REVIEW ARTICLE

Hepatitis C: A Review on Current and Emerging Genotyping Assays

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ABSTRACT

Hepatitis C is a global public health concern that infects millions of people worldwide. The continual discovery of new genotypes and subtypes of hepatitis C virus (HCV) is an indication of a persistent molecular evolution of the virus. This remains a concern in the efforts towards hepatitis C elimination, as effective management of the disease is, in part, dependent on the HCV genotype responsible for the infection. Accurate HCV screening and quantification using rapid but highly sensitive and reliable methods are crucial for the diagnosis and subsequent management of HCV-related diseases. Thus, this article discusses HCV and the common methods employed for HCV detection and genotyping. While nucleotide sequencing and phylogenetic analysis of core/E1 and NS5B region are regarded as the gold standard and the most recommended method used for HCV genotyping, electrochemical sensors are being explored for their rapidity.
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INTRODUCTION

Hepatitis C virus (HCV) was first described by Michael Houghton and colleagues in 1989 through cloning and sequencing of HCV genome collected from infected chimpanzees (1,2). The virus is responsible for hepatitis C, which is an important global disease chronically infecting around 57.8 million people (3,4). The World Health Organization (WHO) estimates that there are around 1.75 million new infections each year, giving a worldwide prevalence of HCV of 23.7 cases per 100,000 people (5). According to the Centers for Disease Control and Prevention (CDC), the number of hepatitis C-related fatalities has increased by 4% in 2020 compared to 2019 (6). The global prevalence of HCV differs widely throughout the world, ranging from 0.5%-6.5%. The reported prevalence in India was 0.9%, 0.5%-1.5% in Western countries, 2.2% in Indonesia, 2.3% in Southeast Asia and eastern Mediterranean countries, 6.5% in Pakistan, and 3.2% in China (4,7). 10 million people have chronic hepatitis C in Southeast Asia and Western Pacific region, according to World Health Organization (WHO) (8). Southeast Asia has the highest number of HCV-related deaths in Asia after the East Asia and South Asia regions (9).

HCV is a main contributor to liver related-mortality and fatality. The risk exposure associated with HCV infection is typically related to direct contact with blood, blood transfusions, improper medical injections, sexual contact and intravenous drug use (IVDU) (10). The transmission from mother-to-child or also known as the vertical transmission is rare with an estimated risk of approximately 6% although it can be two times higher in mothers with HCV-HIV co-infection (11). Other transmission modes of HCV such as piercing, scarification and improper tattooing are also contribute to the significant raise in HCV infection (11,12).

Acute HCV infection lasts up to six months and is considered a silent killer since most of the HCV-infected patients were asymptomatic (10). Hepatitis C becomes chronic after six months of infection and usually resulted in liver failure, cirrhosis, hepatocellular carcinoma (HCC) and death (7). Disease progression is associated with certain factors including male gender, higher age during the diagnosis, obesity, heavy alcohol intake, co-infection with HIV and immunosuppression (11). Untreated chronic HCV may lead to the development of

hepatic cirrhosis and hepatocellular cancer in cirrhotic patients. Generally, the average 5-year survival rate for cirrhotic patients is 50% (10). Therefore, early HCV diagnosis and therapy are necessary to manage the infection.

The continuous documentation of new genotypes and subtypes of HCV is an indication of constant molecular evolution of the virus. This remains a concern in the stride towards the elimination of hepatitis C, as effective treatment and management of the disease are, in part, dependent on the HCV genotype responsible for the infection. The emergence of instruments and sophisticated technology for screening and diagnosis in populations as well as efficient treatments have minimized the disease's impact on society and could eventually lead to the eradication of viral hepatitis by 2030. As such, accurate detection and proper genotyping of HCV are crucial for patient management.

GLOBAL GENOTYPE DISTRIBUTION

HCV demonstrates a high genetic diversity with many genotypes and subtypes reported thus far. Presently, there are eight main genotypes and 90 subtypes of HCV (13). The genomic sequences between the genotypes differ by 30-35% while the subtypes are distinguished by less than 15% (13,14). The number of genotypes and subtypes most probably will expand in future as the virus undergoes further mutations. Studies have shown, genotype 1 accounts for 44%-46% (83.4 million cases) of all HCV incidences globally, with almost a quarter of those cases occurring in East Asia making it the most prevalent (14,15). In the meantime, the second most frequent genotype is genotype 3 which contributes for 30% of the overall occurrences, while genotype 2, 4 and 6 represents up to 20% of all cases. Last but not least, the remaining <1% belong to the rare genotype 5 (14).

