DEVELOPMENT OF A TWO-STEP HCV QUANTIFICATION ASSAY BASED ON THIRD GENERATION EVAGREEN DYE FOR REAL-TIME PCR: A PILOT STUDY

BY

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ABSTRACT

HCV exhibits extreme genome heterogeneity, additionally it is usually present in blood at a low copy number. Thus, high specificity and sensitivity represent a major challenge during development of any HCV detection and quantification assay. The aim of the present study was to establish a highly specific and sensitive semi-nested real time PCR using EvaGreen dye for detecting and quantifying HCV RNA. A total of 50 serum samples, comprising 40 HCV- positive and 10 HCV-negative, were included in our study. RNA was extracted, reverse transcribed, and then subjected to two rounds of PCR amplification. In the first round, conventional PCR was performed using a pair of primers targeting the 5'UTR. In the second round, real time PCR using EvaGreen dye and primers targeting a region within the previously amplified product was carried out for sensitive detection and quantification. Reference samplesnumber 15 and 39with known viral load were treated similarly to the unknown samples and used to create the standard curves.Our method showed a high level of analytical specificity and accuracy, with a low limit of detection (~2 IU/ml). It yielded repeatable results with less than 4% of intra- assay variation. The assay covered a broad dynamic range of quantification, ranging from 0.34 to 6 log IU/ml. The diagnostic sensitivity, specificity, and accuracy were all 100%, indicating neither false positive nor false negative results were obtained. The newly developed semi-nested real time PCR using EvaGreen has demonstrated a high analytical and diagnostic performance, suggesting its potential to have wide applications in clinical diagnosis, therapeutic management, and epidemiological studies of HCV.

خلاصة البحث

التحديات التي تواجه العاملين على تطوير اختبارات كشفية و كمية لفيروس التهاب الكبد سي. يعزى ذلك إلى طبيعة الفيروس المتمثلة في إظهار درجة عالية من التغير الوراثي, بالإضافة إلى وجوده بأعداد قليلة في الدم. تهدف هذه الدراسة إلى تطوير طريقة جديدة مبنية على تفاعل إنزيم البلمرة المتسلسل اللحظي الشبه متداخل باستخدام صبغة الايفا الخضراء, حيث تتميز هذه الطريقة بدرجة عالية من الخصوصية والحساسية في الكشف عن وجود الفيروس وتحديد كميته في الدم. أجريت هذه الدراسة على 50 عينة, والتي تشمل 40 عينة موجبة و 10 عينات سالبة لفيروس التهاب الكبد سي. لقد تم استخلاص الحمض النووي الريبوزي من جميع العينات, متبوعآ بعملية النسخ العكسي لتحضير الحمض النووي المكمل. وقد أجريت عملية مضاعفة الحمض النووي المكمل على مرحلتين. تضمنت المرحلة الأولى تفاعل إنزيم البلمرة المتسلسل التقليدي باستحدام بادئات تستهدف منطقة ثابتة وراثيأ. وأما في المرحلة الثانية فقد تمت عملية التضاعف الناتجة من المرحلة الأولى عن طريق تفاعل إنزيم البلمرة المتسلسل اللحظي باستخدام صبغة الايفا الخضراء للكشف عن وجود الفيروس وتحديد كميته في الدم. و تتم معالجة العينة المرجعية التي تحتوي على تركيز معلوم لكمية الفيروس فيها بنفس الطريقة التي تعالج بها العينة الغير معلومة التركيز. و هذى العينات المرجعية استخدمت لإنشاء المنحنى المعياري لتحديد كمية الفيروس في العينات الغير معلومة التركيز. و قد أظهرت الطريقة المبتكرة في هذه الدراسة مستوى عال من الصحة و الخصوصية مع درجة عالية من الحساسية التحليلية و التي تتمثل في قدرة حساسية الإختبار على كشف 2 وحدة دولية لكل مل كحد أدنى للكشف كمية الفيروس في الدم. بالاضافة الي ذلك , هذه الطريقة قادرة على تغطية مدى واسع للكشف و تحديد كمية الفيروس في الدم ابتداء من لوغاريتم 0.34 الي 6 وحدات دولية لكل مل. بينما تظهر قياس التكرارية على الإختبار المبتكر نتائج متقاربة ,والتي لا تتجاوز 4 %. وبالنسبة لتقييم القدرات التشخيصية للإختبار , لقد كانت حساسية وخصوصية و مدى صحة الإختبار التشخيصي 100% , والتي تدل على عدم وجود نتائج إيجابية أو سلبيةكاذبة. قد أظهرت هذه الطريقة المطوره قدرة عالية من الآداء التحليلي و التشخيصي، والتي قد تشير بأن إمكانياتها لها تطبيقات واسعة في تشخيص و علاج الإصابة بفيروس التهاب الكبد سي.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Master of Medical Sciences.

