Molecular diagnosis of hepatitis C viruses; technologies and its clinical applications Review

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Contents:

Abstract

Hepatitis-C is one of the most common viral diseases caused by hepatitis C virus (HCV). It is responsible for millions of deaths each year in the developing world. The common dissemination paths of HCV include the use of 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, lack of efficient diagnosis, quantification and genotyping of viral strains are the major hindrances in a management of this notorious epidemic. The knowledge of HCV genotype and an amount of virus in patient's blood are pre-requisites to determine the duration and method of treatment. In this review, we discuss the implications of HCV molecular diagnostic methods and their clinical applications. We conclude that while, several commercial and home-brewed methods are available for this purpose, and there is a visible vacuum for cost effective, robust, sensitive assays that can detect multiple viral genotypes in a single reaction. We are of the view that the level of sensitivity offered by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) technique is unequivocal as compared to other techniques. Therefore, researchers may explore further possibilities using this technique in the management of HCV.

Key words: 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 of 1950-60, and was distinguished into so called infectious and serum hepatitis [1]. 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 [2, 3]. 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 viruses [4]. 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 to use chimpanzee as a reliable model [5]. The experiments on chimpanzee involved serial passage of infectious material from human sources and provided important data indicating the presence of multiple NANBH agents. One of such agents demonstrated the formation of characteristic membranous tubules within the chimpanzee liver cells and was known as tubule forming agent (TFA) [5]. 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 a 80 nM pore-sized filter [6, 7]. Eventually, in 1989, researchers from CDC and Chiron laboratories identified the virus as HCV [8]. 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 [9].

HCV remains a major threat to public health, causing both acute and chronic hepatitis infection. It currently affects 130-150 million people (approximately 2% to 3% of the world population) globally, who are living with chronic infection [10]. A significant number of these chronically infected individuals develop liver cirrhosis or liver cancer [11]. HCV infection

annually kills more than 350,000 people across the world because of liver-related diseases [12]. The true burden of HCV infection is not known due to limitations in data collection [13], the situation could be even worse in developing countries [14].

Overall, HCV causes great human and economic loss, which makes the basis to value HCV screening, diagnosis and treatment [15]. 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 [16].

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% [14, 17, 18]. In comparison, the infection rate is more than 3% in many countries in Eastern Europe, Latin America, former USSR including Central Asian States, and certain countries in Africa, the Middle East and South Asia [14, 19, 20]. Egypt has more than 10% of the population at risk [14]. Other African countries have prevalence rates ranging from 2% to >3% [18, 19].

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 [21]. In Pakistan, HCV infection is highly endemic and is attributed to the highest rate of unsafe injections (13 injection/person/year) [20]. A recent report confirmed the association between high chances of transmission and unsafe drug use. They reported that the individuals received injections within 6 months preceding the diagnosis of viral hepatitis [22]. Other practices and events leading to HCV infection are related to traditional healers, unqualified medical professionals, tattooing, and commercial barbershops in Pakistan [20].

2. Hepatitis C viral genome structure

HCV belongs to genus *Hepacivirus* of 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 is composed of 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 viral entry phase, respectively [23].
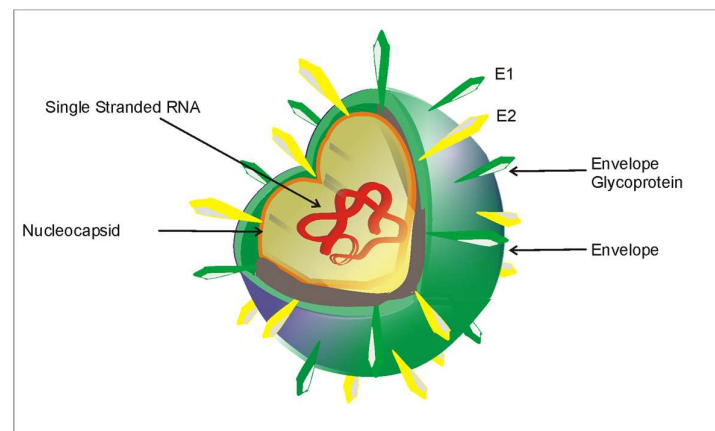
Figure 1. HCV virion structure