Interestingly, certain genotypes are associated with particular regions or countries (16). In Central Sub-Saharan Africa, genotype 1 is uncommon, accounting for only 1.7% of cases, whereas it is quite frequent in Central Europe and Southern America with a prevalence rate of more than 85%. Genotype 3 is highly prevalent in South Asia, Australia and some European countries (17). The prevalence of genotype 3 cases is slightly lower in Africa, in contrast to South Asia where genotype 3 is more common with up to 70% frequency. Meanwhile, genotype 4 is more common in Africa and the Middle East, with prevalence rates of 97.6% and 65.3% reported in Central Sub-Saharan Africa and North Africa/ the Middle East, respectively. In contrast, genotypes 5 predominates in South Africa while genotype 6 is commonly found in East Asia and Southeast Asia (18,19). HCV infection with genotype 7 has been described in Central African immigrants in Canada (14) and genotype 8 in patients from India (20). Among the HCV subtypes, 1a, 1b, 2a and 3a have been previously shown to be widespread around the world and are responsible for a significant proportion of HCV infections in resource-rich countries (14). The updated information of confirmed HCV genotypes and subtypes can be found in the link: https://ictv.global/sg_wiki/flaviviridae/hepacivirus/table1. Hence, a thorough understanding of the HCV genotype and their prevalence is necessary for the formulation of national treatment regimen as well as for an epidemiological perspective.

HCV GENOME ORGANIZATION AND FUNCTION

HCV is an enveloped virus and has a single-stranded RNA with positive polarity. The virus belongs to the genus Hepacivirus within the family Flaviviridae (21). The genome is around 9.6 kb long and contains an open reading frame (ORF) that encodes a single polyprotein of 3000 amino acids and is flanked by 5′ and 3′ UTRs (22,23). The polyprotein is translated and processed into three structural proteins (core, E1 and E2) at the N-terminus of the ORF and seven non-structural proteins (NS1, NS2, NS3, NS4A, NS5A and NS5B (Figure 1) (12,24). The NS1 is a brief 63 amino acid-long protein that suppresses the acidity which is necessary for the formation of HCV particles. NS2 is a hydrophobic transmembrane protein of 217 amino acids which is necessary for HCV infectivity. The NS3/NS4A contribute significantly to viral pathogenicity, and thus represents an attractive target for antivirals. NS5A is a complex protein that has several roles in HCV replication, viral pathogenesis, cell signalling pathway modification, virus circulation and interferon response (12).

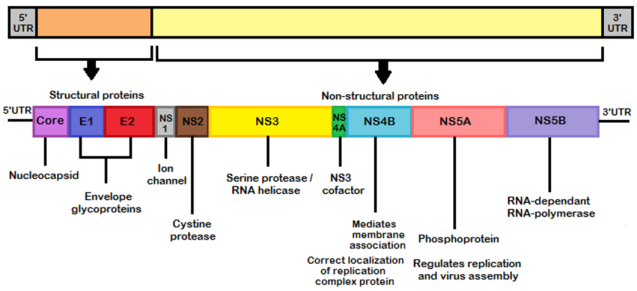

Figure 1: Structure of HCV genome depicting the structural/ non-structural polyprotein

Particular HCV genotypes in infected patients can be identified typically by molecular analyses. A few target regions of the virus genome can be used to differentiate the genotypes, including the 5′-UTR, core/E1 and NS5B regions. The genotyping analysis on E1, core, and NS5B regions is usually sufficient to determine genotypes and subtypes of the virus as they are relatively well-conserved (25). The conserved nature of the 5′-UTR has made it among the primary target for pan-genomic HCV RNA detection assays and sequence analysis of amplicons to genotype HCV in a clinical laboratory setting. In contrast, the differentiation of subtypes benefits greatly from the use of the core region as this region has

more mutation compared with the 5′-UTR (26). Apart from being used to differentiate HCV genotypes and subtypes, the NS5B is, as well, an important target for drug research. Nucleotide sequencing and phylogenetic analysis of the core/E1 and NS5B region are considered the gold standard and the most recommended method used for HCV genotyping (27).