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LIST OF ABBREVIATIONS

ARF	Alternate Reading Frame
bDNA	branched DNA assays
Cq	Quantification Cycle
Ct	Threshold Cycle
CV	Coefficient of Variation
DAA	Direct-Acting Antiviral
EIA	Enzyme-Linked Immunoassays
ELISA	Enzyme-Linked Immunosorbent Assay
ETR	End-of-Treatment Response
EVR	Early Virological Response
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HRM	High Resolution Melting
HTAA	Hospital Tengku Ampuan Afzan
IU	International Unit
NANBH	Non-A, Non-B Hepatitis
NS	Non-structural Proteins
ORF	open reading frame
PCR	Polymerase chain reaction
PEG-INF	pegylated interferon
qPCR	quantitative PCR
RdRp	RNA-dependent RNA polymerase
RIBA	Recombinant Immunoblot assays
RT-PCR	Reverse transcription PCR
RVR	Rapid Virological Response
SD	Standard Deviation
SIA	Strip Immunoblot Assays
SQ	Starting Quantity
SVR	Sustained Virological Response
TMA	Transcription-Mediated Amplification
UTR	Un-translated region
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND AND JUSTIFICATION

Hepatitis C virus (HCV) infection is one of the major causes of chronic liver diseases. The long range effects of chronic HCV infection are greatly changeable ranging from slightvariations to extensive fibrosis and cirrhosis with or without hepatocellular carcinoma. According to the World Health Organization (WHO) the total of HCV chronically infected people worldwide is evaluatedto be more than 160 million. Meanwhile, it is evaluatedthat over 430,000 individuals are chronically infected by HCV in Malaysia alone(Zawawi, 2014; Moradpour & Blum, 2004).

HCV is a single stranded positive sense RNA virus of the Flaviviridae family. The RNA harbours one open reading frame (ORF) flanked by 5' and 3' untranslated regions (UTR). The ORF encodes structural and non-structural proteins. Unlike the other types of viral hepatitis, HCV shows high rate of genomic mutation which permits it to avoid the host's immune response. HCV is classified into 7 major genotypes, each revealing≥30% genetic variations from one another. HCV genetic sequences that differ by 20-25 % are called subtypes, and those that have around 10 % sequence variability are termed quasi-species (Smith et al., 2014).

Patients infected with HCV usually present with nonspecific clinical manifestations including nausea, vomiting and jaundice. The liver enzymes,aspartate aminotransferase (AST) and alanine aminotransferase (ALT), non-specific markers of liver cell damage, are measured to help in the diagnosis. Despite that they are usually elevated in the patients' sera, 40% of the patients may show normal range of those

enzymes (Al Olaby, Reem R & Azzazy, Hassan M E, 2011; Scott & Gretch, 2007). Alternatively, two sensitive and specific types of laboratory methods are utilized for the diagnosis of HCVinfection, which are immunoassays and molecular assays (Ghany, Strader, Thomas, & Seeff, 2009).

The immunoassays such as EIA and RIBA detect the total anti-HCV antibodies against numerous epitopes of HCV in the serum upon seroconversion, which occur2–3monthssubsequent exposure to the HCV(Alter, Kuhnert, & Finelli, 2003; Chevaliez & Pawlotsky, 2006). EIA is usually used to screen for seroconversion to HCV infection. However, high percentage of false-positive and negative results are associated with these assays. While RIBA test is quite specific but does not differentiate a resolved infection from non-resolved one, thus, molecular assays are used to confirm the results obtained by the immunoassays (Al Olaby & Azzazy, 2011).