The first protein to be synthesized inside the host cell is the 191 amino acid long core protein. Core is an RNA binding protein [24] and plays a critical role in encapsidation and packaging of RNA into a newly emerged virion. Core exhibits its crucial role in viral replication and immune-pathogenesis of HCV infection [25]. Other potential functions of core protein include oncogenesis, regulation of cellular signaling, and apoptosis [26]. Recent studies have shown the importance of core protein in diagnosis and genotype determination of HCV [27].

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 (NS) 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).
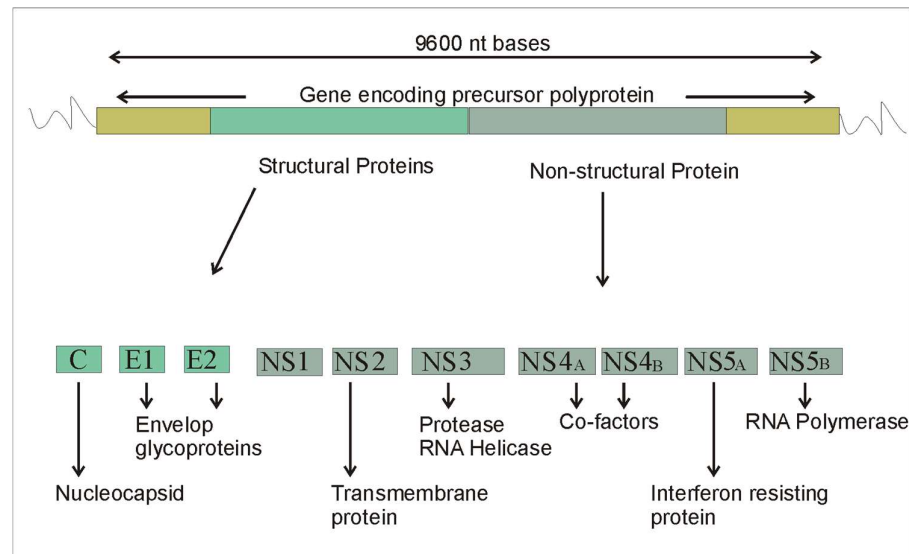
Figure 2. Genomic organization of HCV

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) that lacks proof reading ability, is essential for the replication of the HCV genome [28]. 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 [29]. Thus, a closely related but diverse population of viral variants known as quasi species is produced [30]. In addition, there are certain coding regions in the HCV genome, which impart high mutation rate, such as the hyper variable regions in envelope protein E2, which is responsible for high mutation rate and enables virus

to escape the host immune system [31]. Mutations in the envelope protein can modify the antigenicity of the virus surface, and hence are the most popular immune escape rout in quasi species.

HCV isolates exhibit a notable variation throughout their genome sequence. These variants can be categorized into a number of different types based on sequence comparisons of sub-genomic regions [32]. These sub-genomic regions or variable sequences include encoding NS5 protein [33], the core protein [34], the envelop protein E1 [35] , NS3 [36] and 5´UTR [37]. 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 exist 65% similarity between HCV types [38].

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 as 1, 2, 3, 4, 5 and 6. These genotypes have different serological and/or biological characteristics [39]. Each of these types is further sub-classified into subtypes, which differ in around 20% of nucleotide positions. There are more than 70 subtypes which are assigned lowercase letters such as a, b, c etc. [40]. 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 as quasispecies [41]. The presence of quasispecies imparts a distinct survival advantage, as co-presence of multiple variants viral genomes and a high rate of generation of new variants may allow rapid selection of those variants that have high rates of survival in changing environmental conditions [42].

