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Review Article

Molecular Diagnosis of Hepatitis C Viruses; Technologies and Their Clinical Applications

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Abstract

Hepatitis C is one of the most common viral diseases caused by the hepatitis C virus (HCV). It is responsible for millions of deaths each year in the developing world. The common dissemination paths of HCV include using contaminated water and transfusion of infected blood. Control of this virus has become a challenge for scientists and health professionals due to its versatility and adaptability in different host environments. Along with other problems, the lack of efficient diagnosis, quantification, and genotyping of viral strains are the major hindrances in managing this notorious epidemic. The knowledge of HCV genotype and the amount of virus in the patient's blood are prerequisites to determine the duration and method of treatment. This review discusses HCV molecular diagnostic methods and their clinical applications. We conclude that while several commercial and home-brewed methods are available for this purpose, there is a visible vacuum for cost-effective, robust, sensitive assays that can detect multiple viral genotypes in a single reaction. We believe that the level of sensitivity offered by the Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) technique is unequivocally superior compared to other techniques. Therefore, researchers may explore further possibilities using this technique to manage HCV.

Keywords: Hepatitis C virus, genotyping, mixed infection, fluorescence melting curve analysis, viral load, quantification.

1. Introduction

Viral hepatitis became a matter of attention in the decades 1950-60 and was distinguished into so-called infectious and serum hepatitis (Krugman, Ward, and Giles 1962). Later evidences from two independent research groups of Dr. Stephen Feinstone et al. and Dr. Baruch Blumberg et al. proved that hepatitis A and B viruses (HAV & HBV) were the etiological factors behind hepatitis (Bayer, Blumberg, and Werner 1968, Castaneda et al. 2021). In the 1970s, detection assays based on serology for HAV and HBV were introduced, and surprisingly it was found that parentally transmitted hepatitis was not caused by either type of virus (Feinstone et al. 1975). Hence, the term Non-A Non-B hepatitis (NANBH) was coined to describe the causative agent in these cases. Many groups, notably Dr. Harvey Alter and his colleagues, initiated the work on NANBH and suggested using chimpanzees as

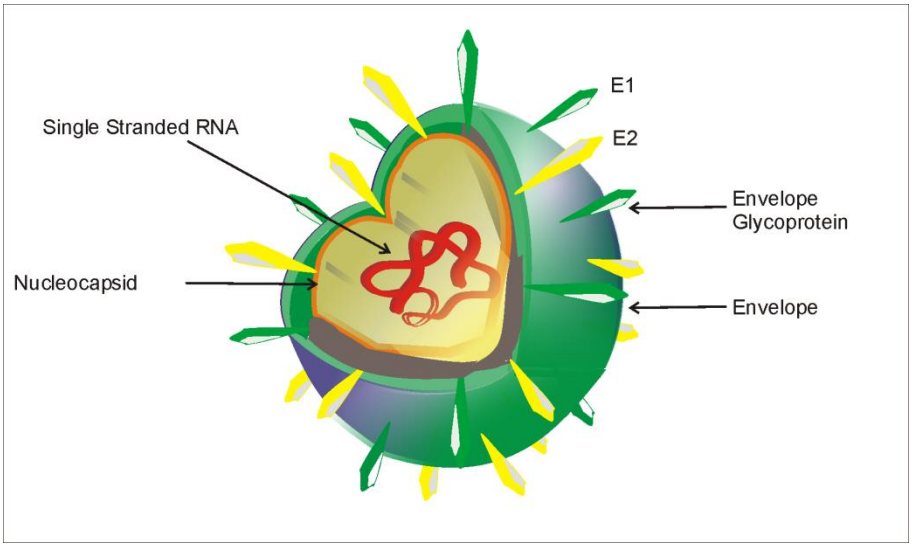

Figure 1. HCV virion structure, E1, E2, envelope glycoproteins, Nucleocapsid

a reliable model (Alter et al. 1978). The experiments on chimpanzees involved the serial passage of infectious material from human sources and provided important data indicating the presence of multiple NANBH agents. One such agent demonstrated the formation of characteristic membranous tubules within the chimpanzee liver cells and was known as a tubule-forming agent (TFA) (Alter et al. 1978). Dr. Bradley's and Dr. Purcell's groups investigated the biochemical nature of this so-called TFA as lipid enveloped agent that could be inactivated by organic solvents and filtered through an 80 nM pore-sized filter (Bradley et al. 1983, Feinstone et al. 1983). Eventually, in 1989, researchers from CDC and Chiron laboratories identified the virus as HCV (Houghton et al. 1981b). In 1990, blood banks started to screen blood for HCV, and in 1992 a blood test was introduced to effectively detect and eradicate HCV from blood transfusion products (Houghton et al. 1981a). HCV remains a major public health threat, causing acute and chronic hepatitis infection. It currently affects 130-150 million people (approximately 2% to 3% of the world population) who are living with chronic infection (WHO July 2011). Many chronically infected individuals develop liver cirrhosis or cancer (Cortez and Kottilil 2015). HCV infection annually kills more than 350,000 people worldwide because of liver-related diseases (Perz et al. 2006). The true burden of HCV infection is unknown due to data collection limitations (Averhoff, Glass, and Holtzman 2012), and the situation could be even worse in developing countries (Sievert et al. 2011)

