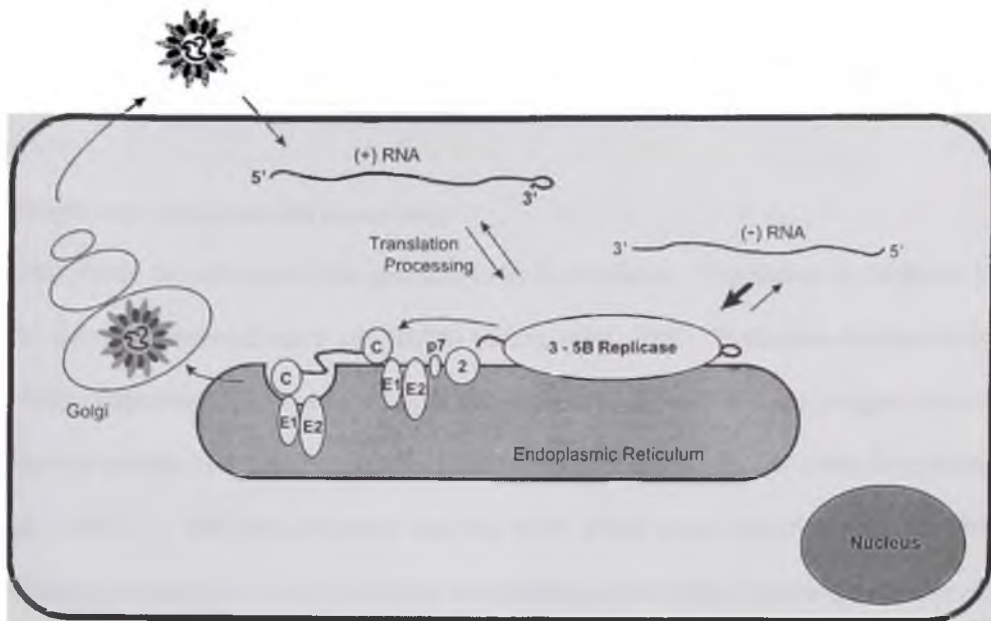


Fig. 3. Hypothetical model of the HCV replication cycle. Upon infection of the host cell (large rectangle) the plus-strand RNA genome (+RNA) is liberated into the cytoplasm and translated. The polyprotein is processed and viral proteins remain tightly associated with membranes of the ER. Minus-strand RNA (-RNA) is synthesized by the replicase composed of NS3-5B and serves as template for production of excess amounts of plus strand. Via interaction with the structural proteins plus-strand RNA is encapsidated. Particles are enveloped by budding into the lumen of the ER and virus particles are exported via transit through the Golgi complex (Bartenschlager *et al.*, 2000).

virus attachment to the host cells (Zibert *et al.*, 1995). The role of E1 is less clear but its presence suggests that it is involved in membrane fusion (Flint *et al.*, 1999).

#### *Polyprotein translation and processing*

Once inside the cytoplasm, the genomic RNA is translated. Translation is mediated by the internal ribosomal entry site (IRES) (Wang *et al.*, 1993; Tsukiyama-Kohara *et al.*, 1992). Experimental evidence suggests that sequences of the core coding region, but not the core protein itself, are required for full IRES activity (Honda *et al.*, 1996; Reynolds *et al.*, 1995). It has been reported that the HCV IRES binds specifically to the 40S ribosome subunit and does not require any additional translation factors (Pestova *et al.*, 1998).

Under the direction of IRES, the polyprotein is translated at the endoplasmic reticulum (ER) and cleaved co- and post-translationally by host cell signalases and viral proteinases (Bartenschlager, 1999; Reed and Rice, 1998). Processing of the polyprotein proceeds through intermediates, notably an E2-p7-NS2 protein. The C-NS2 region is processed by host signal peptidases cleaving at the C/E1, E1/E2, E2/p7, p7-NS2 junctions (Fig. 2). Processing of the NS3-5 region is mediated by the NS3 proteinase with the following preferred order of cleavages: NS3/4a-NS5a/b-NS4a/b-NS4b/5a (Grakoui *et al.*, 1993).

Proteolytic activity of NS3 is greatly stimulated by NS4a, although it is enzymatically active on its own (Bartenschlager *et al.*, 1994; Tanji *et al.*, 1994). In addition to serving as a proteinase cofactor, NS4a further functions to contribute to efficient polyprotein

cleavage and replication. First, it increases the metabolic stability of NS3. For example, in the absence of NS4, NS3 is degraded very rapidly. Secondly, NS4 anchors NS3 to intracellular membranes where most of the HCV proteins are located, thereby increasing the local enzyme:substrate concentration and facilitating the formation of a membrane-associated replicase complex (Wolk *et al.*, 2000; Tanji *et al.*, 1995).

### *RNA replication*

The individual steps underlying RNA replication are largely unknown. Several coprecipitation studies have shown that most or all of the HCV polyprotein cleavage products, in particular NS3-5B, form a replicase complex associated with intracellular membranes. This allows the production of viral proteins and RNA in a distinct compartment (Bolten *et al.*, 1998). In common with other positive-strand viruses, HCV is presumed to replicate its RNA genome through the production of a minus-strand replication intermediate. The virus-encoded NS5B RdRp is the key enzyme that catalyzes the synthesis of minus- and plus-strands either in a primer-dependent initiation manner or a 'copy-back' mechanism in *in vitro* studies (Al *et al.*, 1998; Yamashita *et al.*, 1998). Primer dependent synthesis proceeds by elongation of a primer hybridized to an RNA homopolymer. 'Copy-back' mechanism occurs when using heteropolymeric templates.

Steps in the assembly and release of HCV from the cell are unknown, although it is likely that the mature virus particle is formed by budding of the assembled nucleocapsid

through the lipid membrane of the cell into which the E1 and E2 glycoproteins are inserted (Bartenschlager *et al.*, 2000).

### **Classification of HCV**

A current proposal for the nomenclature of HCV genotypes is to divide HCV into ‘types’, corresponding to the main branches in the phylogenetic tree, and ‘subtypes’ corresponding to the more closely related sequences within the major groups (Simmonds *et al.*, 1994, 1993a; Enomoto *et al.*, 1990). Earlier classification described a total of six major types of HCV, numbered 1 to 6 in order of discovery. The subtypes are denoted by the letters a, b and c, that differ from each other by around 20% (Simmonds *et al.*, 1993a; Stuyver *et al.*, 1993; Chan *et al.*, 1992).

Pairwise similarity applied to either partial or total genome sequences led to the classification of HCV into 11 distinct genotypes (Tokita *et al.*, 1998,1995). However, phylogenetic analysis has grouped HCV-10a with HCV-3, and indicated that HCV-7, -8, -9 and 11a shared a common ancestor with HCV-6 (de Lamballerie *et al.*, 1997; Simmonds *et al.*, 1996). Results from phylogenetic inference methods thus support the existence of six HCV genotypes (Robertson *et al.*, 1998).

### **Genetic Variation in HCV**

A feature of HCV replication is the rapid generation of virus variants. Sequence comparisons of HCV obtained from a wide range of geographic locations have provided evidence for the existence of an extremely large number of variants (Hotta *et al.*, 1997;

Mellor *et al.*, 1995). Even within an individual HCV does not exist as a single entity but rather as a swarm of microvariants of a predominant ‘master sequence’, a phenomenon referred to as quasispecies (Holland *et al.*, 1992). The production of such a large number of variants is primarily due to the high error rate of the viral RNA-dependent RNA polymerase (RdRp) that is expected to be in the range of  $10^{-4}$ . The high variation observed with HCV replication may account for the fact that a significant fraction of virus genomes appear to be defective (Martell *et al.*, 1992).

Phylogenetic analysis of nucleotide sequences of either structural genes, such as the envelope glycoprotein, E1 (Bukh *et al.*, 1993), non-structural genes such as NS5B, the RNA polymerase (Simmonds *et al.*, 1993), or the complete genome (Chamberlain *et al.*, 1997) has provided evidence that the six major genotypes of HCV are approximately equally divergent from each other. This was based on analyses of a worldwide collection of samples from HCV-infected blood donors and patients with hepatitis. Regions that encode the putative envelope proteins (E1, E2/NS1) are the most variable (Hijikata *et al.*, 1991) and the 5' untranslated region is the most conserved (Han *et al.*, 1991). The hypervariable region 1 found within the envelope E2 protein gene is a major site for the genetic evolution of HCV (Kato; 2000).

### **Geographical Distribution of HCV Genotypes**

It has been observed that the genotypes of HCV have particular geographical distribution. Some genotypes are distributed worldwide while others are found predominantly in specific regions (Fig. 4). Genotypes 1, 2 and 3 are found throughout the world whereas

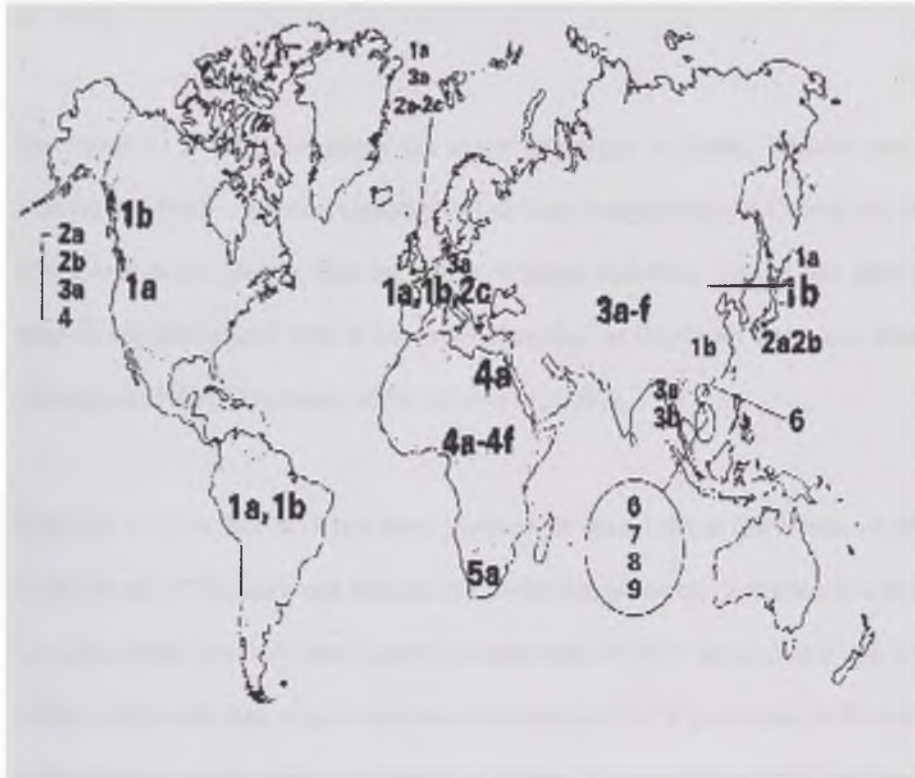


Fig. 4. Geographic distribution of HCV variants (Decker and Troonen, 2001).

genotypes 4, 5, and 6 appear to be more restricted in their distribution. A striking geographical change in genotype distribution is apparent between Europe and the Middle East, West Africa, and parts of North and Central Africa (Bukh *et al.*, 1993; Stuyver *et al.*, 1993).

Genotypes 1, 2 and 3 constitute the major genotypes in Japan, Western and Eastern Europe, and North America. Genotype 4 has been found mostly in Central and Northern Africa and in the Middle East but not in Western countries. Type 5 has been reported from South Africa and type 6 has been identified in Southeast Asia and Hong Kong (Simmonds, 1999; Nousbaum, 1998; Mori *et al.*, 1992).

Hepatitis C virus infection has been reported in West Africa (Develoux *et al.*, 1995; Mellor *et al.*, 1995), although data on its distribution is scarce. Evidence has been found for high genetic diversity and long-term endemicity of HCV genotypes 1 and 2 in West Africa. Available data suggest that the distribution of HCV genotypes in West Africa is different from what occurs in other parts of Africa. A new subtype 2d of genotype 2 has been isolated from a Nigerian patient treated in the Netherlands (Stuyver *et al.*, 1994). Similarly, a new subtype of genotype 1 has been characterized in the serum sample from another Nigeria patient. Genotype 2 has been identified in two samples from The Gambia (Mellor *et al.*, 1995). Recently, genotypes 1 and 2 were identified in Guinea, Burkina Faso and Benin. The genotypes showed high genomic diversity, with 18 different subtypes including 2c, 2d and 16 new subtypes (Dominique *et al.*, 1998).

In Ghana, little data on the prevalence and genotypes of HCV infection is available despite the known high incidence of liver disease, much of which is likely to be caused by hepatitis viruses. A survey of HCV antibody seroprevalence revealed a rate of 5.4% in some school children aged between 6 to 18 years (Martinson *et al.*, 1996). HCV infection has also been observed in both HIV-seropositive and seronegative individuals (Brandful *et al.*, 1999). In another study, a seroprevalence of 2.8% and an occurrence of genotype 2 were observed in blood donors and antenatal clinic attenders in Kumasi in the middle part of the country. HCV genotype 2 was therefore suggested as indigenous to Ghana (Wansbrough-Jones *et al.*, 1998).