4. Geographical distribution of HCV genotypes/subtypes

HCV genotype distribution throughout the world depends on the geographical territory and mode of transmission [37]. Some 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 as a result of blood transfusion, medical procedures and contaminated syringe users among drug users. Genotype 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 [43]. It is noteworthy that more than 70% cases of HCV infection are caused by 1b alone [37].

Genotypes 2a and 2b represent 10-30% of global 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 along with Southeast Asian populations are commonly infected with genotype 3. 3a is specifically prevalent among drug users in Western Europe and the United States [37]. 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 [44]. Only in Vietnam, genotypes 7, 8 and 9 have been identified and genotypes 10 and 11 are identified in Indonesian patients [39]. Since there remains a 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 [45]. In Pakistan, the most common genotype is 3a, which is followed by 3b and 1a [30]. Genotype 1b followed by 2a are predominant in most regions of China [46] (Figure 3).
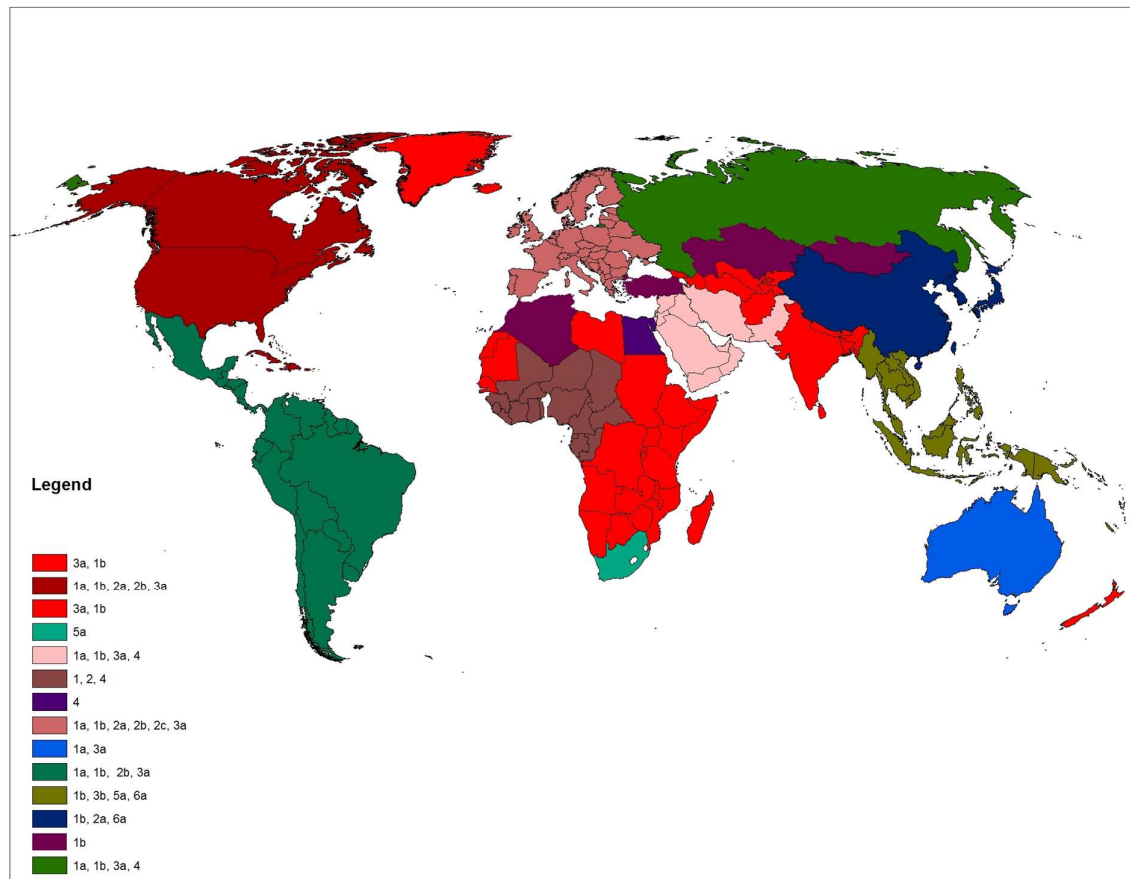
Figure 3. Geographic distribution of HCV genotypes