Overall, HCV causes significant human and economic loss, which makes the basis to value HCV screening, diagnosis, and treatment (El Khoury et al. 2012). Antiviral therapy has significantly improved over time, and research in the related field has made great promises, but access to early and accurate diagnosis is very low (Liang and Ghany 2013). The prevalence of HCV infection is variable throughout the world and ranges from 1% to 10% in different countries. In developed countries like Australia and countries of Western Europe, the HCV disease burden is less than 2% (Alter and Liang 2012b, Sievert et al. 2011). In comparison, the infection rate is more than 3% in many countries in the Eastern

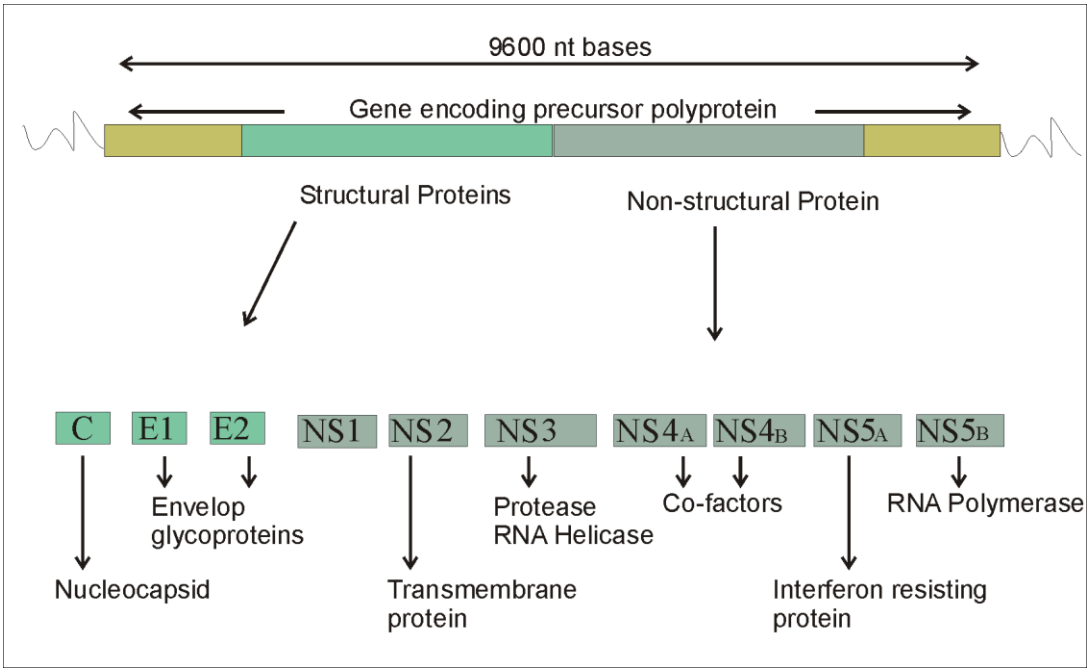

Figure 2. Genomic organization of HCV: HCV viral genome encodes one long polyprotein that is co- and post-translationally processed into mature structural and non-structural proteins. The viral structural proteins include the core protein and envelope glycoproteins E1 and E2; the viral non-structural proteins consist of NS1, NS2, NS3, NS4A, NS4B, NS5A, and NS5B proteins. (Pascut et al., 2021).

Europe, Latin America, the former USSR, including the Central Asian States, and certain countries in Africa, the Middle East, and South Asia (Qureshi et al. 2010, Sievert et al. 2011). For example, Egypt has more than 10% of the population at risk (Sievert et al. 2011). Other African countries have prevalence rates ranging from 2% to >3% (Gower et al. 2014).

The high disease burden in developing countries is mainly caused by poor healthcare settings. China has the highest number of HCV-infected injection drug users, and more than 80% of the population in countries including Pakistan, Thailand, and Mexico is HCV-infected (Nelson et al. 2011). In Pakistan, HCV infection is highly endemic and is attributed to the highest rate of unsafe injections (13 injections/person/year)(Qureshi et al. 2010). A recent report confirmed the association between high chances of transmission and unsafe drug use. They reported that the individuals received injections within six months preceding the diagnosis of viral hepatitis (Ghany et al. 2011). Other practices and events leading to HCV infection are related to traditional healers, unqualified medical professionals, tattooing, and commercial barbershops in Pakistan (Qureshi et al. 2010).

2. Hepatitis C Viral Genome Structure

HCV belongs to the genus *Hepacivirus* of the family *Flaviviridae.* It is a spherical virus of about 55-65 nm diameter (Figure 1). The outer envelope surrounds an inner core encapsulating a positive sense, single-stranded RNA ((+)ssRNA) genome of approximately 9.6 kb. The HCV envelope comprises two envelope proteins, E1 and E2, that are highly glycosylated. E1 and E2 are important during virus-cell fusion and virus-binding to host cell receptors during the viral