### **Pathogenesis of HCV Infection**

The hallmark of HCV is its ability to cause persistent infection that evolves into chronic disease associated with liver damage, characterized by sustained viremia and anti-HCV antibody. Numerous studies involving posttransfusion hepatitis, intravenous drug user-associated hepatitis, and community-acquired hepatitis have shown that viremia may occur in approximately 70 to 90% of HCV-infected persons (Alter *et al.*, 1995, Thomas *et al.*, 1995). Although the majority of HCV- infected individuals experience persistent infection, the disease may be active or quiescent. It has been proposed that differences in the host cellular (Missale *et al.*, 1996) or humoral (Zein *et al.*, 1999) immune responses to HCV are important in spontaneous clearance. HCV infection usually fails to resolve spontaneously in about 80% of individuals who become chronically infected after initial exposure to HCV (Alter *et al.*, 1992). Studies have suggested that disease progression, morbidity and mortality depend on various viral or host factors such as viral load, alcohol

intake, other infecting agents such as HBV and HIV and the length of time of HCV acquisition (Zein, 2000; Dusheiko *et al.*, 1993; Gordon *et al.*, 1993;).

The role of HCV genotypes in persistence of HCV following an acute exposure to HCV has been investigated. Data provided evidence that the genotype of HCV may potentially play an important role in the development of chronic infection. The rate of evolution to chronicity in patients exposed to HCV genotype 1b infection was 92%, while in patients exposed to other genotypes the rate was 33% to 50% (Amoroso *et al.*, 1998). Chronic HCV infection with genotype 1b is also reportedly associated with a more severe liver disease and a more aggressive course than infection with other HCV genotypes (Pozzato *et al.*, 1991). HCV genotype 1b has been found to be prevalent among patients with liver cirrhosis and HCV-associated hepatocellular carcinoma (Abe *et al.*, 1998; Zein *et al.*, 1996; Silini *et al.*, 1995). The role of genotypes in the progression of liver disease cannot be separated from the roles of the cofactors mentioned above (Fong *et al.*, 1991; Mendenhall *et al.*, 1991).

There appears to be significant biologic variation in HCV disease expression in the host over the length of the infection. In a study on the natural history of HCV infection, the mean times from exposure to HCV to the diagnosis of chronic active hepatitis, to compensated liver cirrhosis, to decompensated cirrhosis, and to hepatocellular carcinoma were 11, 18, 23 and 29 years respectively (Zein *et al.*, 2000). Severe complications such as cirrhosis and hepatocellular carcinoma could occur over a short period in some people

whereas others have no complication despite a much longer period of infection (Zein *et al.*, 1996).

### **Clinical Significance of HCV Infection**

Hepatitis C infection causes an indolent and slowly progressive liver disease that is asymptomatic until the development of decompensated liver disease and, often, liver cancer. Chronic liver disease occurs in at least 50% of patients with acute HCV infection and progressive liver injury, fibrosis and cirrhosis develop in one-third of these patients over a period of 20-30 years, while 15% develop hepatocellular carcinoma (Di Bisceglie *et al.*, 1991).

Acute hepatitis C infections are usually associated with subclinical disease. Approximately only one quarter of acute cases result in jaundice (Di Bisceglie *et al.*, 1991). Most of the serious liver disease associated with HCV is a consequence of the chronic, persistent nature of the infections. It has been suggested that posttransfusion cases proceed more aggressively than does infection associated with intravenous drug use, which may be related to the larger viral inoculums received in posttransfusion infections (Di Bisceglie *et al.*, 1991).

Clinically, hepatitis caused by HCV is indistinguishable from that caused by other hepatitis viruses. When acute disease occurs, general symptoms of hepatitis are apparent. The usual signs of the disease are malaise, anorexia, nausea, and occasionally pain in the right upper abdomen. When symptoms occur, fatigue is the most common complaint, but

weakness, wasting, edema, and ascitis also can occur (Dusheiko *et al.*, 1993). Extrahepatic manifestations of the disease include the deposition of immune complexes comprising HCV, viral antibodies and rheumatoid factor within the kidney glomeruli. The deposition of such complexes has been postulated to be the cause of membranoproliferative glomerulonephritis, which typically causes weakness, peripheral edema, hypertension and hepatomegaly (Johnson *et al.*, 1993).

### **Stability and Detection of HCV in Clinical Specimens**

Previous experiments have revealed certain collection procedures as well as handling, transport and storage conditions of samples necessary for the optimal detection of HCV RNA (Halfon *et al.*, 1996). Conventional methods exist for RNA extraction from samples of serum, plasma, peripheral blood mononuclear cells (PBMCs) and whole blood (Schmidt *et al.*, 1995). However, various processing and storage conditions influence the stability and alter the RNA concentration and hence limit the ease of detection of HCV RNA in clinical samples (Halfon *et al.*, 1996).

The type of blood collection tube has been shown to influence the titre of HCV RNA in samples. Recent studies showed that whole blood anticoagulated with EDTA may be stored at up to 25°C for up to five days without any significant loss in plasma HCV RNA level (Grant *et al.*, 2000). Comparative studies showed that whole blood contains significantly more HCV RNA than plasma, which contains more HCV RNA than serum, which also contains more HCV RNA than PBMC, neutrophils, or RBC/ platelets (Schmidt *et al.*, 1997; Wang *et al.*, 1999). However, whole blood extraction methods are

costly and more time-consuming. Besides, Cook *et al* (2000) demonstrated that the use of whole blood specimens for routine detection or quantitation of HCV RNA in the blood does not provide any greater sensitivity than the use of serum or plasma specimens. This makes the use of serum / plasma for HCV diagnosis a valid and reliable procedure.

### **Diagnosis of HCV**

HCV diagnosis is particularly important for the safety of supply of blood and blood products. Serodiagnosis is dependent on the detection of circulating anti-HCV antibodies to specific HCV antigens using commercially available kits. Anti- HCV antibody positivity has been shown to be strongly correlated with persistent viraemia. However, antibodies may not be present in patients with very low viremia. Besides, the titre of circulating virus is generally low, hence currently, serodiagnosis is confirmed by the detection of viral RNA in blood or blood fractions. This is achieved through PCR amplification of reverse-transcribed complementary DNA (cDNA) of HCV (Choo *et al.*, 1994). Thus the methods of choice for the screening of blood samples for HCV should be those that are sensitive, specific and reproducible, ensuring consistency in laboratory assays.

Recombinant DNA techniques have been used to develop structural and non-structural proteins derived from HCV RNA with utility for antibody screening. Anti-HCV assays have evolved from first-generation products incorporating NS4 proteins only, through to third-generation assays by incorporating core (c22-3, structural), NS3 protease / helicase (c33c, non-structural), NS4 (5-1-1 and c100-3, non-structural) and NS5 replicase (non-

structural) proteins. The first-generation assays are affected by the level of circulating globulins in serum, and therefore give a high level of false positivity. It has been suggested that the interpretation of second-generation assays is affected by the HCV genotype (Zein *et al.*, 1997). Third-generation assays for anti-HCV have been shown to have much higher sensitivities and specificities than the second-generation assays and useful for earlier detection of seroconversion. They detect up to 95% of HCV-infected persons (Hosein *et al.*, 1991; McHutchinson *et al.*, 1992). They are much less strongly influenced by the infecting genotype (Courouce, 1998; Kao *et al.*, 1996; Maggi *et al.*, 1995).

Various diagnostic test kits for the detection of antibodies to HCV have been used recently in Ghana. These include the HCV-SPOT assay (Genelabs Diagnostics Ltd., Singapore), the SERODIA passive-particle agglutination assay kits (FUJIREBIO Inc., Tokyo, Japan), enzyme-linked immunoassay (IMUCHECK-HCV C50Ab; International Reagents Corporation, Kobe, Japan) and the third-generation recombinant immunoblot assay (RIBA 3; Ortho Diagnostic Systems, Roissy, France).

The SERODIA-HCV PA (particle agglutination) assay was developed using gelatin particle carriers, sensitized with recombinant antigens c22-3 and c200. The test is based on the principle that sensitized particles are agglutinated by the presence of antibodies to HCV in serum/plasma specimens. The Murex anti-HCV (Version 4.0) enzyme-linked immunosorbent assay (ELISA) utilizes antigens from core, NS3, NS4 and NS5 regions of

the virus genome. It operates on the principle that anti-HCV antibodies in a sample bind to immobilized antigens and are detected subsequently following an enzyme action.

A positive anti-HCV test provides evidence of exposure to HCV. However, seroconversion is generally not detected until 7 to 31 weeks after infection. Therefore, specific antibodies may not be detectable in early acute disease. Moreover, antibody detection does not distinguish between acute, chronic or past HCV infection. Additional information can only be obtained by testing for viral RNA in serum or plasma by PCR or direct hybridization ('branched DNA' test). Besides, individuals vary in their immune response to various HCV proteins (Zein *et al.*, 1996). These have made the detection of HCV RNA by reverse transcriptase (RT)-PCR become essential for the diagnosis of HCV infection.

Usually, the amount of blood HCV, opposed to other viruses, is very small. Besides, HCV RNA level changes with the dynamics of the disease. Thus, the method to extract HCV-RNA from the samples requires good procedures that are effective, simple and quick. Recent technological advances have led to the availability of products such as the SepaGene-RV-R (Sanko Junyaku Co. Ltd., Tokyo, Japan) which extracts target RNA simply and quickly with high collection ratio by utilizing the agglutination partition (AP) method. The AP method uses a non-phenol reagent and a protein agglutinant that form agglutinated partitions according to differences in specific gravity. Target RNA is thus extracted into the aqueous phase while impurities such as proteins are trapped in the agglutinated layer. As a result, the RNA is available in the aqueous phase ready for use in a nested RT-PCR assay for detection (Miyachi *et al.*, 2000; Naito *et al.*, 1999).

The PCR is a sensitive technique that detects as few as tens of viral RNA; besides, viraemia is detected within a few days after acute infection (Choo *et al.*, 1994). RT-PCR amplification targets the 5' leader sequence of the viral genome, which has been shown to be highly conserved among different viral isolates (Bukh *et al.*, 1992; Chan *et al.*, 1992; Okamoto *et al.*, 1990). A positive HCV RNA test suggests viral replication in the liver and validates a diagnosis of either acute or chronic hepatitis C. However, the sensitivity of PCR for HCV RNA detection has been shown to be affected by the choice of primers, handling and storage of preextraction samples (Grant *et al.*, 2000; Bukh *et al.*, 1992; Busch *et al.*, 1992).

Critical parameters affect the performance of PCR assay. Low yield or no amplification product may result from a number of factors including missing reaction component, use of degraded template, presence of an inhibitor (removable by ethanol precipitation), suboptimal reaction conditions, uneven distribution of temperature or template in some positions of thermal cycler and genuine absence of target sequence in target DNA. Multiple, nonspecific amplification products may also result from suboptimal reaction conditions, poor primer design, too high primer concentration or contamination by another target RNA/DNA. Also, degradation of RNA, thermal inactivation of Avian Myeloblastosis Virus (AMV) or Moloney Murine Leukemia Virus (M-MuLV) Reverse Transcriptase, lack of primer specificity or primer annealing and problem with the RNA purification may also lead to low yield or no first strand (RT-PCR) product. Appropriate measures exist for overcoming these problems.

One useful reagent newly developed for PCR technique is the use of 'Ready-To-Go RT-PCR beads (Amersham Pharmacia Biotech Inc., N. J, USA). Ready-To-Go RT-PCR beads provide the reagents for RT-PCR reactions. The components are namely buffer, MgCl<sub>2</sub>, nucleotides, M-MuLV reverse transcriptase, RNAGuard and Taq DNA polymerase, in a convenient ambient-temperature-stable bead. The Ready-To-Go bead format significantly reduces the number of pipetting steps, thereby increasing the reproducibility of the RT-PCR technique and minimizing the risk of contamination and yielding products sufficient for easy detection on agarose gel electrophoresis.

### **Role of Genomic Heterogeneity in HCV Persistence and Vaccine Development**

The mechanisms of HCV persistence are generally unknown. However, several arms of the immune system including humoral and cellular immunity interact to limit viral replication and prevent persistence (Zein, 2000). Neutralizing antibodies are often specific for a particular serologic type of virus (Koziel, 1996). The role of cytotoxic T lymphocytes (CTL), which mediate the cellular immune responses, in protecting against viral persistence is unknown (Koziel *et al.*, 1993). HCV heterogeneity may be important in escaping CTL-induced immunity. Responses to HCV antibody are usually directed against the viral envelope proteins, most likely, the hypervariable region 1 which is located in the E2 region (Farci *et al.*, 1994).