5′-UTR

The most conserved region of the HCV genome is the 5′-UTR which consists of 341 nucleotides and has a secondary structure that is vital during RNA replication and translation (28). Occasionally, the 5′-UTR region is also used for the diagnosis of HCV infection by reverse transcriptase PCR (RT-PCR) before establishing the therapy (18,29). Most of the genotyping assays target the HCV 5′-UTR genome due to its degree of conservation. However, as it is a highly conserved region, the 5′-UTR is not the most accurate region for the identification of all genotypes. It also has limited efficacy in distinguishing between subtypes. From the 10 sequences analysed, El-Tahan and co-workers reported that the 5′-UTR region has a less variability rate than the NS5B (30). Additionally, the VERSANT LiPA assay that uses 5′-UTR-based genotyping demonstrated discrepancy due to mistyping of the genotype 6 variants with genotype 1 (31). Another study showed that the accuracy of the sequenced 5′-UTR region to correctly identify genotypes 1a, 1b and 6 was only 69%, which was much lower compared with the core and NS5B regions (>90% samples) (32). These observations are, as well, consistent with other studies (33–36). Hence, genotyping by the 5′-UTR regions of HCV may not be accurate enough for certain genotypes, particularly genotypes 1a, 1b and 6.

Core

The core region is considered one of the most reliable regions for HCV genotyping other than NS5B due to its high genetic heterogeneity (37). The core region contains 573 nucleotides and codes for RNA-binding protein that forms the nucleocapsid and protects the viral HCV RNA during infection (38). The core is the region of choice for many molecular assays for HCV genotyping, and its high degree of conservation and sensitivity allows PCR amplification for all HCV genotypes (39). A previous study reported that the core region (92.7%) had a higher amplification rate than NS5B (56.4%) (27). Meanwhile, a recent study reported that both the core and NS5B regions had high amplification rates (93.8% and 91.6%, respectively) (27). However, the core region is more suitable for HCV subtyping due to the variability of specific subtypes (21, 35).

NS5B

NS5B is highly conserved among all the recognised RNA-dependent RNA polymerases (RdRp) and is thought to be a significant target for drugs in pharmaceutical research (26). The NS5B RdRp is an important enzyme involved in viral RNA synthesis and an important target region for HCV genotype and subtype discrimination (36, 37). NS5B is the most reliable viral region for determining HCV genotype due to sufficient genetic heterogeneity and thus could be used to differentiate various HCV subtypes (26). A previous study reported successful NS5B sequencing and phylogenetic analysis yielding 92% of genotype 4a as the most prevalent genotype in the study (41). Another study revealed that DNA sequencing on NS5B region was able to precisely detect few HCV subtypes; 1a, 1b, 2a, 3b, 6a, as well as new subtypes; 1b-2a, 1b-2k and 6d-6k (42). NS5B region was successfully sequenced in around 77% of the positive samples reported in the recent study by Anyovi et al. (43). Therefore, NS5B region is among the recommended region for HCV genotyping as well as Core due to the highly informative in phylogeny analysis and identification of HCV strains (43).

Anti-HCV therapy

HCV genotype is an important indicator of the level of severity, treatment response and application of new antiviral therapy (44). HCV infection was treated with interferon (IFN) in the past. The supplementation of ribavirin which has a wide antiviral action, with IFN helped boost the effectiveness of the therapy. Over the years, new drugs called direct acting antivirals (DAAs) were introduced and the majority of the patients receiving DAA treatment have achieved cure rates greater than 90%. DAAs act as inhibitors developed to obstruct the intracellular step of the HCV life cycle with primary viral targets being NS3/4A protease, NS5A protein and NS5B RNA-dependent RNA polymerase (24).

Direct-acting antivirals (DAAs) including protease inhibitors (NS3/4A), NS5A inhibitors, NS5B polymerase inhibitors (nucleotide/nucleoside and non-nucleoside) and several fixed-dose combinations (FDC) DAAs are among hepatitis C treatments that received approval from the Food and Drug Administration (FDA) (45). As of 2018, 13 DAAs had been approved; Glecaprevir, Voxilaprevir, Grazoprevir, Paritaprevir and Simeprevir (protease inhibitors), Daclatasvir, Velpatasvir, Ledipasvir, Ombitasvir, Pibrentasvir, and Elbasvir (NS5A inhibitors), NS5B polymerase inhibitors; nucleotide (Sofosbuvir) and non-nucleoside (Dasabuvir) (45). These DAAs could effectively treat a particular genotype (Table I). The combination of DAAs glecaprevir/pibrentasvir has been used in patients without cirrhosis who were either treatment-naıve or treatment-experienced and both groups achieved sustained virologic response (SVR) including two patients with the rare genotype 5. Additionally, in a different study, sofosbuvir/daclatasvir was administered for 12 weeks to a total of 123 patients with genotype 6 infection and eight patients with genotype 5 infection. SVR rates for genotypes 5 and 6 were 88% and 94%, respectively (45). When DAAs achieved a high treatment efficacy for all HCV genotypes, they are considered pan-genotypic antivirals (45).