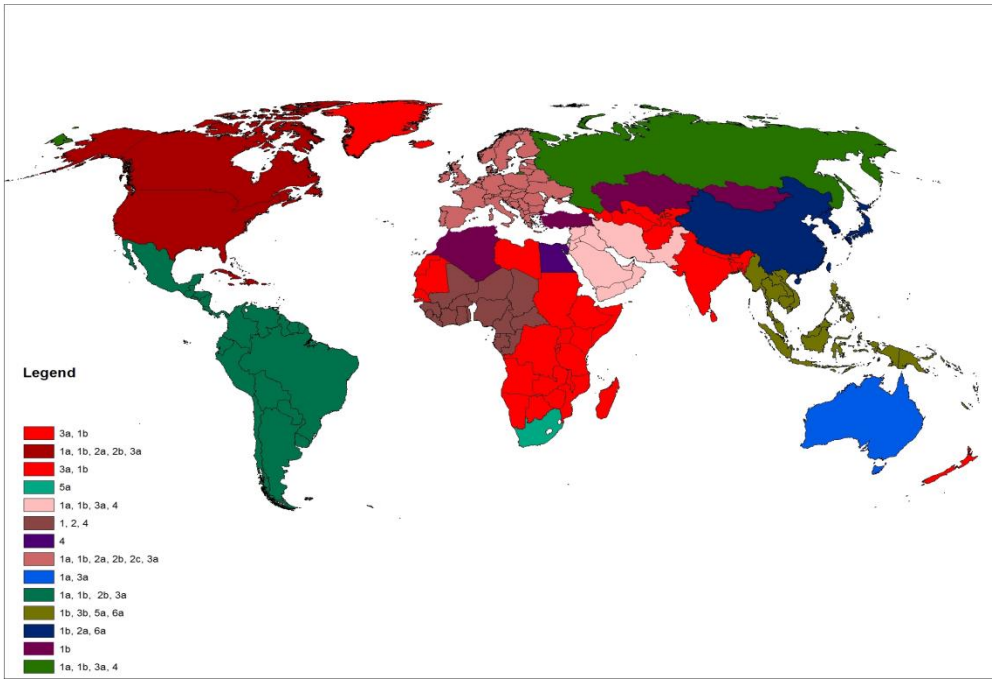

Figure 3. Worldwide geographic distribution of HCV genotypes and subtypes.

entry phase, respectively (Drummer, Maerz, and Poumbourios 2003).

The first protein synthesized inside the host cell is the 191 amino acids long core protein. The core is an RNA-binding protein (Bukh, Purcell, and Miller 1992) and plays a critical role in encapsulating and packaging RNA into a newly emerged virion. Core exhibits its crucial role in viral replication and immune pathogenesis of HCV infection (Irshad and Dhar 2006). Other potential functions of core protein include oncogenesis, regulation of cellular signaling, and apoptosis (Tellinghuisen and Rice 2002). Recent studies have shown the importance of core protein in the diagnosis and genotype determination of HCV (Kanto et al. 1994).

The HCV genome comprises a single open reading frame (ORF), which is flanked by 5' and 3' untranslated regions designated as 5'UTR and 3'UTR. The ORF encodes a single polyprotein of around 3000 amino acids. The polyprotein is further processed into structural and non-structural (N.S.) proteins with the help of host cell-derived and virally encoded proteases. Structural proteins include Core (C), Enveloped (E1 and E2) and p7 while NS2, NS3, NS4A, NS4B, NS5A and NS5B are non-structural proteins (Figure 2).

3. Evolution of HCV and its Genotypes

There is little known about the evolutionary history of most human viruses. Although several RNA viruses, including HCV, have been divided into genotypes and subtypes, there is uncertainty regarding the origin of these variants. The NS5B encoded RNA-dependent RNA polymerase (RdRp) lacks proofreading ability, which is essential for the replication of the HCV genome (Moradpour et al. 2004). Therefore, depending on the site, HCV is a highly mutating virus that evolves at the rate of $1\text{-}3\times10^{3}$ bps mutation/site/year (Kato et al. 2005). Thus, a closely related but diverse population of viral variants known as quasi-species is produced (Khaliq, Jahan, and Pervaiz 2011). In addition, certain coding regions in the HCV genome impart a high

Table 1: Hepatitis C Virus (HCV) Treatment.

HCV Genotype	Treatment Protocol	Treatment Duration	Cure Ratio (sustainable
Genotype-1	Telaprevor Plus Interferon and ribavirin or	24 to 48 weeks	70-75%
	Boceprevir plus Interferon and Ribavirin	28 to 48 weeks	
Genotypes-2 and 3	Interferon plus Ribavirin	24 weeks	80%
Other Genotypes of HCV	Interferon plus Ribavirin	48 weeks	40-70%

mutation rate, such as the hypervariable regions in envelope protein E2, which is responsible for the high mutation rate and enables the virus to escape the host immune system (Khaliq, Jahan, and Pervaiz 2011). Mutations in the envelope protein can modify the antigenicity of the virus surface and hence are the most popular immune escape route in quasi-species.

HCV isolates exhibit a notable variation throughout their genome sequence. These variants can be categorized into several types based on sequence comparisons of sub-genomic regions (Mori et al. 1992). These sub-genomic regions or variable sequences include encoding NS5 protein (Simmonds et al. 1993), the core protein (Chan et al. 1992b), the envelope protein E1 (Stuyver et al. 1993), NS3 (TSUKIYAMA KOHARA 1991) and 5'UTR (Zein 2000). The isolates with >85% sequence similarities are classified into the same subtype, while these subtypes are grouped into types (77-80% sequence similarity). It must be noted that there exists a 65% similarity between HCV types (Simmonds et al. 2005). There are different levels at which the variation in HCV sequences can be described: Almost all HCV isolates have 31-34% variability in their genome sequences, and these sequences can be classified into six distinct groups known as types/genotypes. These genotypes are termed 1, 2, 3, 4, 5, and 6. These genotypes have different serological and biological characteristics (Tokita et al. 1994). Each type is further sub-classified into subtypes, which differ in around 20% of nucleotide positions. There are more than 70 subtypes that are assigned lowercase letters, such as a, b, c, etc. (Murphy et al. 2007). A third level of variability corresponds to the viruses co-circulating in an infected patient's blood. These virus types belong to a population of diverse but closely related variants that differ up to 1.5% and are termed quasi-species (Martell et al. 1992). The presence of quasi-species imparts a distinct survival advantage, as the co-presence of multiple variants of viral genomes and a high generation rate of new variants may allow rapid selection of those variants with high survival rates in changing environmental conditions (Pawlotsky 2006).