Cellular immune responses appear to play a role in protecting against HCV infection. Possible targets for HCV-specific CTL recognition within the conserved core protein and

additional epitopes in the more highly variable region E2 protein have been identified (Koziel *et al.*, 1993, 1992). It has been shown that single amino acid changes in CTL epitopes result in failure of recognition by HCV-specific CTL, although direct evidence for the presence of CTL mutants in human HCV infection is lacking (Koziel, 1996).

Irrespective of the specific type of immune response (humoral or cellular) that is associated with protection and clearance of HCV after an acute exposure, the response appears to be type-specific (Lai *et al.*, 1994). The genetic heterogeneity of HCV is likely to make the development of HCV vaccine difficult. However, a vaccine consisting of recombinant E1 and E2 proteins of HCV-1 (genotype 1a) has been shown to elicit protective immunity. This poses a challenge for the development of a broadly effective vaccine for the prevention of HCV.

### **Clinical Significance of HCV Genotypes**

HCV genotypes have relationship with the epidemiology, pathogenicity and response to interferon therapy. Genotypic characterization of HCV has become an important part of clinical management and public health control of HCV infection (Martinot-Peignoux *et al.*, 1999). Strain differences influence transmission patterns among susceptible hosts as well as the clinical outcome after infection although at varying frequencies in populations throughout the world (Zein, 2000; Amorso *et al.*, 1998; Simmonds *et al.*, 1996).

Studies have suggested that genotypes 2 and 1b may play an important role in hepatocarcinogenesis in Japan, Spain and the United States (Abe *et al.*, 1998). Both

blood donors and patients with chronic hepatitis from countries in Western Europe and the USA all show frequent infection with genotypes 1a, 1b, 2a, 2b and 3a (McOmish *et al.*, 1994). In France, HCV 1b is associated with patients who had received a previous blood transfusion (Nousbaum *et al.*, 1995), whereas HCV 3a is predominantly associated with intravenous drug use (Pawlotsky *et al.*, 1996). In northern Asia, HCV type 6 variants were commonly found among blood donors and blood addicts; while in blood donors, HCV types 1a, 1b and 1d were more strongly associated with elevation of serum aminotransferase levels (Hotta *et al.*, 1997).

HCV genotypes 1a and 1b have been shown to be associated with a more severe liver disease and lower rates of response to interferon therapy than HCV genotype 2a, 2b or 3 (Martinot-Peignoux *et al.*, 1999; Nizar *et al.*, 1996). A low response rate to anti-HCV therapy also occurs in patients infected with HCV type 4 (Remy *et al.*, 1998; el-Zayadi *et al.*, 1996). The association of HCV genotypes with clinical outcome after infection and the level of response to therapy make genotyping of HCV important in the management of HCV-associated hepatitis.

### **Genotyping of HCV**

Reliable methods for determining the HCV genotype are crucial for epidemiological and clinical analyses (Zein, 2000). Data indicate that HCV subtypes can be distinguished by serological and molecular methods (Mathias *et al.*, 1999; Bukh *et al.*, 1992; Okamoto *et al.*, 1992). The reference standard and most definitive method for HCV genotyping is sequencing of a specific PCR-amplified portion of the HCV genome obtained from a

suitable specimen, followed by phylogenetic analysis. However, direct sequencing is impractical on a large scale because of the complexity of the procedure. Besides, sequencing of amplified DNA from a particular region of the genome does not usually identify mixed infections with two different HCV genotypes (Zein, 2000).

The choice of the genomic region to be analysed is crucial. The region must contain type- and subtype-specific motifs, which reliably represent the diversity of the entire genome. Sufficient conservation within the subtype is essential for the development and use of sensitive and specific primers or probes, but sufficient variability is needed to allow discrimination between subtypes. Furthermore, variability of the region to be analysed should be sufficiently low to allow PCR amplification of all HCV genotypes. As such, various regions of the HCV genome, namely, the 5' non-coding region (Chayama *et al.*, 1993; Bukh *et al.*, 1992; Chan *et al.*, 1992), the core region (Chayama *et al.*, 1993; Chan *et al.*, 1992), the envelope region (Simmonds *et al.*, 1994; Stuyver *et al.*, 1993), the NS4 region (Tanaka *et al.*, 1994; Simmonds *et al.*, 1993) and the NS5 region (Sakamoto *et al.*, 1994; Simmonds *et al.*, 1993), have been utilized for grouping of HCV into different types by nucleotide sequencing. However, the low variability of some regions including the 5'-NCR does not allow complete discrimination of HCV genotypes, but the core and NS5B regions, though intermediately conserved, offer greater possibilities for discrimination between different subtypes.

Serologic genotyping is based on specific antibodies directed to epitopes encoded by specific regions of the genome for specific HCV genotype (Machida *et al.*, 1992).

Recently, a recombinant immunoblot assay based on recombinant HCV proteins from the NS4 region has been developed for the differentiation of HCV subtypes 1a, 1b, 2a, 2b, 3a and 4a (Mathias *et al.*, 1999). The serologic genotyping assays have the advantages of simplicity and low risk for contamination. However, serologic typing seems to lack specificity and sensitivity, which limits its usefulness (Zein, 2000). Investigators of HCV genotyping therefore rely on molecular genotyping systems.

A number of HCV molecular genotyping systems have been developed that depend mainly on the amplification of HCV RNA extracted from clinical specimens. Initial HCV RNA amplification is followed by either reamplification with type-specific primers (Chayama *et al.*, 1993, Okamoto *et al.*, 1992) or by digestion of PCR products with restriction endonucleases that recognize genotype-specific cleavage sites (McOmish *et al.*, 1993; Nakao *et al.*, 1991). Without modification, none of the reported methods, with exception of direct nucleotide sequencing, is able to discriminate among the HCV genotype in all six major types and the common subtypes on the basis of the core region of HCV genome. Moreover, all these PCR-based methods have the shortcomings and advantages of PCR. They are expensive and time-consuming and require specialized facilities to prevent contamination and ensure accurate results. However, if performed accurately, they are reliable.

Recently, a convenient genotyping system based on PCR of the core region with genotype-specific primers, which allows for the determination of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a and 6a has been reported (Ohno *et al.*, 1997). The phylogenetic

lineage can be investigated following the sequencing of an informative region of the HCV genome to confirm observed HCV genotypes.

### **Sequencing**

The DNA sequence can be elucidated using chemical (Maxam and Gilbert, 1977) or enzymatic (Sanger *et al.*, 1977) methods. The enzymatic method of sequencing is based on the ability of a DNA polymerase to extend a primer, hybridized to the template that is to be sequenced, until a chain-terminating nucleotide is incorporated. Each sequence determination is carried out as a set of four separate reactions, each of which contains all four deoxyribonucleotide triphosphates (dNTPs) supplemented with a limiting amount of a different dideoxyribonucleotide triphosphate (ddNTP). Because ddNTPs lack the 3-OH group necessary for chain elongation, the growing oligonucleotide is selectively terminated at G, A, T or C, depending on the respective dideoxy analog in the reaction. The resulting fragments, each with a common origin but ending in a different nucleotide, and also differing from one another by the length of one nucleotide, are separated according to size by high resolution denaturing polyacrylamide gel electrophoresis. The final nucleotide on each strand is read by an automated sequencer using fluorometric methods that depend upon the labelling of either the primer or the terminators.

Thermal cycle sequencing offers several advantages over conventional, non-cycled sequencing strategies (Saluz and Jost, 1989). First, the protocol yields a linear amplification of the template DNA, reducing the amount of template required to yield a detectable ladder. Second, the high temperature employed during each denaturation,

helps to circumvent problems associated with rapid reannealing of linear double stranded DNA (dsDNA) templates such as PCR products. Third, high annealing temperatures increase the stringency of primer hybridization.

Several critical factors affect the sequencing results. Template denaturation, as well as primer annealing temperatures; purity, amount and the quality of the template DNA; the quality of the gel; and the sequencing temperature among others, do affect the results of sequencing. Poor performance of any of the steps mentioned normally results in lack of clear peaks, instead of a kind of picture seen in Appendix III, and hence sequence is unreadable. Band compressions are most often associated with GC-rich sequences that form secondary structures that are not completely denatured during gel electrophoresis (Mills and Krammer, 1979). These secondary structures result in the anomalous migration of DNA fragments, which in turn can complicate the interpretation of sequencing results.

## CHAPTER TWO

### MATERIALS AND METHODS

#### 2.1 MATERIALS AND REAGENTS

Insulated box, Ice packs, were from Polar Tech Industries, Inc., Genoa IL60135. Autoclave, freezer, were from Sanyo Electric Biomedical Co., Ltd., Japan. Microtitre plate was from Greiner Labortechnik,. Tray mixer (Mini-orbital shaker) was from Bellco Glass. Inc., Vineland, N. J, USA. Multichanel pipette (Finnpipette Digital Multichanel) was by Labsystems Inc., Finland and single chanel pipette (Pipetman) was from Gilson Co., Ltd., France. Incubator was from Yamato Scientific Co., Ltd., Tokyo, Japan. Automatic strip washer (Immunowash), Microplate reader, were from BIO-RAD Laboratories, 20090 Segrate, Italy. Centrifuge was from Kokusan Corporation, Tokyo, Japan. Perkin-Elmer GeneAmp PCR System 2400 was from Norwalk CT 06859, USA. Vacuum chamber (CentriVap Concentrator) was from LABCONCO Corporation, Kansas City, Missouri 64132, USA. Water bath was from Julabo Labortechnik GMBH, D-77960 Seelbach, Germany.

SERODIA HCV kit was obtained from FUJIREBIO INC., Tokyo, Japan. Murex anti-HCV (Version 4.0) was obtained from ABBOTT Diagnostics UK. SepaGene RV-R RNA extraction kit was obtained from Sanko Junyaku Co. Ltd., Tokyo, Japan. DNA purification kit was from QIAGEN GmbH, Germany. Ready-To-Go RT-PCR and To-Go PCR kits, Thermo Sequence Cy<sup>TM</sup> 5.5 Terminator Cycle Sequencing Kit, RapidGel Cartridge, and the SEQ4x4 Sequencer were obtained from Amersham Pharmacia Biotech Inc., N. J, USA. QIAquick Plasmid DNA Purification Kit was obtained from QIAGEN,

Germany. Restriction enzyme, buffer and ligase were obtained from Roche Diagnostics GmbH., Mannheim, Germany. DNA size markers were obtained from Promega Corporation, Madison, USA.

Absolute ethanol was from Riedel-deHaen (ph. Eur). Ethylenediaminetetraacetic acid (EDTA) sample collection tubes were obtained from Vacutainer Systems, Frank Lakes, N. J., USA. Diethyl pyrocarbonate (DEPC), isopropyl alcohol, glacial acetic acid, tris base (Tris[hydroxymethyl]aminomethane), EDTA, calcium chloride, ethidium bromide were obtained from SIGMA Chemical Co., Steinheim, Germany. Boric acid was obtained from Kanto Chemical Co. Inc., Tokyo, Japan. Agarose, LB Agar, LB Broth Base and pCR 2.1 TOPO vector were obtained from Invitrogen, Life Technologies, Paisley, Scotland. MicroCell™ 300 Cassette was from Visible Genetics Inc., Canada.

## **2.2 METHODS**

### **Samples Collection**

Random samples from blood donors were collected at the Korle-Bu Teaching Hospital. Blood was collected in four milliliters EDTA tubes. The samples were transported to the study laboratory in a cold box (an insulated box containing frozen ice packs to keep samples cold). Within 2 hours of drawing the blood, the samples were centrifuged at 2,500g for 15 minutes at room temperature. After centrifugation, aliquots of plasma were made in duplicates into freezing vials and immediately frozen at  $-70^{\circ}\text{C}$  until needed for serological testing and virus extraction.

## **Assays for Antibody to HCV**

### *Particle Agglutination Assay*

The SERODIA-HCV particle agglutination (PA) assay was performed on the plasma samples to detect the presence of antibodies to HCV. The test was performed according to the manufacturer's instructions. Plasma samples were declared as containing anti-HCV antibodies by visual observation of agglutination between reconstituted HCV-sensitized particles and HCV antibodies present in a sample as described below.

Using a micropipette, 75 $\mu$ l of sample diluent were placed in the first well of a 'U' shaped microplate and 25 $\mu$ l into each of wells two to four. To the diluent in the first well, 25 $\mu$ l of specimen were added and mixed. Two-fold serial dilutions were made in wells two and three, starting with 25 $\mu$ l of dilution in the first well and discarding 25 $\mu$ l of solution remaining in the pipette after mixing in the fourth well. Twenty-five microlitres each of reconstituted control and sensitized particles were placed in wells two and four to give final dilutions of 1:16 and 1: 64 respectively. The contents of the wells were well mixed on a tray mixer for roughly 2 min. The plate was then covered with a plate sealer and placed on a level, vibration-free surface, allowed to stand at room temperature for two hours after which the result was read. A sample was considered reactive if either a large ring / multiform agglutination occurred, or firmly agglutinated particles that spread out covering bottom of wells uniformly occurred. Samples that showed reactivity at 1:64 dilutions were further tested at a higher final dilution of 1:512 as a way of reducing non-specific reactivity. Both reactive and non-reactive samples were screened by ELISA to aid selection of possible HCV positive samples for virus extraction.