Table I: Summary of HCV genotypes with currently available DAA treatments

Targeted HCV genotypes	DAA regimen	SVR rates	Ref.
1, 2, 3	(SOF + DCV ± RBV), (SOF + DCV/SMV ± RBV), (SOF + RBV), and (SOF + DCV ± RBV)	> 90%	
1, 2, 4, 5, 6	SOF-VEL	99%	
1, 4, 6	GZP-EBV	95%	
1	(ABT-450/r–OBV + DAV + RBV)	> 90%	(24)
4	OBV + PTV-r ± RBV	> 90%	
4	LDV–SOF	100%	
1	SMV + SOF	83% (8 weeks) 97% (12 weeks)	
1, 2, 3	DCV + SOF ± RBV	> 88%	
3	DCV + SOF + RBV	90%	
1, 2, 3, 4, 5, 6	GLE + PIB	> 94%	
1, 2, 3, 4, 5, 6	SOF + DCV	> 92% (GT 1-4) 88% (GT 5) 94% (GT 6)	(45)
1, 2, 3, 4, 5, 6	SOF + VEL	90% (GT 1) 86% (GT 2) 88% (GT 4) 97% (GT 3) 100% (GT 5, GT 6)	

SOF: sofosbuvir; DCV: daclatasvir; SMV: simeprevir; RBV: ribavirin; GZP: grazoprevir; EBV: elbasvir; OBV: ombitasvir; ABT-450/r: ABT-450/ritonavir; DAV: dasabuvir; LDV: ledipasvir; VEL: velpatasvir; PTV–r: paritaprevir–ritonavir; OBV: ombitasvir; GLE: glecaprevir; PIB: pibrentasvir; DAA: direct acting antiviral; SVR: sustained virologic response; GT: genotype

HCV genotype 1 infection has a high incidence rate but a poor response to interferon treatment, making it difficult to cure in the past compared with genotypes 2 and 3. However, the use of direct-acting antivirals (DAAs) has significantly enhanced treatment in individuals with genotype 1 (46). In contrast, infection with HCV genotype 3 has become comparatively difficult to treat. Furthermore, infection with HCV genotype 3 is linked to an increased risk of fibrosis, cirrhosis, and hepatocellular carcinoma, suggesting that this genotype is more virulent than other genotypes (46). Various genotypes and subtypes have been demonstrated to react differently to antiviral therapy (46). Hence, the efficacy of HCV treatment is thought to be affected by the genotype that is infecting the patient.

HCV GENOTYPING METHODS

HCV genotyping is an essential tool in predicting the duration of treatment as well as monitoring patients' responses to antiviral drugs (47). As such, the accuracy of HCV genotypes and subtypes discrimination is pertinent for treatment, vaccination and a better understanding of epidemiological studies (47). Several molecular diagnostic techniques are available or currently being developed for the detection and genotyping of HCV. The most common methods used are commercial tests that use complementary probes to report the specific genotype present in a sample or through sequencing of genomic nucleotide sequences (40). These genotyping methods are further elaborated on below.

In-house nucleic acid amplification assays

One of the first HCV genotyping assays used is the restriction fragment length polymorphism (RFLP) analysis of the 5'-UTR regions. In this particular method, the restriction endonucleases enzyme that recognizes the cleavage sites specific to each genotype digests a PCR-amplified DNA fragment into fragments of varying lengths. However, the RFLP method is no longer widely used as it requires the use of several restriction enzymes for digestion which makes it time-consuming. More importantly, the method has a limitation in the number of HCV genotypes it can detect (46).