4. Geographical Distribution of HCV Genotypes/Subtypes

HCV genotype distribution throughout the world depends on the geographical territory and transmission mode (Zein 2000). Some

Table 2: Commercially available real-time PCR assays:

Assays	Vendor	[1]US-FDA Approval for Clinical Applications	Amplification type	Low limit of detection (IU/ml)	Dynamic Range of quantification
COBAS AmpliScreen 2.0HCV Systems	Roche molecular system	Approved for Blood Screening	Target	15 (EDTA plasma, serum	15-1x10^8
COBAS AmpliScreen 2.0	Roche molecular system	Approved for Blood Screening	Target	<50	
AMPLICOR HCV 2.0	Roche molecular system	For confirmation of active infection	Target	50(Serum)	
COBAS AMPLICOR HCV 2.0	Roche molecular system	Approved for confirmation of active infection	Target	50(Serum)	
Versant HCV RNA	Bayer healthcare	Approved for confirmation of active infection	Target	7.5	
Procleix HIV-1/HCV assay	GeneProbe	Approved for Blood Screening	Target	<50	
Procleix Ultrio assay	GeneProbe	Approved for blood donor, organ donors,	Target	<50	
BAYER VERSANT HCV RNA 3.0	Bayer Healthcare	Approved for viral load determination [4]HCV and therapy management	Signal	520	615 - 6.69x10^6
AMPLICOR HCV MONITOR 2.0	Roche Molecular System	Approved for viral load determination HCV and therapy management	Target	600	600-5 x10^5
COBAS AMPLICOR HCV MONITOR 2.0	Roche Molecular System	Approved for viral load determination HCVand therapy management	Target	600	600-5 x10^5
COBAS AmpliPrep/COBAS TaqMan HCV	Roche Molecular System	Monitoring of the response to antiviral therapy	Target	15	4.3 x 10-6.9 x 10^7

[1]United state of America-Food and Drug authority, [2]Reverse Transcription Polymerase Chain Reaction, [3]Transcription Mediated Amplification, [4]Hepatitis C Virus,

HCV subtypes are spread globally through needle sharing (1a and 3a) by drug users or infected blood products (1a and 2b). For example, during the last 5-7 decades, genotypes 1 and 2 were widely distributed in Western countries due to blood transfusion, medical procedures, and contaminated syringe users among drug users. Genotypes 1a and 1b are most commonly associated with drug users, and blood transfusions, respectively, and have a high prevalence in the United States, Europe, and Japan. Genotypes 4-6 are less common; however, they are becoming more frequent because of the cultural diversity within the United States (Nainan et al. 2006). Notably, more than 70% of cases of HCV infection are caused by 1b alone (Zein 2000).

Genotypes 2a and 2b represent 10-30% of globally distributed HCV infections and are the most frequently detected types in North America, Europe, and Japan, while 2c is common in Northern Italy. People from the Indian subcontinent and Southeast Asian populations are commonly infected with genotype 3. 3a is specifically prevalent among drug users in Western Europe and the United States (Zein 2000). Genotype 4 is prevalent in North Africa and the Middle East, while 5 and 6 are most commonly reported in South Africa and Hong Kong, respectively (Tanaka et al. 2004). Only in Vietnam, genotypes 7, 8, and 9 have been identified, and genotypes 10 and 11 are identified in Indonesian patients (Tokita et al. 1994). Since there remains controversy over the number of genotypes to be classified, people have proposed that genotypes 7-11 may be considered as variants of the same group and classified as single genotype 6 (Simmonds et al. 2005). In Pakistan, the most common genotype is 3a, which is followed by 3b and 1a (Khaliq, Jahan, and Pervaiz 2011). Genotype 1b, followed by 2a, is predominant in most regions of China (Lu et al. 2005) (Figure 3).

5. Clinical Relevance of HCV Genotypes and Their Response to Treatment

HCV genotype determination is an extremely important step in response to any available treatment or therapy. If a population has predominant HCV genotypes that do not respond efficiently to a particular treatment, it may pose problems to long-term disease management, and there will be a burden on healthcare resources. These issues may be resolved when HCV genotypes are responsive to antiviral treatment. The standard treatment for chronic HCV infection comprises standard or pegylated interferon-α (IFN-α) administered as a monotherapy or in combination with ribavirin (McHutchison and Fried 2003). Genotype 1 does not respond to IFN-α treatment, while genotypes 2 and 3 respond efficiently (Tsubota et al. 2003). Also, higher rates of hepatitis reactivation and steatosis have been reported for genotypes 2 and 3 using a combined dose of ribavirin and pegylated IFN-α as compared to patients infected with genotype 1. Recently, US Food and Drug Administration (FDA) has approved two direct-acting antivirals (DAAs) and recommended in combination with pegylated-α plus ribavirin, which holds great potential in the management of HCV-infected patients (Alter and Liang 2012a). As compared to the dual therapy involving peg interferon-α and ribavirin, triple combination therapy, including either the first of the two direct-acting antivirals, telaprevir and boceprevir, is currently recommended for HCV genotype 1. Besides a success rate of cure of more than 70%, the duration of therapy has also reduced from twelve to six months (Table 1)(Ghany et al. 2011). However, these drugs have side effects and require the expertise of healthcare

professionals to deal with them. Unfortunately, these drugs are costly and are not equally available worldwide. Therefore, peg-interferon and ribavirin remain the therapy of choice in most parts of the world, particularly in developing countries (Asselah and Marcellin 2014).