### Enzyme Linked Immunosorbent Assay (ELISA)

A third-generation Enzyme-linked Immunosorbent Assay (ELISA) using the Murex anti-HCV (version 4.0) kit was used to evaluate all sera both reactive and non-reactive by PA. The assay was performed strictly according to the manufacturer's instructions. Plates with antigen coated wells and reagents were brought to room temperature before the assay. Substrate and conjugate were reconstituted and the wash solution was diluted to working strength (1X) 20 min before they were used.

A multichannel pipette was used to add 180  $\mu$ l of sample diluent to each well. Twenty microliters of samples and controls were added to the diluent and mixed. The wells were covered with a plate sealer and incubated 1 hr at 37°C to enable any anti-HCV antibodies in the sample bind to the immobilized antigens in the wells. The plates were washed at the end of the incubation period to remove unbound materials. Five wash cycles were performed with an automatic strip washer. Immediately after washing, 100 $\mu$ l of conjugate solution containing peroxidase-conjugated monoclonal anti-human IgG was added to each well. The plates were then covered and incubated at 37°C for 30 min to allow binding between any captured anti-HCV antibody and the conjugate. The wells were washed as before to remove excess conjugate. After washing the plates, bound enzyme was detected by the addition of 100 $\mu$ l of substrate solution containing 3,3',5,5'-tetramethylbenzidine (TMB) and hydrogen peroxide to each well. The wells were covered and incubated at 37°C for 30 min. At the end of the incubation period 50  $\mu$ l of stop solution (0.5 N sulphuric acid) were added to each well. Within 15 min, the absorbance was read at 450 nm against a reference of 630 nm. The assay was declared

valid when the mean absorbance of negative controls (N) at 450 nm (A 450) was less than 0.25 (that is  $N < 0.25$ ) and the positive control A450 (P) was more than 0.8 above the negative control (that is  $P > N + 0.8$ ).

A sample was declared reactive if its absorbance value at 450 nm was greater than the cut-off value (CV) calculated by adding 0.6 to N (that is  $CV = N + 0.6$ ). Sera that were reactive by both PA (high dilution) and Murex anti-HCV ELISA were selected for HCV extraction, amplification and genotyping.

### **HCV RNA Extraction**

The nucleic acid was extracted from 100 $\mu$ l of plasma by using the SepaGene RNA extraction kit as follows: Three hundred microliters of reagent 1 containing guanidine thiocyanate for dissolving HCV membrane and protein denaturation, were pipetted into 1.5ml sterile eppendorf tubes. To the reagent in the microtube, 100 $\mu$ l of plasma sample were added and mixed. This was followed by the addition of 300 $\mu$ l of reagent 2 and 600 $\mu$ l of reagent 3 to the tube and mixed well by shaking vigorously vertically. Reagent 2 contains sodium acetate for salting out protein while reagent 3 contains chloroform protein agglutinant for removing protein or polysaccharide. The mixture obtained was left at  $-20^{\circ}\text{C}$  for 10 mins after which it was centrifuged for 15 mins at 12,000g in a refrigerated centrifuge ( $4^{\circ}\text{C}$ ). The upper phase was transferred into another microtube by pipetting. An equal volume of isopropyl alcohol were added and mixed gently by inverting the tube, and then left for 45 mins at  $-20^{\circ}\text{C}$ . The mixture was then centrifuged as before. The supernate was removed by aspiration. One millilitre of 70% ethanol was

added gently to the blue pellet in the tube and centrifuged at 4°C for 10 mins at 12,000g. The supernate was aspirated; the pellets were dried in a vacuum chamber and resuspended in 40µl of ribonuclease-free water. This extract was used for HCV detection.

### **Detection of HCV**

In order to optimize the nested PCR, the best condition for the reaction was established. Reverse transcription-PCR was performed by the one-step method combined with the cDNA synthesis reaction followed by the PCR as described below.

#### ***Reverse Transcription-Polymerase Chain Reaction to Generate cDNA***

The first PCR was combined with the reverse-transcriptase (RT) step in the same Ready-To-Go RT-PCR tube containing bead optimized for first strand cDNA synthesis and PCR. Each bead contained 10 units of RNAGuard, 100 units of M-MuLV reverse transcriptase, 200 µM each of deoxynucleotides, 2 units of AmpliTaq Gold DNA polymerase and 1.5 mM MgCl<sub>2</sub>. The bead was dissolved in 43.7 µl of RNase-free water on ice; 0.65µl of 10 µM of each HCV-specific outer primer and 5 µl of RNA template were added to make a final reaction volume of 50 µl. The sequences of primers used for the RT-PCR were designed from 5'-untranslated region of HCV genome (HC-J1 isolate: GeneBank accession number D10749). The sequence of the HCV-specific primers (sense, number 19 and antisense, number 20) were respectively 5'-GCGACACTCCACCATAGAT-3' and 5'-GCTCATGGTGCACGGTCTA-3' for the outer primer pairs (that gave a product size of 329 base pairs).

The thermocycler (2400 Perkin-Elmer Thermal Cycler) was programmed first to incubate the samples for 30 mins at 42°C for the initial RT step and then preheat them at 95°C for 5 mins to activate *AmpliTaq* Gold DNA polymerase. The first round amplification was achieved in a reaction of 40 cycles consisting of 94°C for 30 secs, 50°C for 45 secs, and 72°C for 1 min in a Perkin-Elmer GeneAmp PCR System 2,400 (Norwalk CT, USA). The complementary DNA (cDNA) product obtained in the first reaction was used in a second round of amplification.

In the second PCR, 2 µl of the cDNA product generated in the first PCR were amplified for 40 cycles using a Ready-To-Go PCR tube in a Perkin-Elmer 2,400 thermal cycler. The Ready-To-Go PCR tube contained bead which was dissolved in 22.3µl RNase-free water and 0.35µl each of the pair of HCV PCR inner primers was added. The formulation of the bead was such that when dissolved and brought to a final volume of 25µl, it contained 1.5 units of *Taq* DNA Polymerase, 10 mM Tris-HCl (pH 9.0 at room temperature), 50mM KCl, 1.5mM MgCl<sub>2</sub>, 200µM of each dNTP and stabilizers including bovine serum albumin (BSA). The reaction mixture was prepared on ice. The sequences of primers used for the nested PCR are listed in Table 1 below along with the nucleotide position and PCR product size expected by the primer combinations.

Sample amplification was performed under the following conditions: Preheating at 94°C for 2 mins to activate *Taq* DNA Polymerase, a preliminary 20 cycles of amplification at 94°C for 30 secs (denaturation), 53°C for 45 secs (annealing), and extension at 72°C for 1

min. Additional amplification was performed as follows: 20 cycles at 94°C for 30 secs, 55°C for 45 secs and 72°C for 1 min. The PCR products were detected by electrophoresis on 2% agarose gel in 1X Tris-Acetic Ethylenediaminetetraacetic acid (1X TAE). The gel was stained with ethidium bromide and evaluated under UV light. The sizes of the PCR products were estimated according to the migrating pattern of a 100-bp DNA ladder. The PCR assays were done under strict precautions to prevent cross-contamination that would produce false positive results.

TABLE 1: Primers used for HCV detection

Region	Primer code	Sequence (5'-3')	T <sub>m</sub> (°C)	Product length
5'UTR	HCV19 outer/f	GCGACACTCCACCATAGAT	60.1	
	HCV20 outer/r	GCTCATGGTGCACGGTCTA	65.0	329 bp
	HCV21 inner/f	CTGTGAGGAACTACTGTCT	51.1	
	HCV22 inner/r	ACTCGCAAGCACCTATC	63.4	268 bp

F =forward (sense) primer, r = reverse (antisense) primer (Konomi *et al.*, 2000)

### Genotyping of HCV

HCV PCR positive samples were genotyped by the method of Ohno *et al* (1997). Table 2 shows the sequences and nucleotide positions of the primers used. The concentrations of primers used were adjusted to 2.5µM. RNA was reverse-transcribed to generate cDNA, amplified and typed by PCR using type-specific primers and Ready-To-Go RT-PCR Beads. Two rounds of amplification were carried out as described below.

RNA was first reverse-transcribed into cDNA with M-MuLV reverse transcriptase contained in the Ready-To-Go RT-PCR bead and with the random hexamer pd(N)<sub>6</sub>. The bead was dissolved in 40.5µl of RNase-free water on ice, followed by the addition of 2.5µl of 1µg/ul pd(N)<sub>6</sub> and 5µl RNA template. For the initial RT step, the samples were first incubated at 42°C for 25 mins, preheated at 95°C for 5 mins in a Thermal Cycler (Perkin-Elmer 2,400) and then put on ice while the first round PCR reaction mix was prepared.

The first round PCR utilized the primers Sc2 and Ac2, and *Taq* DNA Polymerase contained in the reaction tube. One microliter of each primer was added to the reaction tubes on ice. Amplification conditions included preincubation at 94°C for 1 min followed by 40 cycles with the following parameters: a preliminary 20 cycles of amplification at 94°C for 1 min denaturation, annealing at 45°C for 1 min, and extension at 72°C for 1 min; followed by an additional 20 cycles of 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. The product obtained in the above reaction was further amplified in a second round of HCV genotyping PCR.

#### *PCR for Genotyping of HCV*

Two different second-round PCRs were performed for each sample using Ready-To-Go PCR Bead. Two different primer mixtures (Table 2), one containing the S7, S2a, G1b, G2a, G2b and G3b primers (mix 1) and another containing the S7, G1a, G3a, G4, G5a and G6a primers (mix 2), were prepared and used for the two different reactions.

TABLE 2. Oligonucleotide primers used for PCR, sequencing and genotyping

Primer	PCR round	Sequence (5'-3')	Nucleotide position
Sc2	1	GGGAGGTCTCGTAGACCGTGCACCAT	- 24 – 3
Ac2	1	GAG(AC)GG(GT)TACCCCATGAG(AG)TCGGC	417 – 391
S7	2s	AGACCGTGCACCATGAGCAC	- 12 + 8
Ac2	2s	GAG(AC)GG(GT)TACCCCATGAG(AG)TCGGC	417 – 391
Mix 1			
S7	2g	AGACCGTGCACCATGAGCAC	12 +8
S2a	2g	AACACTAACCGTCGCCACAA	40-60
G1b	2g	CCTGCCCTCGGGTTGGCTA(AG)	222-203
G2a	2g	CACGTGGCTGGGAATCGCTCC	178-159
G2b	2g	GGCCCCAATTAGGACGAGAC	325-306
G3b	2g	CGCTCGGAAGTCTTACGTAC	164-145
Mix 2			
S7	2g	AGACCGTGCACCATGAGCAC	- 12-8
G1a	2g	GGATAGGCTGACGTCTACCT	196-177
G3a	2g	GCCCAGGACCGGCCTTCGCT	220-201
G4	2g	CCCGGGAACCTAACGTCCAT	87-58
G5a	2g	GAACCTCGGGGGGAGAGCAA	308-289
G6a	2g	GGTCATTGGGGCCCCAATGT	334-315

The primers used are denoted as follows: S = sense, A or G = antisense, and c = core region; 2s denotes second-round PCR for sequencing, 2g denotes second-round PCR for genotyping; the notations 1a to 6a are in accordance with HCV genotype nomenclature proposed by Simmonds *et al* (1994). Pairs of nucleotides inside parentheses being degenerate nucleotides (Ohno *et al.*, 1997).

The total reaction volume was 25 $\mu$ l and made up of 18.5 $\mu$ l RNase-free water, 6 $\mu$ l of 2.5 $\mu$ M PCR primer Mix. 1 or Mix. 2 and 0.5 ul template DNA. Amplification was performed with preheating at 94°C, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 62°C for 45 secs, and extension at 72°C for 1 min. Ten microliters of the second-round PCR product was electrophoresed on a 2% agarose gel in 1X TAE, stained with ethidium bromide, and evaluated under UV light. The HCV genotype was determined by identifying the genotype-specific cDNA bands in the gel with their expected sizes as shown in Table 3.

**TABLE 3: HCV Genotypes and Expected sizes in Agarose gel Electrophoresis**

Pimer	Genotype	Expected band size (bp)
Mix 1	1b	234
	2a	190, 139
	2b	337
	3b	176
Mix 2	1a	208
	3a	232
	4	99
	5a	320
	6a	336

### **HCV cDNA Cloning and Sequencing**

To confirm HCV genotype and to determine inter- and intra-patient subtype differences, the nucleotide sequences of HCV from the PCR positive samples were determined. A semi-nested PCR was performed with Sc2 and Ac2 as first-round primers and S7 and Ac2 as second-round primers. The PCR product was excised from the gel after the electrophoresis, purified and cloned into a TA cloning vector; and sequenced.