The molecular assays identify HCV RNA in the patients' sera as early as 11 days postinfection (Gallarda & Dragon, 2000; Richter, 2002). Dissimilar HCV immunoassays, molecular assays can differentiate between the active and past infections, and exhibit a very high sensitivity and specificity, serving as an independent method for confirming active HCV infection. Thus, it is not surprising that molecular assays are widely used as a clinical and research tool (Gallarda & Dragon, 2000; Giachetti et al., 2002)

HCV infection is typically mild in early stage, and rarely clinically detected until it has caused significant damage to the liver. However, early detection of the infection may allow the patients to start antiviral intervention that may reduce adverse consequences to the liver, thus reduce mortality. Although not all infected individuals will successfully respond to the antiviral therapy and clear the virus, pegylated

interferon and ribavirin remained as the mainstay for standard combination for HCV treatment despite the development of other specific antiviral drugs. The ultimate goal of the treatment is to achieve sustained virological response (SVR), which is defined as permanent eradication of the virus. This is the stage where the patients are considered cured from the infection. One of the prerequisites for SVR is undetectable RNA after completion of therapy or defined as end-of-treatment response (ETR)(McHutchison et al., 1998). However, not all ETR-patients will achieve SVR status because recurrence of infection may occur. Thus, the viral RNA level in the patients should be monitored periodically. Furthermore, at acertain stage of the hepatitis c, the level of viremia maysuddenly decrease under the detection limit of the utilized assays.Consequently,one negative result from HCVmolecular assays does not eliminate the probability of active HCV infection (Richter, 2002). Therefore, a highly sensitive molecular method, such as nested or semi-nested PCR, is required to diagnose the very low levels of HCV RNA in the blood.

Real-time PCR is a quantitative technique that has the ability to quantify accumulated PCR products in real-time by monitoring the detection of the fluorescence signals at each cycle of PCR reaction. Current advances in real-time PCR technology for the quantification of HCV RNA has allowed linear amplification over a broad dynamic range with low detection limits. Current commercially available real-time quantitative PCR are COBAS Ampliprep/CobasTM TaqMan HCV assay (CAP/CTM; Roche Diagnostics, Pleasanton, CA) and Real-time HCV (Abbott Molecular Inc., Abbott Park, IL). However, several considerations must be taken by low-resources laboratories for utilizing these commercial assays, which includes expensive cost per run, dependent close data, and delay in specimen and result delivery as specimens may have to be exported to another country for performing the

test. Furthermore, the assays are calibrated based on HCV genotype 1 as proposed by the WHO for standardization that would under or over quantify other HCV genotypes (Vermehren et al., 2008). The ideal HCV RNA assay should have a lowerdetection limit ofabout 5 to 50 IU/ml (Caliendo et al., 2006; Le Guillou-Guillemette & Lunel-Fabiani, 2009). This is allowingdiagnostic laboratories to use a single method for therapeutic management instead of using two assays, qualitative and quantitative.

Current widely used techniques in the real-time PCR technology are using fluorescence-labelled probes or fluorescent intercalating dyes (e.g. SYBR green 1) (Table 1.1). The probe relies on hybridization to the correct amplicon sequence, while fluorescent dye binds non-specifically to double-stranded DNA. Both techniques have contrasting advantages and disadvantages. Intercalating dyes are cheap and easily used on large region of DNA template. The only disadvantage of this chemistry is that it is of less specificity than the probe. The solution to this disadvantage includes good primers design and good methodology that would allow reproducible and melting curve analysis at the end of the amplification. Other problem with SYBR Green dye is the difficulty to guarantee saturation of the PCR product as only limited concentrations can be used before it inhibits PCR. Recently, other dyes, so called third generation dyes (e.g. EvaGreen, SYTO9 and LCGreen) have become available (Reed, Kent, & Wittwer, 2007). These dyes do not cause PCR inhibition, thus are better for DNA melting curve analysis to discriminate actual target from contamination. The dyes are so specific that allow application of high resolution melting (HRM) analysis to discriminate single-base variants and small deletions(Tajiri-Utagawa, Hara, Takahashi, Watanabe, & Wakita, 2009).

In a previous study involving two tertiary hospitals and haemodialysis patients, reported that HCV genotype 3 is the most prevalent followed by genotype 1 (Hadzri

MH., Hairul AH., Mustafa MIA., & Azril HY., 2009; Hairul Aini, Mustafa, M I A, Seman, & Nasuruddin, 2012). The primers used targetedthe highly conserved region of all HCV strains found in the studies (genotypes 1, 3, 4 and 6) using conventional RT-PCR. The primers could also be applied for a new technique for HCV quantification using real-time quantitative PCR (RT-qPCR) which aimed for sensitive, cost effective and reliable quantification of HCV.