6. Molecular Diagnosis of HCV

Since the discovery of HCV in 1989, the advances in developing assays to detect HCV RNA have progressed tremendously. It has become routine to conduct virological and serological assays to diagnose the infection accurately. Furthermore, these assays are essential in managing the infection through antiviral therapy. Molecular assays are direct tests that quantify or characterize the HCV RNA virus.

Molecular detection assays are based on amplifying a signal or target genome sequence using either classic polymerase chain reaction (PCR), real-time PCR, or transcription-mediated amplification (TMA). Molecular assays offer detection, quantification, and genotyping of HCV RNA in a patient's blood serum, with a true reflection of active infection. These assays can detect the virus at very low concentrations, usually within 1-3 weeks following exposure (Kamili and Qadri 2020). These assays are either qualitative, to discriminate acute infection from chronic; quantitative, to determine the baseline viral load; or genotype assays to determine the genetic nature/type of HCV. For the detection of HCV RNA, diagnostic laboratories often use commercially available kits (Table 2) as well as methods developed and optimized in local settings.

Since the amount of HCV RNA in the infected patient's serum is limited, amplification of RNA is required. Therefore, molecular assays for the detection and quantification of HCV RNA may belong to two general methods; these are signal amplification, e.g., branched DNA (bDNA), and amplification of the target genome transcription-mediated amplification (TMA), reverse transcription PCR while real-time PCR is used for RNA detection and quantification.

6.1. RT-PCR Assays

Reverse transcription converts RNA into cDNA by an RNA-dependent DNA polymerase enzyme. The resulting cDNA then serves as a template for PCR. RT-PCR makes use of sequence-specific or random primers or oligo-dTs. The oligo-dTs attach to the poly-A tail of mRNA and cannot attach to rRNA or tRNAs as they lack a poly-A tail. Random primers start cDNA synthesis from all RNA species of the cell. In contrast, specific primers initiate cDNA synthesis from a particular mRNA sequence, allowing more sensitive and accurate detection and quantification (Deprez et al. 2002). When RT-PCR is used to determine relative gene expression or quantification of RNA, it is known as quantitative RT-PCR (qRT-PCR). RT-PCR can be accomplished either in one or two steps. One-step RT-PCR combines the cDNA synthesis and quantification in a single step, and thus, reduces the chances of cross-contamination and is less laborious and less time-consuming (Aatsinki et al. 1994). Two steps RT-PCR, however, offers higher sensitivity and specificity; In this method, cDNA synthesis and amplification steps are performed separately with specific optimized conditions.

Multiplex PCR is a method of amplifying more than one gene fragment simultaneously, using more than one pair of primers. It reduces the time and effort, needs less amount of sample, and provides broader sensitivity. It uses fluorescent dyes with different emission

spectra such as FAM, ROX, and Cy5, etc., combined with probes.

The most conserved sequence of HCV RNA Viruses, such as the 5'UTR and the core region, is amplified during PCR by sequence-specific primers (Bukh, Purcell, and Miller 1992). Several factors influence reverse transcription PCR, including the quality of the template, design of the primers, reaction efficiency, and post-amplification detection system (Busch et al. 1992). Many commercial kits are available for the detection of HCV RNA, quantification, and genotyping analysis (Table 2). A modified version of the Amplicore assay is more specific (97% to 99%) and can detect less than 100 copies/mL of HCV RNA in serum and has a lower limit of detection of 50 International Units Per Milliliter (IU/mL) (Richter 2002).

6.2. Transcription Mediated Amplification (TMA)

TMA is an isothermal nucleic acid process that uses two enzymes, reverse transcriptase and T7 RNA polymerase. Target RNA is converted into DNA, and a promoter sequence is added to the newly generated DNA specific for T7 polymerase by a specially designed primer. T7 polymerase then transcribes this DNA into a detectable amount of RNA (Scott and Gretch 2007).The TMA-based VERSANT® HCV RNA assay amplifies the conserved region within the 5'UTR. This is a qualitative assay that detects HCV RNA levels as low as 5-10 IU/mL, which may not be detected with the RT-PCR (Azzazy and Mansour 2009). The entire process, sample preparation, target amplification, and amplicon detection, are performed in a single tube. This assay detects all genotypes at 9.6 IU/mL, except 2b, which is detected at 14.4 IU/mL (Al Olaby and Azzazy 2011).

6.3. Branched DNA Assays

Branched DNA (bDNA) uses a series of hybridization steps to detect and amplify HCV RNA. As a result, the bDNA does not need amplification of the target genomic sequence. This reduces contamination, and the chances of false positive results are significantly reduced (Richter 2002). The VERSANT HCV RNA 3.0 assay is a bDNA assay for quantifying HCV RNA in human serum and plasma. In this assay, viral RNA is released from the virions and then captured by specific synthetic oligonucleotides (capture probes). A series of subsequent hybridizations follow in which target probes hybridize to both the viral RNA and preamplifier probes, respectively. The capture probes and the target probes bind to the 5'UTR and core regions of the HCV genome. Amplifier probes subsequently hybridize with the preamplifier probe, forming a bDNA complex. Alkaline phosphatase-labeled probes then hybridize to these bDNA complexes. A chemiluminescent substrate is then added, and the intensity of the emitted light corresponds to the amount of RNA in the sample (Al Olaby and Azzazy 2011).