#### ***cDNA Extraction from Agarose Gel***

DNA extraction from gel was performed using QIAquick Gel purification protocol, designed to extract and purify DNA of 70 bp to 10 kb from low-melting agarose gels in TAE or TBE. With a scalpel the DNA fragment, approximately 429 bp long, was excised from the agarose gel and weighed in a 1.5 ml eppendorf tube. Three volumes (300µl) of gel solubilization buffer were added to each 100 mg of gel and incubated in a water bath at 50°C until gel slice completely dissolved in approximately 10 min. One gel volume of isopropanol (100µl for each 100mg gel slice) was then added to the sample and mixed. The solution was transferred onto a QIAquick column and span down for the DNA to bind to the column. The column was washed with 750µl of wash buffer. The column was transferred into a clean 1.5ml microcentrifuge tube; 40µl of autoclaved Milli Q water were added to the column and span to elute DNA into the tube. Eluted DNA was stored at -20°C.

### ***Competent Cell Preparation***

Competent cells were prepared by modifying the method described by Sambrook and Russell (2001). Fifty microliters of stock bacterial (*E. coli*) cells were added to 20ml of Luria Bertani (LB) medium in a 100ml flask. The cells were grown at 37°C overnight with vigorous shaking (150rpm). Five millilitres of the subculture were poured into 250ml LB in a one-liter flask and incubated for 3hr. The culture was then put on ice for 30min after which it was dispensed into 50ml centrifuge tubes and centrifuged at 3,000g for 15min in a refrigerated (4°C) centrifuge. The pellets were resuspended in half volume of ice cold 0.1 M CaCl<sub>2</sub> and again left on ice for 30min. The cells were pelleted and resuspended in a total volume of 8ml 0.1M CaCl<sub>2</sub> and left on ice overnight. Two milliliters (1/5 volume) of sterile glycerol was added and mixed by pipetting gently. The competent cells prepared were dispensed in 200µl aliquots into sterile eppendorf tubes and immediately put in dry ice and absolute ethanol mixture before storage at -70°C.

### ***Ligation and Transformation***

Nested RT-PCR products purified from agarose gel were inserted into a 2.1 pCR plasmid vector (Appendix I) to which a topoisomerase was covalently attached and which had a ligase activity. The ligation reaction mix was made up of 1µl each of salt solution (1.2 M NaCl and 60 mM MgCl<sub>2</sub>), vector and 1µg/µl insert DNA; and 3µl water (total reaction volume was 6 µl). The reaction proceeded at 23°C in a refrigerated water bath for 30min. Two microliters of the recombinant plasmid DNA reaction were used to transform bacterial cells as follows: Ninety-eight microliters of competent *E. coli* cells that had been thawed on ice were added to 2µl of ligated DNA in sterile reaction tubes and

immediately put on ice for 20 mins. The cells were heat-shocked at 42°C for 45 secs in a water-bath and then put on ice again for 10 mins. The transformed bacteria were spread on an agar plate containing ampicillin as selective agent by using glass beads. The plates were incubated at 37°C overnight.

### ***Miniprep Plasmid DNA Isolation and Purification***

A single colony of about 2 mm diameter was picked from a plate freshly grown overnight at 37°C and transferred into 2 ml of LB medium in a 10 ml culture tube. The culture was incubated overnight at 37°C with vigorous shaking in a water-bath with shaker. Plasmid DNA was purified by using QIAGEN Plasmid Purification kit. One milliliter of the culture was taken into a sterile eppendorf tube and spun at 1,000g for 1 min. The supernatant was completely discarded. Resuspension buffer, 150µl, were added and vortexed to completely resuspend the cells. To the suspension, 150µl of lysis buffer were added and mixed by gently inverting the tube 15 times. That was followed by the addition of 150µl of neutralization buffer and mixing by slowly inverting the tube 10 times. The lysate was spun at 13,000g for 3 min. The supernatant was carefully taken into a fresh tube containing 200µl of phenol chloroform isoamyl alcohol, vortexed and spun as before. The upper layer was carefully taken into fresh tube, two volumes (approximately 900µl) of absolute ethanol were added, shaken, and the pellet spun down at 1,300g as before. The supernate was aspirated. The pellets were washed with 250µl of absolute ethanol, dried in a vacuum chamber and resuspended in 40µl of DNase-free water. The purity and the concentration were measured and the concentration was adjusted to 1µg / µl.

Two micrograms of the above miniprep were digested with restriction with Eco RI and the electrophoresis run to determine positive clones. The remaining 1ml of the mini culture of the positive clones was used for a largeprep plasmid DNA culture.

### ***Largeprep Plasmid DNA Isolation and Purification***

One hundred milliliters of LB medium were transferred into a 250 ml culture flask and inoculated with 1 ml mini prep culture and incubated at 37°C overnight with vigorous shaking. Plasmid DNA was purified using QIAGEN Plasmid Purification kit in almost the same procedure as for mini prep.

The cells were aseptically transferred to sterile 50-ml polypropylene tubes. The cultures were cooled to 0°C by storing the tubes on ice for 10 mins. The cells were recovered by centrifugation at 5,000g for 15 mins at 4°C in a refrigerated centrifuge. Cell resuspension, lysis and neutralization were done as for mini prep using 10ml each of the cell resuspension, lysis and chilled neutralisation buffers. A QIAGEN-tip 500 column was equilibrated 10 ml equilibration buffer and the column allowed emptying by gravity flow. After centrifugation, the supernate was filtered through the resin of the column and washed two times with 30 ml wash buffer. DNA was eluted with 15 ml elution buffer into a fresh tube and precipitated by adding 10.5 (0.7 volume) room-temperature isopropanol to the eluted DNA, mixing and centrifuging as before. The DNA was washed with 5ml 70% ethanol, dried and resuspended in 100µl DNase-free water. The purity was measured and the concentration adjusted to 1µg /µl. The purified DNA was digested with Eco RI to check for the presence of insert DNA before sequencing.

### ***Restriction Enzyme Digestion***

The restriction enzyme digestion reaction mix was made up of the following: 16.5µl Milli Q water, 2µl of 10 X digestion reaction buffer, 0.5µl of enzyme (Eco RI) and 1µl plasmid DNA (1µg/µl) in a 1.5 ml eppendorf tube. The reaction components were mixed by tapping the reaction tube gently. The reaction was proceeded with incubation at 37°C for 2 hours in a water bath. Digestion products were run on 1% agarose in 1X TAE.

### ***PCR for HCV cDNA Sequencing***

Sequencing PCR reactions were performed using Cy5.5<sup>TM</sup> dye-labelled dideoxynucleotide triphosphate (ddNTP) terminators. Four tubes were labelled A, C, G, and T for the respective termination reactions. For each template that was sequenced, a master mix of DNA template (2µg DNA obtained in 4µl of 0.5µg/µl DNA concentration), reaction buffer (3.5µl), 1 µM sense or antisense primer (2.0µl), ThermoSequence DNA polymerase (10 U/µl, 2µl) and distilled water up to total reaction volume of 31.5µl was prepared. Into the appropriately labelled tube, 1µl of the corresponding Cy5.5 ddNTP termination mix was dispensed. After thorough mixing, 7µl of the master mix were aliquoted into the reaction tubes, thoroughly mixed again and cycled in a thermocycler with the following parameters: one-minute pre-incubation at 95°C, 30 cycles consisting of 30 secs denaturation at 95°C, 30 secs annealing at 55°C and a 2-min extension at 72°C. The PCR products were purified for sequencing as described below.

### *Purification of PCR Products*

Unincorporated dye terminators were removed by ethanol precipitation method. The PCR products were transferred to a 1.5 ml eppendorf tubes. To each tube, 2 $\mu$ l of 7.5 M ammonium acetate was added and 30 $\mu$ l (approximately 3 times the reaction volume) of chilled 100% ethanol. The tubes with their contents were centrifuged in a refrigerated microcentrifuge at 12,000g for 30 mins at 0°C. The supernatant was carefully removed and the pellets washed with 200 $\mu$ l of chilled 70% ethanol through 5 mins centrifugation at 0°C. The pellets were vacuum-dried for 10mins in a vacuum centrifuge. Each pellet was completely resuspended by adding 6 $\mu$ l formamide loading dye and vortexing gently for 10 secs.

### *Sequencing Electrophoresis*

DNA was sequenced in an acrylamide gel electrophoresis. A SureFill Gel Cartridge and a MicroCell Cassette were used for the electrophoresis. The SureFill Gel Cartridge contained an aqueous solution of unpolymerized acrylamide and a number of components to aid electrophoresis. The MicroCell Cassette was designed to be easily filled with acrylamide solution containing a photoinitiator. A comb was inserted into the MicroCell Cassette and was properly placed into a fill fixture. By using an injector, the cassette was filled with gel from the cartridge. The cassette was then loaded into a gel toaster and polymerised ready for use.

Two microlitres of each sample were loaded into separate lanes of prepared sequencing gel and sequenced in SEQ 4 x 4 Sequencer with 1X TBE (Appendix I) as running buffer. Nucleotide sequences obtained were aligned and compared with previously reported

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sequences. The inner sense primer (2s AGACCGTGCACCATGAGCAC) used for HCV sequencing was used to blast the Internet to retrieve HCV sequences from the gene bank. The sources of sequence data used for comparison with those obtained in this study were as shown in Table 4.

TABLE 4: Sources of sequence data for core region used for comparison with those obtained in this study.

Genotype	Isolate	Accession no.
1b	HCJ 2	D10074
	HCV-S1	AF356827
	HCV-A	AJ000009
	HCV-N	AF139594
	MD33	AF207774
2a	HCJ 6	D00944
	NDM59	AF1690005
2b	MA	AB030907
5a	INDIA	AY051292
	EUH1480	Y13184
Outgroup	SynCon	

## CHAPTER THREE

### RESULTS

#### Sample Collection, Serological Testing and Confirmation

A total of 200 blood samples were collected from donors at the National Blood Transfusion Service, Korle-Bu Teaching Hospital, Accra for the studies. The samples were screened for the identification of seropositive samples for virus extraction as described in materials and methods. Out of the 200 samples screened, 54 (27%) were found positive by low dilution (1 in 64) PA assay, 12 (6%) were positive by high (1 in 512) dilution PA assay. Only 1 of the 12 samples also reacted on ELISA, 1 sample reacted on ELISA alone but was found negative by PCR. HCV RNA was detected by PCR in 2 samples – HCV 152 and HCV 173 as seen in Fig. 5. HCV 152 was both PA and ELISA reactive while HCV 173 was reactive by PA only. The reactivity pattern is summarized in Table 5 below.

Table 5: Reactivity profiles for thirteen subjects positive for anti-HCV by the SERODIA particle agglutination (PA) assay, ELISA and confirmation by PCR (n = 200)

	PA 1:512*	ELISA
PA 1: 512*	10 (2) <sup>P</sup>	1
ELISA	1(1) <sup>P</sup>	2
Total Positives	13	2

<sup>P</sup>- indicates number of tested samples confirmed positive by PCR

\*PA 1:512 indicates the dilution of plasma sample in the SERODIA assay

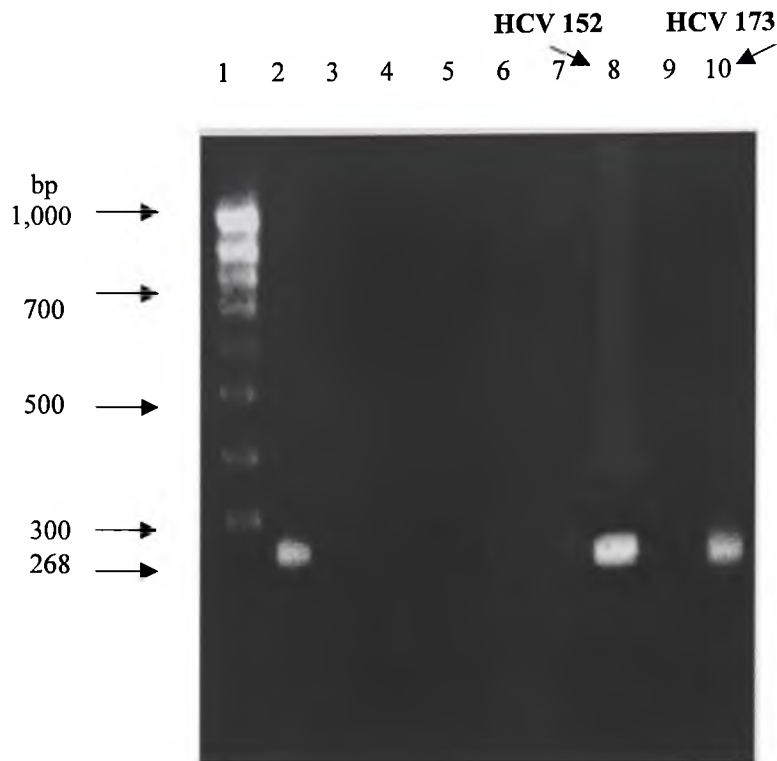


Fig. 5. HCV detection in plasma samples. Electrophoretic mobility of HCV cDNA product in 2% agarose gel.