5. Clinical relevance of HCV genotypes and its response to treatment

HCV genotype determination is extremely important step in the 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 the 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) that is being administered as a monotherapy or in combination with ribavirin [47]. The genotype 1 does not respond to IFN-α treatment, while genotypes 2 and 3 respond efficiently [48]. Also, higher rates of hepatitis reactivation and steatosis have been reported for genotype 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 been 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 [49]. As compared to the dual therapy involving peg interferon-α and ribavirin, triple combination therapy including either first of the two direct-acting antivirals, telaprevir and boceprevir, are currently recommended for HCV genotype 1 and the success rate of cure more than 70%, the duration of therapy has also reduced from twelve to six months (Table 1) [22]. These drugs have side effects require expertise of the health care professionals to deal the drugs. Unfortunately these drugs are extremely expensive and are not equally available worldwide. Therefore, in most parts of the world peg-interferon and ribavirin still remains the therapy of choice, particularly in the developing countries [50, 51].

Table 1: Hepatitis C Virus (HCV) Treatment

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

6. Molecular diagnosis of HCV

Since the discovery of HCV in 1989, the advances in developing assays to detect HCV RNA has 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 with respect to anti-viral therapy. Molecular assays are direct tests, which quantify or characterize HCV RNA virus.

6.1. *Molecular assays* for HCV detection and quantification

Molecular detection assays are based on the amplification of 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 patient's blood serum, with a true reflection of active infection. These assays can detect virus at very low concentrations, usually within 1-3 weeks following exposure [52]. These assays are either qualitative, to discriminate acute infection from chronic; quantitative, determining 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.

Table 2: Commercially available real-time PCR assays:

Assays	Manufacturer	Instruments	IVD Approval	Limits of detection (IU/ml)	Range of quantification
COBAS TaqMan HCV Test v2.0 For Use With The High Pure System (HPS/CTM)	Roche molecular diagnostics	COBAS TaqMan	CE (Europe) FDA (USA	8.8-9.3 (Europe gentype 1), 20,(All USA genotypes)	25-3.91×10^8 (Europe genotypes 1), 25-3x10^8(All USA genotypes)

COBAS AmpliPrep/COBAS TaqMan HCV Test, v2.0 (CAP/CTM)	Roche molecular diagnostics	COBAS TaqMan	CE (Europe) FDA (USA	15	$15\text{-}1.0\times10^{8}$[8]
RealTime HCV	Abbott molecular	m2000RT	CE (Europe) FDA (USA)	12	$12\text{-}1.0\times10^{8}$[8]
Artus HCV QS-RGQ assay	Qiagen	Rotor-Gene Q	CE (Europe)	36.2	$67.6\text{-}17.7\times10^{6}$
Versant HCV 1.0 kPCR Assay	Siemens healthcare	kPCR amplification and detection (AD) module	CE (Europe)	15	$15\text{-}1.0\times10^{8}$
Xpert HCV Viral Load	Cepheid	GeneXpert system	CE (Europe)	4-6.1	$15\text{-}1.0\times10^{8}$
Aptima HCV Quant Dx Assay	Hologic	Panther system	CE (Europe)	3.9-4.3	$10\text{-}1.0\times10^{8}$
VERIS HCV Assay	Beckman coulter	DxN VERIS molecular diagnostics System	CE (Europe)	12	$12\text{-}1.0\times10^{9}$
COBAS HCV test	Roche molecular diagnostics	COBAS 6800/8800 systems	CE (Europe)	10	$15\text{-}1.0\times10^{8}$

Since the amount of HCV RNA in infected patient's serum is limited, an 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 and, real-time PCR are used for RNA detection and quantification.