Several "in-house" genotyping assays based on nested/semi-nested RT-PCR, followed by HCV genome sequencing have been established by amplifying a combination of two or three distinct genomic regions: 5'UTR, core and NS5B. At present, HCV genome sequencing is the gold standard as it could accurately identify various genotypes (27). However, different laboratories are known to target different regions and even utilise different sets of primers within the same regions due to various reasons such as cost and sensitivity (Table II).

Tagnouokam-Ngoupo and co-workers (27) demonstrated a similar detection rate when using the core (93.8%) and NS5B regions (91.6%). Of the 369 samples collected in their study, it was revealed that 129 (39.6%) were genotype 4, 132 (3.8%) were genotype 1, while 89 (24.1%) were genotype 2 in both regions of the core and/or NS5B. Using specific primers, discrimination of various HCV genotypes was also possible from the core (1b, 1e, 1h, 4c and 4f) and NS5B (1b, 1c, 1h, 4c, 4f, 4o, 4p and 4t) regions. However, it has been suggested that NS5B is a better target region for genotyping due to high genetic variability than the core region, which enables the discrimination of more HCV subtypes. The study was performed monoplex nested RT-PCR targeting the 5'-UTR, core and NS5B region using in-house primers sets (Table II) (18). In their study, the 5'-UTR region was used to identify HCV infection, while core and NS5B regions were analysed for viral genotyping. Four genotypes (1, 2, 3 and 6), including subtypes (1a, 1b, 3a, 3b, 2a, 6c, 6f, 6i, 6j, 6m, 6n, 6v and 6xa) were obtained through this assay.

Yang et al. developed a new in-house method for

Table II: List of notable primers used for Hepatitis C virus (HCV) genotyping by nested RT-PCR assay

Region: 5′-UTR			
Primers	**Sequences 5′-3′**	**Reported genotypes identified**	**Ref.**
OC1 OC2 IC3 IC4	GCCGACACTCCACCATGAAT CATGGTGCACGGTCTACGAG GGAACTACTGTCTTCACGCAG TCGCAAGCACCCTATCAGGCA	1a, 1b, 3a, 3b, 2a, 6c, 6f, 6i, 6j, 6m, 6n, 6v and 6xa	(18)
5′UTR ExF 5′UTR ExF 5′UTR InF 5′UTR InR	CCCTGTGAGGAACTWCTGTCTTCACG GGTGCACGGTCTACGAGACC TCTAGCCATGGCGTTAGTRYGAG CACTCGCAAGCACCCTATCAGGCAG	6a, 6b, 6n, 6i, 6j, 6h, 6k, 6l, 6m, 6f, 6g, 6o, 6d and 6e	(49)
Region: Core			
Primers	**Sequences 5′-3**		**Ref.**
954F 410R 953F 951R	ACTGCCTGATAGGGTGCTTGCGA ATGTACCCCATGAGGTCGG AGGTCTCGTAGACCGTGCATCAT CACTGTRAGGGTATCGATGA	1a, 1b, 3a, 3b, 2a, 6c, 6f, 6i, 6j, 6m, 6n, 6v and 6xa	(18)
GEN6_EXF1 GEN_EXR2 GEN6_INTF GEN6_INTR	ATCACTCCCCTGTGAGGAACTACTGT CCCTGTTGCATARTTRATCCCGTC ACTGCCTGATAGGGTGCTTGCG ATGTACCCCATGAGGTCGG	6a, 6b, 6n, 6i, 6j, 6h, 6k, 6l, 6m, 6f, 6g, 6o, 6d and 6e	(49)
Core outer f Core outer r Core inner f Core inner r	ACTGCCTGATAGGGTGCTTG ATGTACCCCATGAGGTCGC AGGTCTCGTAGACCGTGC CATGTGAGGGTATCGATGA	1b, 2a, 3a, 3b and 6a	(35)
CoreOS CoreOAS CoreIS CoreIAS	ACTGCCTGATAGGGTGCTTGCGAG ATGTACCCCATGAGGTCGGC AGGTCTCGTAGACCGTGCATCATG CAYGTRAGGGTATCGATGAC	1b, 1e, 1h and 4f	(27)
Region: NS5B			
Primers	**Sequences 5′-3′**		**Ref.**
NS5BF1 NS5BR1 NS5BF2 NSB5R2	CAATWSMMACBACCATCATGGC CCAGGARTTRACTGGAGTGTG GATGGGHHSBKCMTAYGGATTCC CATAGCNTCCGTGAANGCTC	1a, 1b, 3a, 3b, 2a, 6c, 6f, 6i, 6j, 6m, 6n, 6v and 6xa	(18)
ENO2_ NS5BF1 ENO4_NS-5BR1 NS5S3_ NS5BF2 NS5A5_NS-5BR2	TGGGSTTYKCSTATGAYACYCGMT-GYTTTGA ARTACCTRGTCATAGCCTCCGTGAA TATGATACCCGCTGCTTTGACTCCAC GTCATAGCCTCCGTGAAGGCTC	6a, 6b, 6n, 6i, 6j, 6h, 6k, 6l, 6m, 6f, 6g, 6o, 6d and 6e	(49)
NS5B outer f NS5B outer r NS5B inner f NS5B inner r	CNTAYGGITTCCARTACTCICC GAGGARCAIGATGTTATIARCTC TATGAYACCCGCTGYTTTGACTC GCNGAR TAYCTVGTCATAGCCTC	1b, 2a, 3a, 3b and 6a	(35)
SO755 ASO1121 SO755 ENO2BIS	TATGAYACCCGCTGYTTTGACTC GCNGARTAYCTVGTCATAGCCTC TATGAYACCCGCTGYTTTGACTC GCTAGTCATAGCCTCCTG	1a, 1b, 1c, 1h, 1l, 2a, 4c, 4f, 4o, 4p and 4t	(27)