Lane 1 - 100 bp molecular weight markers.

Lane 2 - cDNA of globin mRNA as HCV positive control.

Lanes 3, 4, 5, 6, 7 and 9 – RT-PCR amplified products showing no bands suggestive of HCV.

Lanes 8 and 10 RT-PCR amplified products of plasma HCV RNA extracts showing product bands of size 268 bp indicating presence of HCV.

The electrophoretic patterns of PCR products from different genotyping PCR reactions are shown in Fig. 6. Each sample was analysed twice by PCR with primer 'Mix 1' and 'Mix 2', electrophoresed on a 2% agarose gel and stained with ethidium bromide. Primer 'mix 1' amplification allows for the specific detection of PCR products for HCV genotypes 1b, 2a, 2b and 3b while primer 'mix 2' allows for the specific detection of HCV genotypes 1a, 3a, 4, 5a and 6a. The sizes of the HCV genotype-specific products are given in Table 3. Lane 1 shows a 100bp DNA molecular weight marker. Lanes 2 and 3 contain the samples HCV 152 and HCV 173 respectively, amplified with primer 'mix 1'. Lanes 4 and 5 contain the same samples amplified with primer 'mix 2'. In 'mix 1' (left half). Two specific bands of sizes 190 and 337 corresponding to genotypes 2a and 2b respectively, were detected in lane 2, while a 190 bp band corresponding to genotype 2a was detected in lane 3. However, no such bands were detected in the corresponding lanes of 'mix 2' (lanes 4 and 5).

Figure 7 shows the electrophoretic pattern of a semi-nested PCR amplification products obtained from the HCV infected samples for cloning. A sequence in the core region of the HCV genome was RT-PCR-amplified with Sc2 and Ac2 as first-round PCR primers and S7 (nucleotide position -12) and Ac2 (nucleotide position 417) as second-round PCR primers. The products (429 bp) were electrophoresed on a 2% agarose gel and stained with ethidium bromide

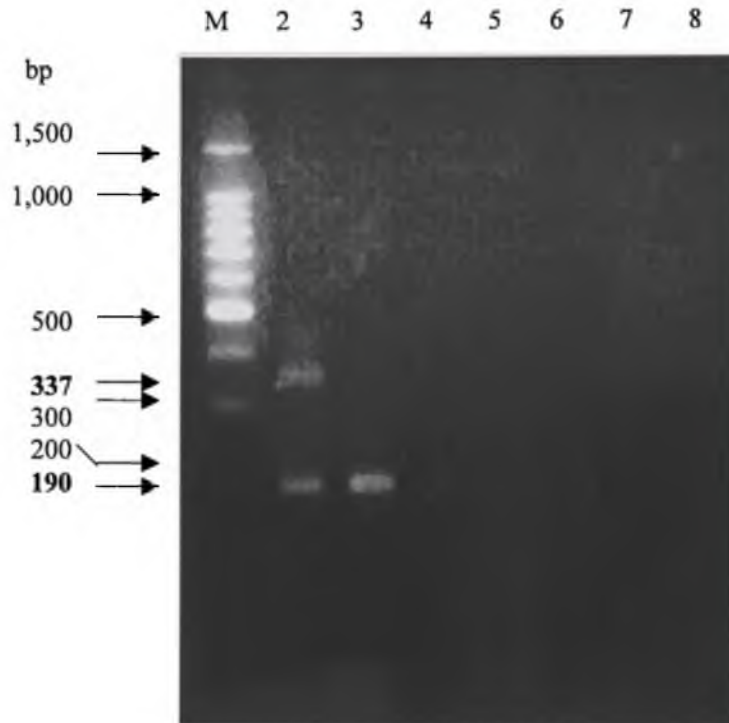


Fig. 6. Electrophoretic patterns of PCR products from different genotyping primer mix systems. Migration positions of PCR products of two different HCV types are shown in bold.

Lane 1- 100bp DNA molecular weight markers

Lanes 2 and 3 Two PCR reactive samples HCV 152 and HCV 173 respectively amplified with 'primer mix 1' system.

Lanes 6 and 7 - Same PCR reactive samples HCV 152 and HCV 173, respectively, amplified with primer 'mix 2' system.

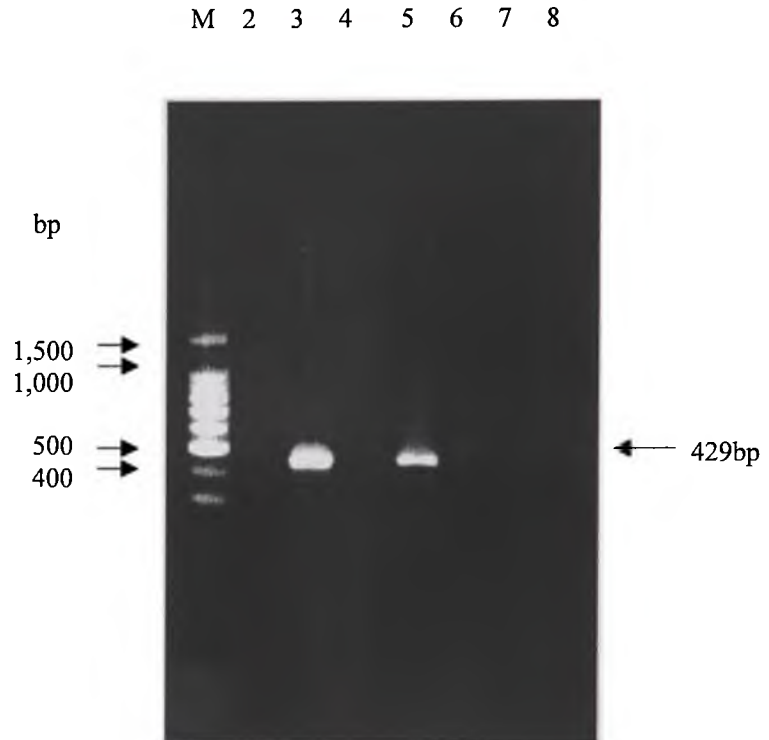


Fig. 7. Semi-nested PCR amplification products obtained from HCV infected samples to be purified for cloning.

Lane 1 contains 100 bp DNA size markers.

Lanes 3 and 5 products of HCV 152 and 173 respectively with size of 429 bp corresponding to the amplified HCV core region.

Figure 8 shows the electrophoretic pattern of the rDNA (4.35 kb) obtained from cloning HCV cDNA (429 bp) purified from semi-nested PCR electrophoretic gel into pCR 2.1 TOPO vector (3.921 kb). The rDNA obtained was used to transform competent *E. coli* cells. DNA was purified from overnight cultures of *E. coli* colonies. In the figure, DNA bands of about 4.35kb (corresponding to the size of the expected rDNA) appeared in all the sample lanes. The sizes of the purified products (lanes 2 to 6) were estimated according to the migration pattern of a 1 kb molecular size marker. The numbers in parentheses are the clone numbers.

In Fig. 9, the Eco RI digestion products of the five clones HCV 173 (1), 173 (2), HCV 152 (4), 152 (5) and HCV 152 (8) are shown in 2% agarose gel electrophoresis. DNA cleavage was achieved due to the presence of Eco RI recognition sequence about eight base pairs before and after the PCR product insert site of the pCR 2.1-TOPO vector (Appendix II). Positive clones showed two bands of digestion products: the bigger band is that of the vector DNA (~ 3.9 kb) and the smaller band shows the insert DNA (~ 429 bp).

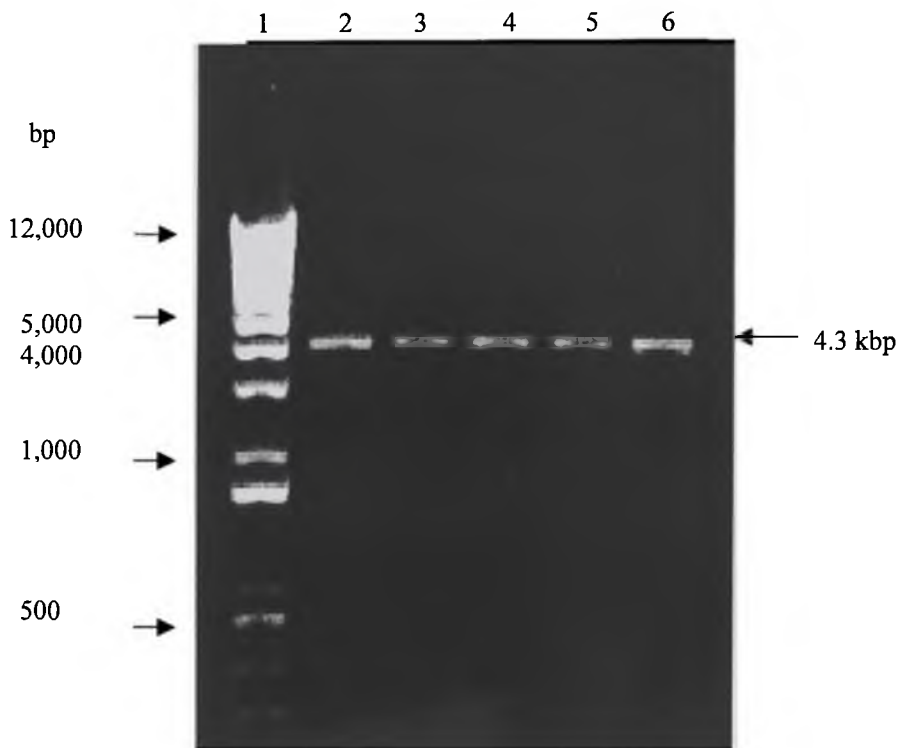


Fig. 8. Agarose gel (2%) electrophoresis of recombinant DNA (rDNA) purified from cultured bacterial colonies.

Lane 1 - migration positions of a 1 kb molecular size markers.

Lanes 2, 3, 4, 5 and 6 - rDNA bands of about 4.35kb corresponding to the clones HCV 173 (1), 173 (2), HCV 152 (4), 152 (5) and HCV 152 (8).

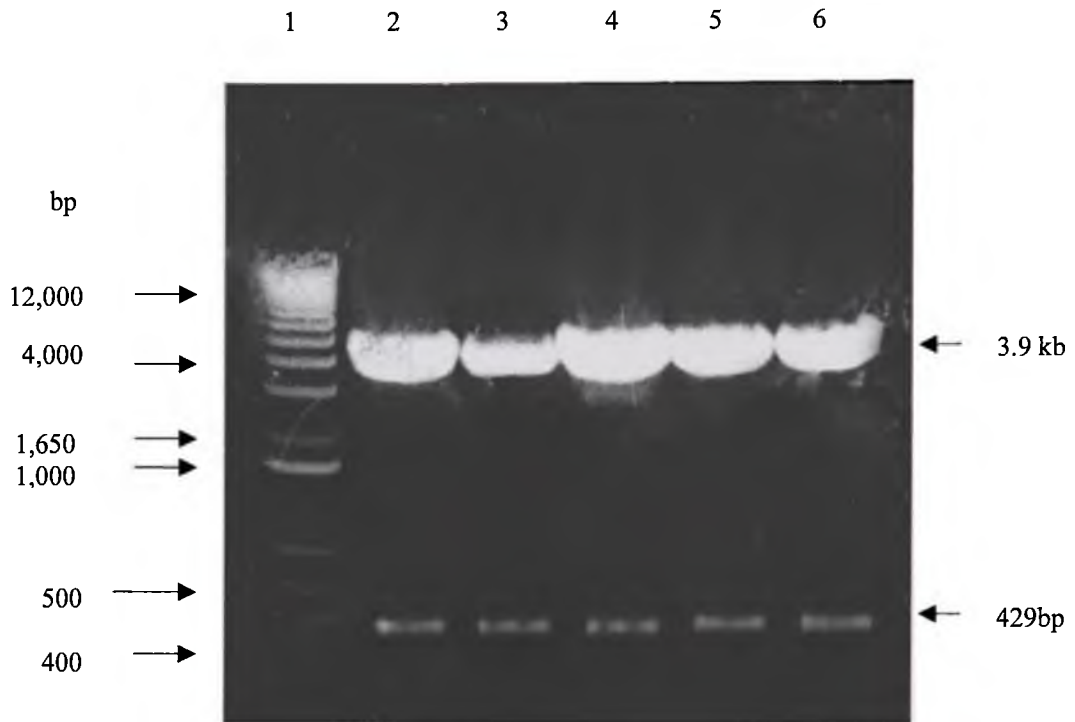


Fig. 9. Eco RI digestion of DNA clones purified from cultured colonies of transformed *E. coli*.

Lane 1 - 1kb molecular weight markers

Lanes 2 to 6 - Eco RI digestion products of HCV 173 (1), 173 (2), HCV 152 (4), 152 (5) and HCV 152 (8) respectively. Two product bands of sizes ~ 3.9 kb (bigger band) and ~ 429 bp (smaller band) corresponding to vector and insert DNA, respectively, were observed in each lane.