Table 1.1 Available HCV real-time PCR assay for commercial and in-house application

Assay	Technique	Availability	Manufacturer/Author
Cobas TaqMan assay	TaqMan probes	Commercial	Roche Molecular system, CA, USA
Abott RealTime HCV	Hybridization probes	Commercial	Abott Molecular Inc., Abott, IL, USA
HCV quantification and genotyping	Hybridization probes	In-house	(Casanova et al., 2014)
HCV quantification and genotyping	Hybridization probes	In-house	(Davalieva, Kiprijanovska, & Plaseska-Karanfilska, 2014)
HCV detection	SYBR Green I	In-house	(Shahzamani et al., 2011)
HCV genotyping	SYTO9	In-house	(Nazemi A., Tazehabadi ES., Jafarpoor M., & Sharifi S., 2011)
HCV quantification	Hybridization probes	In-house	(Shahzamani et al., 2010)
HCV quantification	Duplex scorpion primer	In-house	(Xu et al., 2007)
HCV quantification	Molecular beacon	In-house	(Yang et al., 2002)
HCV quantification	SYBR Green I	In-house	(Nozaki & Kato, 2002)
HCV quantification	SYBR Green I	In-house	(Schroter, Zollner, Schafer, Laufs, & Feucht, 2001)

Any laboratory equipped with real-time PCR machine can perform quantitative PCR. However, to use third generation dyes, the system has to be equipped with the HRM system. In this study, a new in-house protocol for HCV

quantification based on semi-nested real-time PCR with the third generation EvaGreen detection dye was developedto detect and quantify HCV-RNA from serum specimens.

In this study, semi-nested PCR was devised to improve sensitivity and specificity the PCR assay, (Zhang & Ehrlich, 1994). EvaGreen is a newly developed dye which can be used for both real-time PCR and melting curve analysis.

The assessments of newly developed assay must be accompanied by analytical and diagnostic performancedata. Both performances are required to determine the fitness of an assay, which is accomplished by statistical analysis and interpretation. Determination of the analytical performance includes limit of detection, analytical specificity, and accuracy, which areimportant to ensure quality operational procedures and to identify inaccuracy of the test. Estimated diagnostic performance characteristics such as diagnostic sensitivity and specificity are required to discriminate between infected and uninfected individuals (Flatland, Friedrichs, & Klenner, 2014; OIE, 2013). Additional validation assessments of a new developed assay areavailability of reproducibility (inter-assay variability) and repeatability (intra-assay variability) data. Reproducibility is the ability of the assay to illustrate consistent results, which is estimated by testing a panel of samples in different days by different operators in multiple laboratories. While, repeatability is the variation in measurements taken by a single operator using the same method, equipment and laboratory (OIE, 2013).

1.2 RESEARCH QUESTION

What is the analytical and diagnostic performance of the developed semi-nested real time PCR using EvaGreen dye? And can it become a useful method for HCV RNA detection and quantification?

1.3 RESEARCH OBJECTIVES

- To establish a semi-nested real-time PCR using EvaGreen dye for detection and quantification of HCV RNA.
- To assess the analytical specificity, sensitivity, and accuracy ofthe established method.
- To assess the intra-assay variability ofthe established method.
- To assess the diagnostic specificity, sensitivity, and accuracy ofthe established method.

CHAPTER TWO

LITERATURE REVIEW

2.1 HISTORICAL BACKGROUND OF HCV

Blood screening tests were first introduced in 1963 to detect hepatitis B and for hepatitis A, it was started in 1973. However, many cases of post-transfusion associated hepatitis were negative for both hepatitis A and B, leading to classification of unidentified cases as non-A, non-B hepatitis (NANBH) (Mosley, Redeker, Feinstone, & Purcell, 1977; Stoliarova & Burlev, 1973). The aetiological agent causing NANBH was identified as virus by filtration studies with approximately 30-60 nm diameter (He et al., 1987). In 1983, it was shown to be inactivated by chloroform, indicating that it was an enveloped virus (Feinstone et al., 1983).

In 1989, researchers used molecular tools to recognize the NANBH agent in detail. They isolated total nucleic acid from the plasma of an infected chimpanzee. Then, complementary DNA (cDNA) was generated using reverse transcriptase with random primers and then screened with patients' serum as a source of NANBH antibodies. Isolation of the cloned DNA which encoded an antigen specifically associated with NANBH and was the basis for the first serologic assay for NANBH antibodies and the virus was consequently named hepatitis C virus (HCV) (Choo et al., 1989; Kuo et al., 1989).