6.1.1. RT-PCR assays

Reverse transcription converts RNA into cDNA by a 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 prime cDNA synthesis from all RNA species of the cell. In contrast, specific primers initiate cDNA synthesis from a particular sequence of mRNA and allow more sensitive and accurate detection and quantification [53]. When RT-PCR is used for the determination of 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 [54]. 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 fragments simultaneously, by using more than one pair of primers. It reduces the time and effort, needs less amount of sample and provides broader sensitivity. It makes use of flourophores/fluorescent dyes that have different emission spectra such as FAM, ROX and Cy5 etc. that are combined with probes.

The most conserved sequence of HCV RNA Viruses, such as the 5'UTR and the core region are amplified during PCR by sequence specific primers [55]. A number of factors influence reverse transcription PCR including quality of template, design of the primers, reaction efficiency and post-amplification detection system [56].

Many commercial kits are available for 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) [57].

6.1.2. Transcription mediated amplification (TMA)

TMA is an isothermal nucleic acid process and it makes use of two enzymes namely 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 detectable amount of RNA [58].

The TMA based VERSANT® HCV RNA assay amplifies the conserved region within the 5´UTR. This is a qualitative assay that detects the HCV RNA levels as low as 5-10 IU/mL, which may not be detected with the RT-PCR [59]. The entire process including 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 [60].

6.1.3. Branched DNA assays

Branched DNA (bDNA) uses a series of hybridization steps to detect and amplify HCV RNA. The bDNA does not need amplification of target genomic sequence. This reduces contamination and, therefore, the chances of false positive results are significantly reduced [57].

The VERSANT HCV RNA 3.0 assay is a bDNA assay for the quantification of 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 HCV genome. Amplifier probes subsequently hybridize to 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 [60].

6.1.4. Real-time PCR assays

As compared to conventional PCR that 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 amplicon produced at each PCR cycle.

In principle, 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 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 hydrolysis probe that employs 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/flourophores, respectively (Figure 4). When the probe is hybridized to 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 [61].
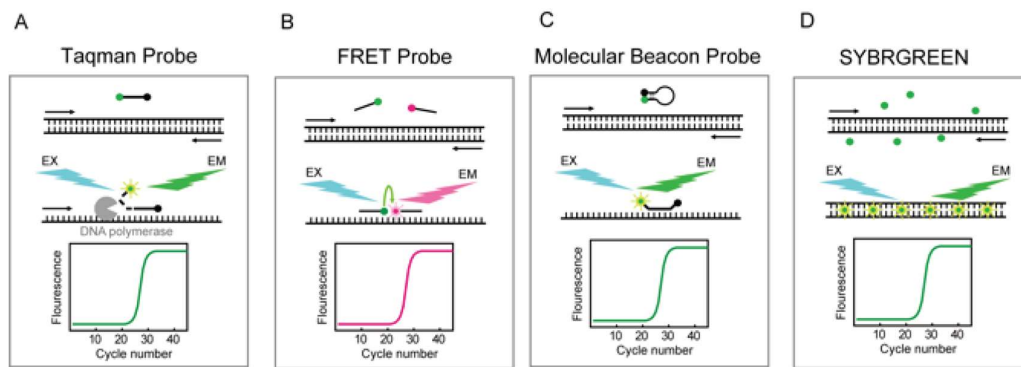
Figure 4. A representation of different molecular probes for the detection of PCR products.

(A) 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 yield precise results. The FRET probe **(B)** and Molecular beacon probe **(C)** 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 the 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. **(D)** 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

An other 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 quenching of the fluorescence. The loop contains a probe sequence that is complementary to a target sequence, and a stem that is formed by the annealing of complementary arm sequences that are located on either side of the probe sequence. [62]. As 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 sequences changes and PCR product specificity, allows comparison of melting temperature of specific and non-specific products, if any [63]. 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 [53].

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 [64]. 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 a qualitative one. 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, fluorescent signal increases and reaches a threshold level that correlates with the amount of original target sequencing.