### cDNA Nucleotide Sequences and Analyses

In all five clones were obtained and the core regions of four, comprising two from each of the two PCR positive HCV extracts, were successfully sequenced. Consensus sequences of the 4 clones were obtained and aligned together with references available for the core region. Table 6 shows the sequence identity matrix performed to compare the sequences obtained. There was poor homology (69.9%) between one pair of clones from HCV extract that showed dual genotype, 2a and 2b. The homology between the other pair that showed a single genotype, 2b was greater than 95%. The intertype homology varied from 70% to 95% depending on the clones compared.

Table 6. Sequence identity matrix of four HCV clones from two HCV positive specimens

Seq->	HCV152 (4)	HCV 152 (8)	HCV173 (1)	HCV 172 (2)
HCV 152 (4)*	1.000	0.699	0.704	0.704
HCV 152 (8)†	---	1.000	0.940	0.945
HCV 173 (1)†	---	---	1.000	0.962
HCV 173 (2)†	---	---	---	1.000

† All HCV 173 clones and one clone of HCV 152 (clone 8) show high similarity (> 92 %) to one another but different from one other clone of HCV 152 (clone 4) \*.

The core HCV cDNA sequence of nucleotides of Ghanaian HCV extracts are shown in Fig. 10 together with some other isolates of HCV whose core sequences are available. The first twenty nucleotides at the 5' end show the inner forward primer sequence used for the PCR amplification prior to cloning and sequencing. The same sequence was used to obtain available sequences from the Gene bank for comparison. Homologous nucleotides are highlighted on a black background.

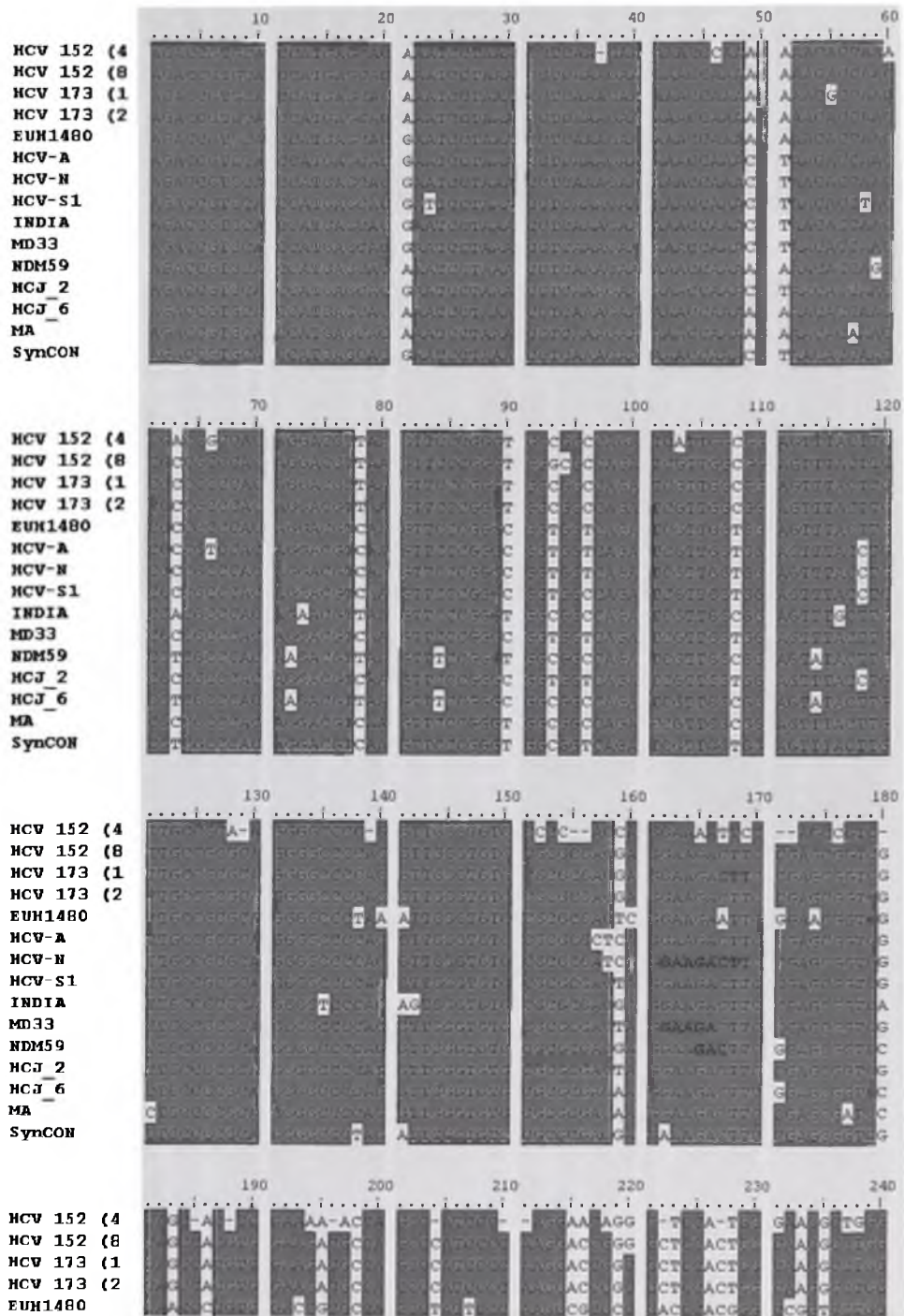
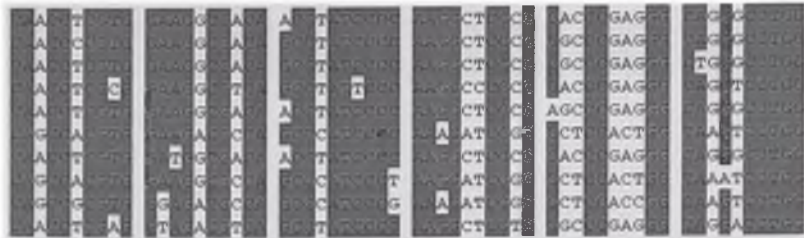
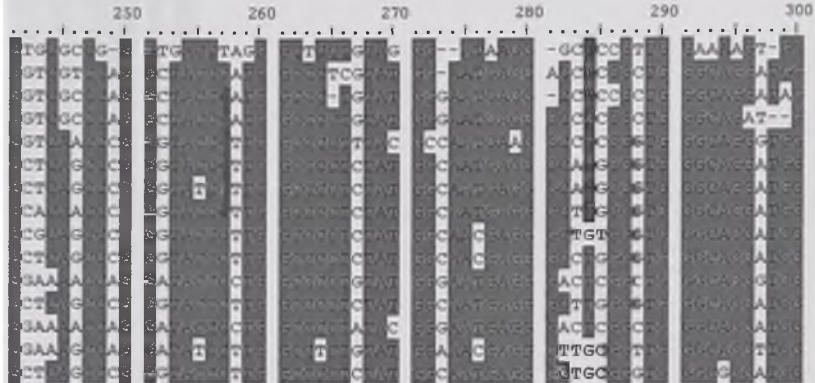


Fig. 10. Core nucleotide sequences of HCV cDNA.

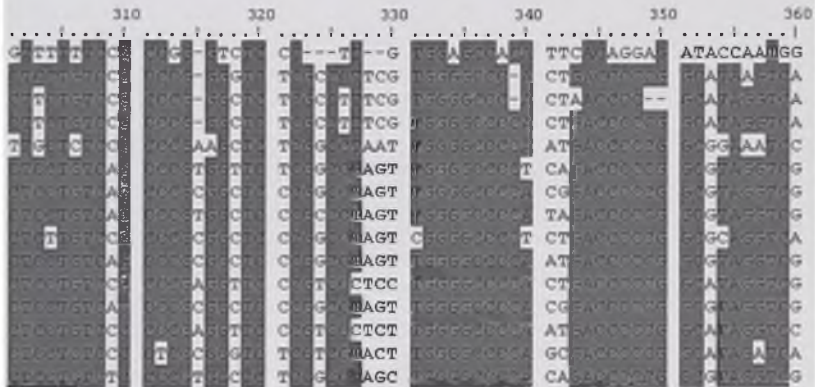
HCV-A  
HCV-N  
HCV-S1  
INDIA  
MD33  
NDM59  
HCV\_2  
HCV\_6  
MA  
SynCON



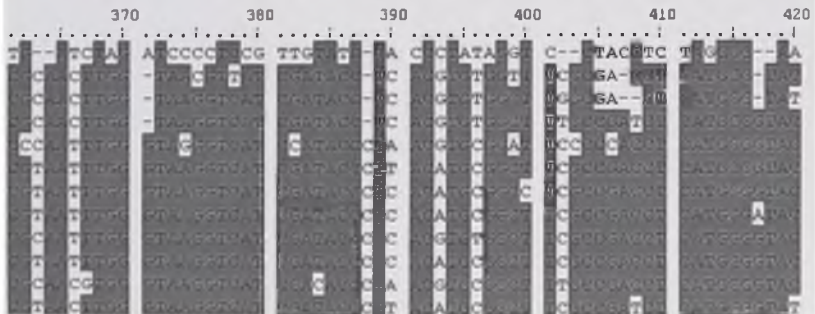
HCV 152 (4)  
HCV 152 (8)  
HCV 173 (1)  
HCV 173 (2)  
EUH1480  
HCV-A  
HCV-N  
HCV-S1  
INDIA  
MD33  
NDM59  
HCV\_2  
HCV\_6  
MA  
SynCON



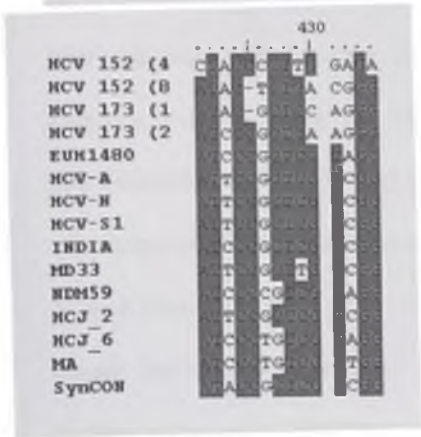
HCV 152 (4)  
HCV 152 (8)  
HCV 173 (1)  
HCV 173 (2)  
EUH1480  
HCV-A  
HCV-N  
HCV-S1  
INDIA  
MD33  
NDM59  
HCV\_2  
HCV\_6  
MA  
SynCON



HCV 152 (4)  
HCV 152 (8)  
HCV 173 (1)  
HCV 173 (2)  
EUH1480  
HCV-A  
HCV-N  
HCV-S1  
INDIA  
MD33  
NDM59  
HCV\_2







Phylogenetic tree (neighbour joining) of HCV core sequences of four Ghanaian HCV cDNA clones (HCV 152 –4, HCV 152 –8, HCV 173 –1 and HCV 173 – 2) and available reference sequences from the Gene bank using a synthetic construct (Syn CON) as an outgroup is shown in Fig. 11. The tree was constructed by the neighbour-joining method. The Ghanaian extracts are highlighted on a faint black background. The percentage of bootstrap replications (within branches at relevant nodes) supporting the branches leading to each clade is indicated. Bootstrap values are reported when greater than 55%. The branch leading to the group of Ghanaian extracts is 99% supported. Also the branch leading to the group of Ghanaian extracts and the group to which the HCJ 6 of genotype 2a belongs is 100% supported.

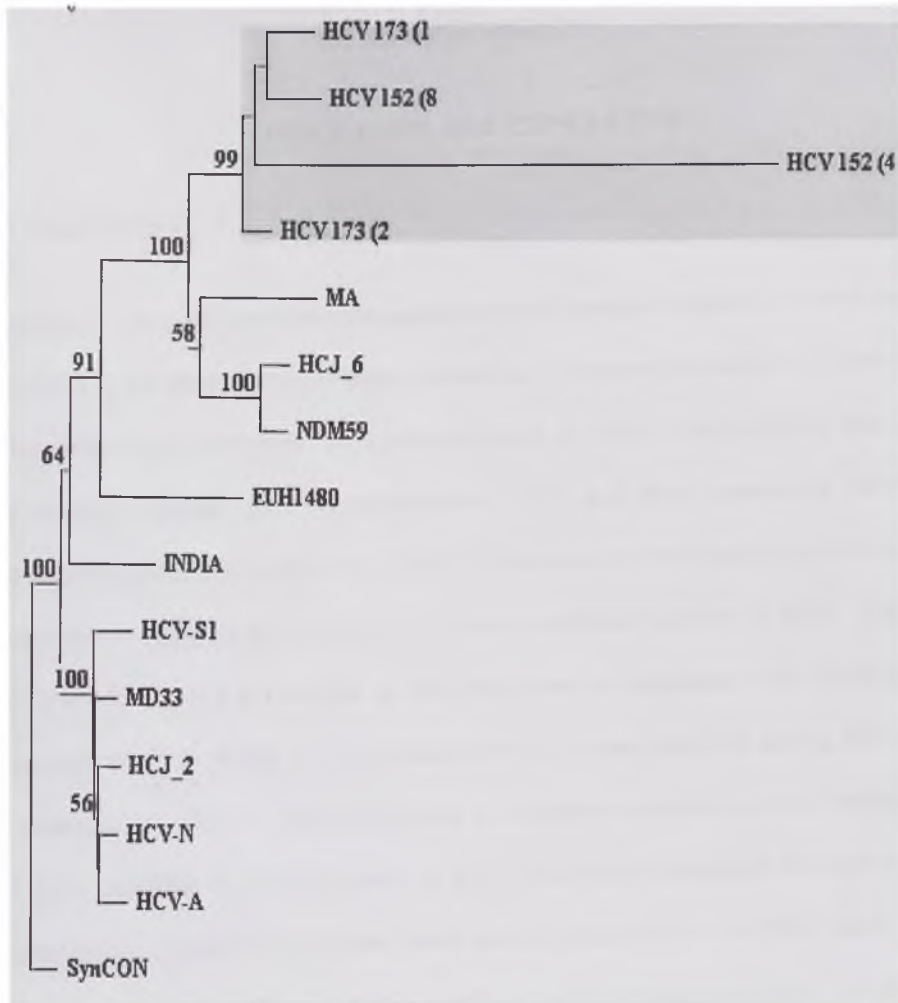


Fig. 11. Phylogenetic tree of HCV core cDNA sequences of Ghanaian extracts (highlighted) and other available isolates.