Investigation of the particles recovered from infectious sera by using immunoelectron microscopy revealed the present of circular particles of 33- 70 nm in diameter (Kaito et al., 1994; Li et al., 1995). However, 30-40 nm icosahedron-shaped particles generated in infectious specimen treated by detergent consisted of the HCV core protein and HCV RNA (Takahashi et al., 1992). Based on these finding, HCV

has been classified as a member of the Flaviviridae family. Eventually, it has been classified into a new genus hepacivirus under that family, as a result of the low sequence homology compared to other flaviviruses (Robertson et al., 1998).

2.2 HCV PROPERTIES AND VARIABILITY

HCV is a positive-sense, single-stranded enveloped RNA virus approximately 9600 nucleotides in length. The HCV particle encapsulates viral RNA genome by an icosahedral protective shell of protein in which 2 viral glycoproteins (E1 and E2) are attached to the cell-derived double-layer lipid envelope.HCV belongs to the Flaviviridae family and classified as the first member of the genus Hepacivirus. (Choo et al., 1991; Seng-Lai, 2006). Recently developed cell culture systemhas been able to generategreatamounts of infectious HCV particles. Two types of viral particles have been pictured by immune-electron microscopy;particles of 50-60 nm width are likely to be the infectious virions , and particles of 30-35 nm width are likely to correspond to the viral nucleocapsids (Lindenbach et al., 2005; Wakita et al., 2005).

HCV appears in several forms in the serum of patients, including: viruses bound to lipoproteins, which represent the infectious fraction, bound to immunoglobulin, or circulating free in the serum. Furthermore HCV exhibits a wide range of densities, while the most infectious element has a density of 1.15-1.17 g/mL(Penin, Dubuisson, Rey, Moradpour, & Pawlotsky, 2004; Thomssen, Bonk, & Thiele, 1993; Wakita et al., 2005).

Current classification of HCV is based on recognizedvariations of HCV nucleotide databases collected from around the world. Diverse regions of the viral genome show different levels of genetic heterogeneity. This is due to the HCV RNA-dependent RNA polymerase, which lacksproofreading capability. Due to a high rate of

error-prone replication, HCV reveals significant genetic diversity,thus allowing it to avoid host's immune response (Kim & Chang, 2013).

The terminal regions of the viral genome, the 5UTR and theinitial coding region (the core gene) that are involved in virus replication and proteins translation are highly conserved among genotypes. While, other regions, such as coding region for the envelope glycoproteins (E1 and E2) are highly mutable between genotypes (Kolykhalov, Feinstone, & Rice, 1996; Simmonds, 2001). For nomenclature, HCV has been classified into 7 confirmed genotypes exhibiting 30% sequence variation from one another. The genotypes corresponding to the main branches in the phylogenetic tree, which are further divided into 67 subtypes. HCV subtypes differ among themselves by about 20%identical to the more closely related sequences within a main group. Those that have around 10% genetic variability are termed quasi-species. So far, there are around 21 additional coding regions of unassigned subtypes. The types are numbered from 1 to 7 while the subtypes designated by alphabetic letters such as genotype 1a, 1b (Simmonds et al., 2005; Smith et al., 2014).

The HCV genotype distribution was available from different parts of the world. Globally, genotype 1 was the most common worldwide approximately 46%, around one-third of which are in East Asia, followed by genotype 3 (30%) (Messina et al., 2015). However, a previous study that involved two tertiary hospitals and haemodialysis patients in Pahang, Malaysia has reported that HCV genotypes 3 is the most prevalent followed by genotype 1 (Hadzri MH. et al., 2009; Hairul Aini et al., 2012). Genotypes 2, 4, and 6 are responsible for around 22.8% of all patients, and genotypes 5 and 7 comprises the remaining <2% (Messina et al., 2015).

2.2.1 HCV genome

The genome of the HCV is a positive-sense, single-stranded RNA, consisting of one open reading frame (ORF) that is approximately 9600 nucleotides in length. The ORF encodes a large single polyprotein of about 3000 amino acids(Choo et al., 1991; Kato et al., 1990). This polyprotein is the primary product of HCV translation which contains all ten HCV structural and non-structural proteins required for HCV replication. During HCVmultiplication, the viral polyprotein is cleaved by cellular enzymes and viral proteases at the level of the endoplasmic reticulum (ER) membrane to createnon-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B), and structural proteins (core protein, E1 and E2). The structural organization of HCV genome is shown schematically in Figure 2.1 (Penin et al., 2004).