6.2. HCV genotyping assays

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

6.2.1. Nucleic acid sequencing

The genetic variability of the HCV genome complicates the processes of amplification, sequencing, and genotyping. These processes typically rely upon the use of primers and probes (e.g., PCR amplification primers, sequencing primers, and site-specific probes) that are complementary to and are 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 definite method. In general, target sequences in the 5'UTR or NS5 regions of HCV genome are amplified by RT-PCR and amplicons obtained are subjected to nucleic acid sequencing [65]. 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 Simmonds classification of HCV genotypes is based on the NS5 region [37].

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 genotypes.

6.2.2. 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 amplification of 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. These immobilized biotin-labeled amplicons are then detected by an enzymatic detection system. Although LiPA requires multiple steps to complete and is time consuming, it requires fewer technicalities as compared to nucleic acid sequencing. A modified version of INNO-LiPA has been developed that has higher sensitivity, and is able to differentiate

genotypes 1a, 1b, 2a to 2c, 3a to 3c, 4a to 4h, 5a, and 6a [66]. However, HCV genotyping assays including INNO-LiPA are unable to distinguish genotypes 1a from 1b in about 5% to 10% cases, and genotype 2a from 2c [67]. Another LIPA commercial kit, VERSANT HCV Genotype 2.0 is based on probes hybridized to the core region, and discriminate between the subtypes of types 1-6 [68].

6.2.3. PCR-restriction fragment length polymorphism (RFLP)

RFLP, also known as cleaved amplified polymorphic sequences (CAPS), involves digestion of PCR products with restriction enzymes that recognize and cut at type-specific cleavage sites (polymorphic sites) in the target sequence [69]. 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 [34, 70]. Advantages of RFLP include simplicity, reliability, cost and time efficiency. The potential disadvantage of RFLP is non-specific restriction that produces unwanted DNA bands, which ultimately reduce the reliability of the assay [71]. It is important to upgrade the RFLP on a regular basis as the virus is highly mutating and the heterogeneity of its genome is continuously increased [71].

6.2.4. 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 [72]. The melting profile of a PCR product is best monitored in the presence of saturating dyes that produce fluorescence when attached with double stranded DNA. The target sequence within DNA is amplified and as the concentration or copies of amplicon increase in the reaction tube,

the intensity of fluorescence 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 are 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 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 [73, 74].

7. Conclusions

Molecular assays have a growing role in management of the Hepatitis C virus infected patients. As new therapeutic anti-HCV agents, such as HCV polymerase and protease inhibitors, are develop and 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 also used as an indicator of the response to antiviral therapy [42]. High viral load in patients with chronic infection is an indication of reduced response to the antiviral therapy [75] and whereas a decrease in the viral load during the early phase (2-12 weeks of treatment) may predict an effective treatment [76]. Therefore, the ideal assay for the determination of 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, precise and reproducible.

The standard therapy for chronic hepatitis C infection is treatment with pegylated interferon-α that is administrated as a monotherapy or in combination with ribavirin [77]. The

response rate to this therapy differs among patients because; the genotype of HCV is a strong prognostic factor for sustained virological response [78, 79]. It is observed that the patients with genotype 2 and 3 have two to three fold higher response rate than the patients infected with genotype 1 [80]. 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 to be the gold standard for genotyping [81]. 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 [82], restriction fragment length polymorphisms [83], line-probe testing [84] and hetero-duplex mobility analysis [85]. More recently, high throughput method based on Matrix-Assisted Laser Desorption Ionization Time Of Flight (MALDI-TOF) technology [86], melting curve analysis using LightCycler® systems [87] were also developed. So far, these methods have only found limited application in the developing countries due to the need of 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 (Realtime 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 [88], which has increased significantly in the past years in China [89].

These assays show significant variations, and 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 [90]. The ideal assay for HCV RNA detection should have a lower limit of detection that ranges from 5 to 50 IU/mL and should 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 end-of-treatment response or sustained virological response [91].

Antiviral treatment administration and HCV disease management is 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 should work to exploit the sensitivity of real time PCR, and develop methods for detection and quantification of 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 countries in South Asia and Africa.

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