## CHAPTER FOUR

### DISCUSSION AND CONCLUSION

#### 4.1 DISCUSSION

Hepatitis C virus infection has been associated with repeated exposure to blood and blood products in the absence of serological screening. Non-specific reactivity of anti-HCV in Ghanaians has been reported in a previous study, in which false positivity was resolved by a high dilution particle agglutination (PA) and third generation recombinant immunoblot assay (Ampofo *et al.*, 2002). In this current investigation, out of 200 blood samples screened for anti-HCV, 2 (1%) were confirmed positive by PCR. The overall seroprevalence of 1% recorded in this study was in agreement with findings of the previous study in which HCV prevalence of 0.9% was observed among blood donors (Ampofo *et al.*, 2002). Such prevalence is moderate compared to what occurs in other African countries (2 – 14%) as seen in Fig. 1, but high if compared with the prevalence levels in the United Kingdom and some parts of Japan (0.04 – 0.09%); North America, Western Europe and Israel (0.15 – 0.5%). HCV antibody screening might help to practically eliminate any transfusion-associated hepatitis.

Positive anti-HCV test provides evidence of exposure to HCV. Blood donors are clearly not representative of the entire population, but scrutiny of HCV positive donors provides some insights into the epidemiology of this infection (Alter, 1995; Mansel and Locarnini, 1995). The low specificity of the PA screening assay (Table 5) in this study could be attributed to non-specific reactivity with antibodies to the closely related viruses of the

*Flaviviridae* family. The RT-PCR results shown in Fig. 5 confirmed the presence of HCV.

Based on the first and second PCR primer pairs used, the second amplification product was expected to give a distinct band of 268 bp regardless of the genotype, when run on agarose electrophoretic gel. The products observed in Fig. 5 were in agreement with this. The very high band intensity of one of the PCR reactive samples (HCV 152) could be due to high viral load since the same amount of DNA extract was used for all the samples. Such information concerning the extent, or especially the apparent quantification of viraemia, is necessary to assist with the identification and monitoring of patients prior to or during an anti-viral therapy. It is also relevant for health care workers implicated in “injury-prone” activities such as surgery, other invasive diagnostic or therapeutic interventions, dental procedures and for chronically infected women planning pregnancy.

The results from the determination of HCV genotypes using type-specific primers in RT-PCR techniques (Fig. 6) showed evidence that HCV type 2 is present in Ghana. This observation supports previous findings in a study conducted in Kumasi in which HCV type 2 was observed in pregnant women and blood donors (Wansbrough-Jones *et al.*, 1998). It had been suggested that HCV genotypes 1 and 2 might be endemic in West Africa (Stuyver *et al.*, 1994). The diagnosis of type 2 HCV, especially genotype 2a among blood donors in this study complements the proposal by Wansbrough-Jones *et al.* (1998) that type 2 HCV might be indigenous to Ghana.

Reports have shown that co-infection with more than one HCV subtype is common among intravenous drug abusers (De Socio *et al.*, 1996). There is also some evidence that infection with different virus genotypes is associated with differences in the clinical course of disease, and the likelihood of developing hepatocellular carcinoma (Abe *et al.*, 1998). Repeated exposure to blood and blood products, in the absence of serological screening and / or intravenous drug abuse might account for HCV infections observed since they are largely associated with a high risk of HCV exposure and infection (Alter *et al.*, 1998, Thomas *et al.*, 1995).

HCV genotypes have been shown to be associated with specific geographical areas, specific routes of transmission and specific clinical implications (Zein, 2000; Amorso *et al.*, 1998; Simmonds *et al.*, 1996; Bukh *et al.*, 1993). Genotypes 2 and 3 are predictive of a better response to interferon- $\alpha$  therapy than type 1, which is suggested to be associated with more severe liver disease than other genotypes (Feraÿ *et al.*, 1995; Martinot-Peignoux *et al.*, 1995). Subtyping will be required not only for the initiation and monitoring of therapy, but also for the choice of vaccine and further follow up of vaccinees. Besides, genotyping is required for the correct interpretation of HCV antibody diagnosis.

HCV genotypes 2a and 2b are most frequently found in Taiwan, East Asia, parts of China, (Decker and Troonen, 2001) and Japan (Okamoto *et al.*, 1992). Overwhelming lines of epidemiological evidence have indicated direct involvement of hepatitis C virus infection, particularly genotype 2, in hepatocellular carcinoma (Koike *et al.*, 2002; Abe *et*

*al.*, 1998). Perhaps a similar situation may exist in Ghana since HCV is rarely screened for in clinical practice and it may be necessary to do routine screening particularly for patients having liver and related problems to determine the contribution of HCV infection in such cases.

Considering the geographical distribution of HCV genotypes, and the demonstrated presence of HCV type 2 in Ghana, there is the possibility that this genotype originated from countries in East Asia and USA, which are associated with genotypes 2a and 2b. Another possibility may be that these genotypes have been introduced across neighbouring West African countries namely Nigeria, Guinea, Burkina Faso, Gambia, and Benin due to the high rate of migration in the sub-region.

A comparative analysis of nucleotide sequences of multiple clones of an RT-PCR fragment obtained from the core region indicated phylogenetic relatedness of HCV in Ghanaian extracts. The detection of the primer sequences (first twenty nucleotides) in the core HCV sequences compared as shown in Fig. 10 confirmed the validity of the procedure followed and the reliability of such primers used in this region for the detection HCV RNA. The pattern of Ghanaian HCV nucleotide sequences obtained in this study appeared to belong to a common phylogenetic group as seen (with shaded background) in Fig. 11; although they were also similar to sequences of some of the available isolates they were compared with. This was demonstrated by an inter-donor nucleotide similarity of at least 94%. There was 30% difference in cDNA nucleotide sequences of the sequences of the Ghanaian HCV extracts representing HCV genotypes 2a and 2b. The

high genetic distance observed in one of the Ghanaian HCV extracts, HCV 152 (4), in the phylogenetic tree shown in Fig. 11 may indicate a more rapid process of diversification than other subtypes and perhaps, the possible existence of strains of unusual genotypes in asymptomatic Ghanaians (Mellor *et al.*, 1995).

The need for routine screening of all blood donations for all transfusion-relevant viruses, with priority going to HCV, HBV and HIV, cannot be overemphasized. Also, the distribution of HCV genotypes needs to be periodically monitored since they may result in serious modifications of patterns of disease and responses to therapy in the future.

## 4.2 CONCLUSION

This study has demonstrated that:

- (i) The current seroprevalence of HCV among healthy Ghanaians in Accra is 1%.
- (i) HCV genotype 2 is probably the prevalent HCV type common among blood donors in Accra.
- (ii) Intra- and intertype differences exist in Ghanaian HCV strains. However, these HCV strains appear to belong to a common phylogenetic group and seem to vary significantly from strains from other geographical regions. Overall, the endemicity of type 2 HCV in Ghana is evident.

This local nucleotide sequencing procedure is one of the first attempts at molecular diagnosis of HCV infection in Ghana and also reports a novel genotype 2a/2b dual infection (Fig. 6) in Ghana. It presents the possibility of interferon- $\alpha$  treatment for HCV

infections in Ghana. The results also support the previous suggestion by Wansbrough-Jones *et al* (1998) that HCV genotype 2 is indigenous to Ghana.

### **Recommendations**

This study illustrates primarily the current transfusion-transmissible risk of HCV in Ghana. It is recommended that:

1. Effective routine HCV diagnosis is required not only for the safety of blood supply prior to transfusion but also for identifying carriers for initiation of treatment
2. Developing appropriate methods for HCV diagnosis will require evaluation of cost-effectiveness.
3. Results of this study stress the need to continue surveillance of circulating HCV genotypes to provide data for vaccine application in the country.
4. Complete subtype differentiation of the prevailing genotypes will be particularly useful in determining the route of infection and also reflect the past epidemiology of HCV transmission.
5. Further, there is the need to investigate the association of HCV types with clinical disease in an extended series of patients and symptom-free carriers, and in various populations.
6. Finally, isolation and determination of full-length HCV genomic sequences in Ghana may be helpful in designing vaccine and for serological investigations of possible group- and type-specific antibodies.

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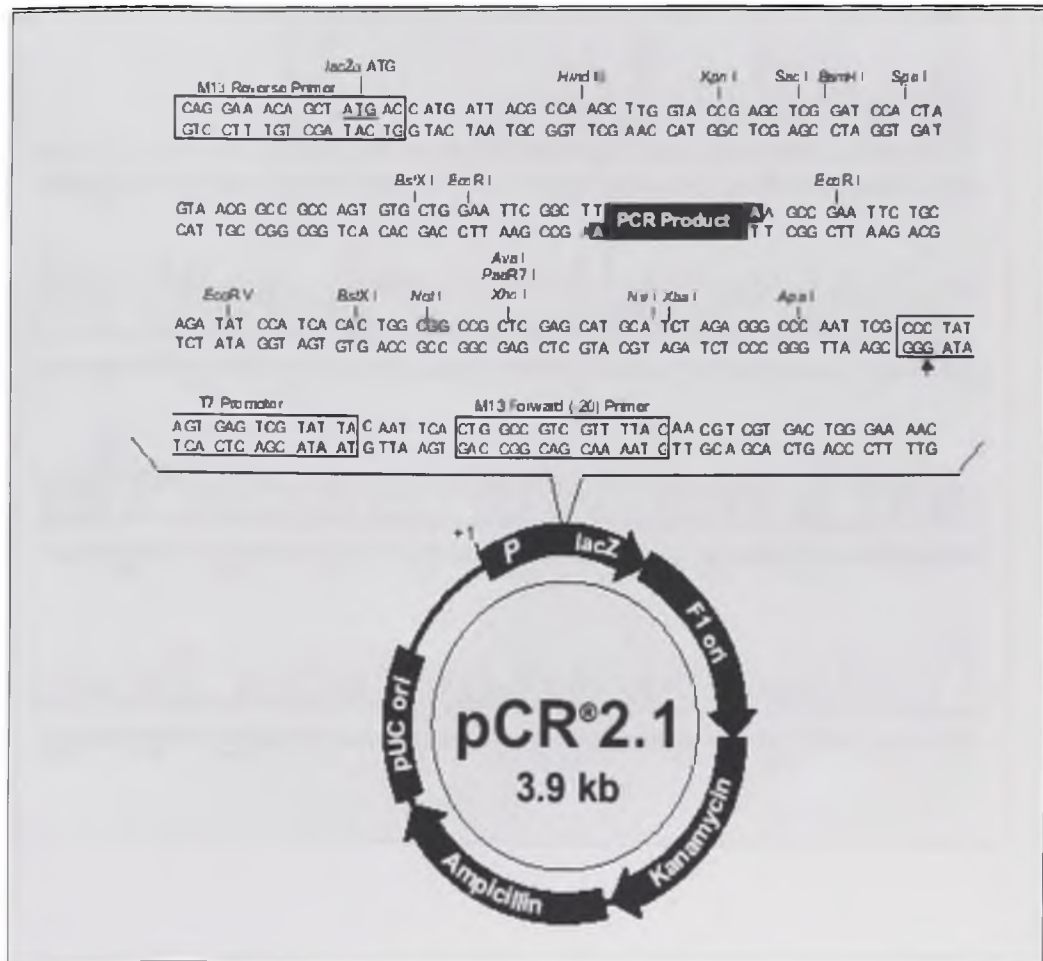
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APPENDICES

APPENDIX I

Restriction map of pCR 2.1 TOPO Vector



## APPENDIX II

### Complementary DNA sequence graph of Ghanaian HCV extract