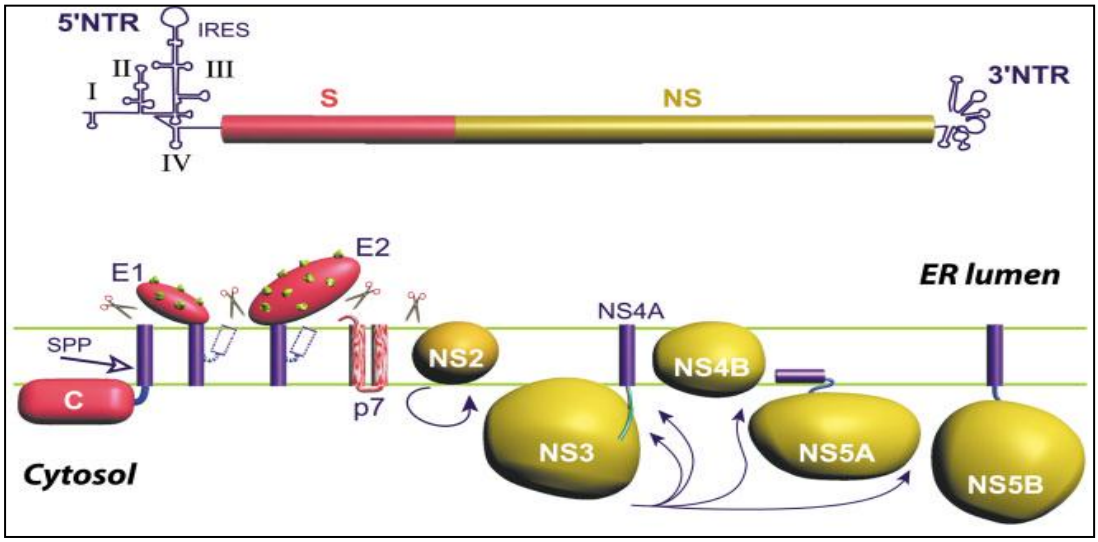

Figure 2.1HCV genome organization and polyprotein processing(Penin et al., 2004).The 5'UTR consists of 4 domains and Internal Ribosome Entry Site (IRES). The 3'UTR consists of stem-loop structures. The central 9.6-kb ORF codes for a polyprotein. S and NS correspond to regions coding for structural and nonstructural proteins. Intramembranous arrow (SPP) signal peptide peptidase; Scissors indicate peptidase cleavage sites; arrow indicate autocatalytic and protease cleavage sites.

The structural proteins are necessaryingredients to compose the HCV particle, while the non-structural proteins are involved in virion morphogenesis, RNA replication and polyprotein processing. Additionally, alternate reading frame (ARF)or protein F is expected as a product of ribosomal frame shifting during viral replication and proteins translation within the core region of the genomic RNA. Antibodies to this proteindetected in the patients' sera indicates that the protein is expressed during infection in vivo (Branch, Stump, Gutierrez, Eng, & Walewski, 2005; Walewski, Keller, Stump, & Branch, 2001).

The 5' and 3' ends of the RNA are the highly conserved, untranslated regions (5' and 3'UTRs; also called non-translated regions, NTR or noncoding regions, NCR), that are not translated into proteins but involved in multiplication and translation of the HCV RNA. Additionally, the highly structured and conserved properties of these regions makethem candidates for improved molecular diagnostics, targets for antiviral therapy, and targets for an anti-HCV vaccine.

2.3 EPIDEMIOLOGY AND NATURAL HISTORY OF HEPATITIS C VIRUS

HCV is one of the most important causes of chronic liver diseases, cirrhosis, and hepatocellular carcinoma (Al Olaby, Reem R & Azzazy, Hassan M E, 2011; Moradpour & Blum, 2004). Most patients acquire HCV infection by intravenous drug use or blood transfusion. Therefore, the most efficient means of HCV transmission is by parenteral exposure. According to the World Health Organization (WHO), 3–4 million people are newly infected with HCV every year and, currently 130–170 million people are chronically infected. It is estimated that over 350,000 people die each year from hepatitis C-related liver diseases (World Health Assembly, 63, 2010). HCV has different prevalence in different countries of the world, ranging from a low