6.4. Real-Time PCR Assays

Compared to conventional PCR, which relies on end-point analysis, real-time PCR uses the conventional PCR process and measures/monitors the amplification progress through fluorescent dyes/probes that can be measured in real-time. This is achieved through special chemistry and instrumentation. Fluorescent dyes become intercalated with dsDNA, whereas probes are modified oligonucleotides that produce fluorescence upon hybridization with complementary DNA. The fluorescence is a direct indication of the amplicon produced at each PCR cycle.

In principle, a probe-based real-time PCR system relies on energy transfer from a high-energy reporter dye to a neighboring low-energy quencher dye through a mechanism

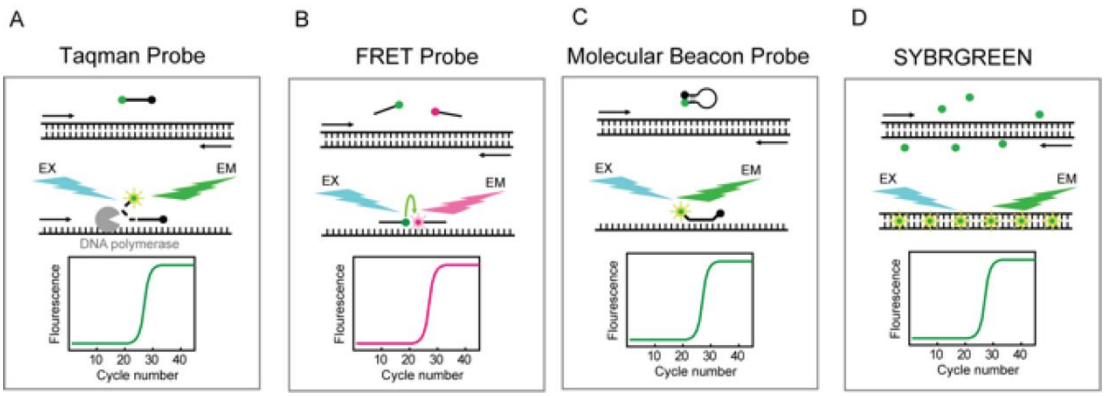

Figure 4. A representation of different molecular probes for the detection of PCR products.

that is known as Forster Resonance Energy Transfer (FRET). There are three general methods in which different probes or dyes are used: 1) hydrolysis probes, 2) hybridization probes, and 3) DNA binding agents. For example, TaqMan probe is a hydrolysis probe that employs the 5'-3'exonuclease activity of the *Taq* polymerase to measure the amount of product during PCR amplification. The probes are single-stranded DNA, dually labeled at the 5′ and 3′ ends with reporter and quencher dyes/fluorophores, respectively (Figure 4). When the probe is hybridized with its complementary target, the *Taq* polymerase cleaves the reporter from the quencher dye, and there is no more FRET. As a result, the fluorescence of the reporter dye increases in each PCR cycle, which is directly proportional to the amount of probe cleaved and displaced (Heid et al. 1996).

In figure 4A, the TaqMan probe format uses an oligonucleotide with a fluorescent label (reporter dye) at its 3′ end and a different fluorescent label (quencher dye) at its 5′ end. The TaqMan probe anneals to the target DNA. During elongation, the 5′ exonuclease activity of the polymerase excises the reporter dye. When the reporter dye is separated from the quencher dye, the reporter dye emits fluorescent light at a certain wavelength. In contrast to all other detection formats, complete hydrolysis of the probes by the DNA polymerase is essential to yielding precise results. The FRET probe (4B) and Molecular beacon probe (4C) remain intact throughout the PCR reaction and rebind to the target at every cycle. As the PCR products and the molecular beacon are denatured at high temperatures, the hairpin structure is disrupted, and FRET no longer occurs. As the temperature cools for the next round of primer annealing, the molecular beacon anneals with the appropriate strand of the PCR products. (4D) The SYBRGreen dye intercalates into double-stranded DNA. During the PCR, DNA polymerase amplifies the target sequence, which creates the PCR products, or "amplicons." The SYBRGreen dye then binds to each new copy of double-stranded DNA. As the PCR progresses, more amplicons are created. Since the SYBR Green dye binds to all double-stranded DNA, the result is an increase in fluorescence intensity proportionate to the amount of PCR product produced.

Another example of a hybridization probe is a molecular beacon. These are also single-stranded DNA probes that utilize FRET and are labeled with reporter and quencher dyes at their 5′ and 3′ ends, respectively. They form a

stem-loop structure, which brings the two dyes close to each other, which results in the quenching of the fluorescence. The loop contains a probe sequence that is complementary to a target sequence and a stem that is formed by annealing complementary arm sequences on either side of the probe sequence. (Bustin and Nolan 2004). Compared to TaqMan probes, molecular beacons remain intact and only anneal to the target, as shown in figure 4. TaqMan probes and molecular beacons are examples of self-quenched probes.

Dye-based real-time PCR systems are based on non-specific dyes, such as SYBRGreen that bind to double-stranded DNA. The amount of fluorescence is increased as the amount of amplicon is accumulated and can be monitored in real-time. SYBRGreen has several merits, such as melting curve analysis can be performed after the PCR reaction. The melting curve analysis describes the target sequence changes and PCR product specificity and allows comparison of melting temperature of specific and non-specific products, if any (Ririe, Rasmussen, and (Wittwer 1997). However, a demerit is that the dye binds to all double-stranded products, including primer dimers, which may result in false positive results and overestimation of the amount of product (Deprez et al. 2002). Real-time PCR assays represent high throughput, reliable, cost-effective nucleic acid testing methods and have distinctive advantages. For example, they permit real-time kinetic detection of amplicons as reflected by the change in fluorescence intensity in a closed tube system and require no post-amplification processing (Saunders 2004). In addition, they provide the least variation between assays and thus generate reliable and reproducible results. When combined with qRT-PCR, real-time PCR assays are a potential quantitative tool rather than qualitative. Now a day, the terms "quantitative" and "real-time" are used interchangeably as real-time PCR becomes the first choice to quantify nucleic acids. The primary objective of real-time PCR is to discriminate and measure the amount of a specific nucleic acid sequence. During real-time PCR, as amplification progresses, the fluorescent signal increases and reaches a threshold level that correlates with the amount of original target sequencing.

7. HCV Genotyping Assays

HCV genotype determination is an important predictor of disease progression, aggressiveness, type, duration, and response to antiviral therapy. Most importantly, the identification and characterization of HCV genotypes have greater implications for vaccine development. Over the years, several methods have been used for HCV genotyping.

7.1. Nucleic Acid Sequencing

The HCV genome's genetic variability complicates the amplification, sequencing, and genotyping processes. These processes typically rely upon primers and probes (e.g., PCR amplification primers, sequencing primers, and site-specific probes) that are complementary to and capable of hybridizing to corresponding nucleic acid sequences of the HCV genome. As a result of the high degree of variability of the HCV genome, primers and probes complementary to one species of HCV may not be complementary to another species. Primers and probes must therefore be designed for specificity to highly conserved regions. Alternatively, assays must use mixtures of degenerate primers and probes that are complementary to all species.

Direct sequencing of PCR-amplified target sequences followed by phylogenetic analysis is considered the gold standard and the most definitive method. In general, target

sequences in the 5'UTR or NS5 regions of the HCV genome are amplified by RT-PCR, and amplicons obtained are subjected to nucleic acid sequencing (McHutchison et al. 1998) The sequencing results are blasted against HCV genotype databases to assess the genotype. When the 5'UTR is sequenced, 1a, 1b, 1c, 2, 2a, 2b, 2c, 3, 3a, 3b, 4a-h, 5a, and 6a including novel genotypes can be determined. Sequencing of the NS5 regions is preferred as Simmond's classification of HCV genotypes is based on the NS5 region (Zein 2000).

Direct sequencing, however, is impractical for high throughput analysis due to the complexity of the procedure that requires highly skilled labor. It is not cost-effective, especially in low-income laboratory settings, as it requires expensive sequencing apparatus and fluorescent cycle-sequencing kits. Additionally, direct sequencing is unable to identify mixed infections involving more than one HCV genotype.

7.2. Line Probe Method (LiPA)

Several assays based on type-specific probes have been described and are commercially available for HCV genotyping. InnoLipa, developed by Innogenetics (Zwijndre, Belgium), is based on type-specific probes that hybridize to 5'UTR amplicons and is referred to as line-probe assay (LiPA). LiPA generally involves RT-PCR amplifying target sequences with biotinylated primers that label the amplicon. The biotin-labeled amplicons can hybridize with a set of types- or subtype-specific probes that are immobilized to nitrocellulose membrane. An enzymatic detection system then detects these immobilized biotin-labeled amplicons. Although LiPA requires multiple steps to complete and is time-consuming, it requires fewer technicalities than nucleic acid sequencing. A modified version of INNO-LiPA has been developed with higher sensitivity and can differentiate genotypes 1a, 1b, 2a to 2c, 3a to 3c, 4a to 4h, 5a, and 6a (Maertens G 1997). However, HCV genotyping assays, including INNO-LiPA, are unable to distinguish genotypes 1a from 1b in about 5% to 10% of cases and genotype 2a from 2c (Smith et al. 1995). Another LIPA commercial kit, VERSANT HCV Genotype 2.0, is based on probes hybridized to the core region and discriminates between the subtypes of types 1-6(Bouchardeau et al. 2007).

7.3. PCR-Restriction Fragment Length Polymorphism (RFLP)

RFLP, also known as cleaved amplified polymorphic sequences (CAPS), involves the digestion of PCR products with restriction enzymes that recognize and cut at type-specific cleavage sites (polymorphic sites) in the target sequence (McOmish et al. 1993). RFLP results in disparate electrophoretic patterns unique for every genotype. Different regions of the HCV genome, such as the NS5 and the 5'UTR, have been targeted for RFLP analysis (Bukh, Purcell, and Miller 1992, Chan et al. 1992a). Advantages of RFLP include simplicity, reliability, cost, and time efficiency. The potential disadvantage of RFLP is a non-specific restriction that produces unwanted DNA bands, which ultimately reduces the reliability of the assay (Nakao et al. 1991). It is important to upgrade the RFLP regularly as the virus is highly mutating, and the heterogeneity of its genome is continuously increasing (Nakao et al. 1991).

7.4. Melting Curve Analysis

An additional advantage of real-time PCR is that the final product can be further characterized by determining the "melting" of the double-stranded product by increasing the temperature. The melting temperature/ point is an exceptional character that depends on product length, sequence, nucleotide composition/ GC contents, and heterozygosity

(Mark A. Valasek 2005). The melting profile of a PCR product is best monitored in the presence of saturating dyes that produce fluorescence when attached to double-stranded DNA. The target sequence within DNA is amplified, and as the concentration or copies of amplicon increase in the reaction tube, fluorescence intensity also increases. Following amplification, the double-stranded amplicons are heated from ~45°C to ~90°C. As the temperature increases, the double-stranded amplicons are melted apart and converted into single strands. The dye only binds to double-stranded nucleic acid and gives fluoresce. By plotting the negative derivative of fluorescence over temperature (-dF/dT), a "melting curve" is obtained. As the melting profile of the amplicon depends on the sequence of the sample, even a single base pair change (as in the case of SNPs) affects the melting curve. Different genetic sequences (e.g., different HCV genotypes) will melt at different melting temperatures and can be observed, compared, and detected using melt curves (Reed, Kent, and Wittwer 2007, Athar et al. 2015).

8. Conclusions

Molecular assays have a growing role in the management of Hepatitis C virus-infected patients. As new therapeutic anti-HCV agents, such as HCV polymerase and protease inhibitors, are developed, the demand for molecular tests to monitor the response to these new agents will increase. Qualitative commercial assays used to detect and quantify the HCV are either based on RT-PCR or TMA. HCV viral load in acute and chronic infections is important to determine the progress and management of disease and is also used as an indicator of the response to antiviral therapy (Pawlotsky 2006). High viral load in patients with chronic infection is an indication of reduced response to antiviral therapy (Yamada et al. 1995). In contrast, a decrease in the viral load during the early phase (2-12 weeks of treatment) may predict an effective treatment (Zeuzem et al. 1998). Therefore, the ideal assay for determining HCV genotype for clinical use should have characteristics to give accurate genotype in clinical samples regardless of genomics variability that may affect primer binding site, sensitivity, precision, and reproducibility.

The standard therapy for chronic hepatitis C infection is treated with pegylated interferon-α that is administrated as a monotherapy or in combination with ribavirin (Jun et al. 2012). The response rate to this therapy differs among patients because HCV's genotype is a strong prognostic factor for sustained virological response(Blum 2003). It is observed that the patients with genotypes 2 and 3 have two to three-fold higher response rates than the patients infected with genotype 1 (Marc G. Ghany 2009). Therefore, a rapid, accurate, and affordable HCV genotyping assay for treatment management and ultimate patient care is necessary. Currently, sequencing analysis of specific regions (NS5, core, E1, and 5'UTR) is considered the gold standard for genotyping (Ohno and Lau 1996). However, alternative methods that offer rapid and cost-effective genotyping could be better suited for clinical use. These methods include amplification with type-specific primers or probes (Qu et al. 1994), restriction fragment length polymorphisms (Park et al. 1998), line-probe testing (Stuyver et al. 1996), and hetero-duplex mobility analysis (Margraf et al. 2004). More recently, a high throughput method based on Matrix-Assisted Laser Desorption Ionization Time Of Flight (MALDI-TOF) technology (Ilina et al. 2005), melting curve analysis using LightCycler® systems (Haverstick, Bullock, and Bruns 2004) were

also developed. So far, these methods have only found limited application in developing countries due to the need for special and expensive instruments. Two commercial assays, the Versant HCV genotype 2.0 assay (line probe assay [LiPA] 2.0), based on reverse hybridization, and the Abbott Realtime HCV genotype II assay (real-time II), based on genotype-specific real-time PCR, have been widely used to analyze hepatitis C virus (HCV) genotypes. These two assays, however, have been found limited in identifying HCV genotype 6 (Yang et al. 2014), which has increased significantly in the past years in China (Rong et al. 2014).

These assays show significant variations. To ensure the accuracy of the quantification, the dynamics of each assay should be optimized for appropriate dilutions of HCV RNA. For these assays, the lower limit of quantification may be in the range of 600 to 615 IU/mL, while the upper limit ranges from 8.5×10^5 to 7.7×10^6 IU/mL (Pivert and Lunel 2006). The ideal assay for HCV RNA detection should have a lower limit of detection that ranges from 5 to 50 IU/mL and display a linear curve up to a concentration of 6 to 7 $\log_{10}$ IU/ml. Therefore, these traditional commercial assays do not provide sufficient information about the end-of-treatment or sustained virological response (Neiva Sellan Lopes Gonçales 2007).

Antiviral treatment administration and HCV disease management are now well established. However, clinicians and physicians still need robust, reproducible, and sensitive nucleic acid testing-based molecular assays that have a wide range of detection and quantification. Furthermore, we recommend that researchers work to exploit the sensitivity of real-time PCR and develop methods for detecting and quantifying multiple HCV RNA types and subtypes in a single reaction. This multiplex PCR approach will save time and cost to suit the patients of low-income South Asia and African countries.

Conflict of Interest

The authors declare that they have no competing interests.

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Study Approval

NA

Consent Forms

NA.

Authors Contribution

MAA conceptualized the study and wrote the final manuscript, VA critically reviewed and edited the manuscript, IU critically reviewed the manuscript and made editing and language improvements. FF and SM helped in the analysis and figures, SW was responsible for supervision, critical review and finalized the manuscript writing.

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