*UTR – untranslated region

quantitative RT-PCR (qRT-PCR) using TaqMan probes with primers (Table III) that were designed to detect five genotypes/subtypes of HCV; 1b, 2a, 3a, 3b and 6a (50). The real-time ABI 7500 PCR system was used in their study in which the fluorescence detector channel was set to fluorescein FAM™ and fluorescein JOE™ to discriminate the subtypes, and a cycle threshold (Ct) value was set at ≤26.5 to indicate a positive result. From this study, 45% of 1b subtype,18% of 3b subtype, 15% of 6a subtype, 13% infections of 3a, and 9% of 2a were successfully detected. However, the limitation of this method is that it requires multiple probes for detection specificity and only detects certain genotypes according to the probes used.

Alternative to molecular HCV detection or genotyping is the Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP). RT-LAMP is an assay that amplifies the target RNA sequence using multiple sets of primers under isothermal conditions within a short incubation time (51). This is a very specific assay as it uses multiple pairs of primers that recognize the specific sites on the DNA or RNA targets (52). Additionally, RT-LAMP generates a much higher copy of amplified DNA, up to a billion copies, compared with PCR assay which generates only a million DNA copies (52). In addition, RT-LAMP can easily be carried out in a heating block, water bath or incubator, avoiding the need for advanced laboratory equipment such as a thermal cycler. LAMP also generates results faster than PCR as it is readily visible by naked-eye observation, either by turbidity or dye staining (51). Nyan & Swinson reported a rapid, naked detection and genotyping of HCV using RT-LAMP by targeting the conserved and sparse polymorphism in the HCV 5′ untranslated region (53). The HCV detection and genotyping were performed using an RNA template in a 25µL reaction mixture with manually designed primers (Table III) and was incubated at 63.5°C for 30-60 minutes. The study showed that RT-LAMP assay could successfully determine HCV genotypes 1 to 6 as individual positive amplification ladder-like banding patterns formed in parallel and optically detected using the GelGreen fluorescent dye. The assay specificity was assessed by testing HCV primer against human immunodeficiency virus (HIV), West Nile virus (WNV), dengue virus (DENV) and hepatitis B virus (HBV). Interestingly, the assay achieved sensitivity and specificity of 91.5% and 100%, respectively. Furthermore, Hongjaisee and co-workers developed RT-LAMP for HCV detection using clinical samples in Thailand (51). The primer set used in this study was originally developed by Nyan and Swinson but was adjusted to detect various genotypes of HCV prevalent in Thailand (53). Using 200 clinical samples previously confirmed by real-time RT-PCR, the RT-LAMP could detect 97.5% of genotype 1, 91.1% of genotype 3, and 100% of genotype 6. The overall assay's sensitivity and specificity were 95.5% and 100%, respectively, with no evidence of viral cross-reactivity.

Table III: Designed primers and probes for HCV genotype-specific qRT-PCR and in-house RT-LAMP primers developed for HCV genotyping

Primers/probes for qRT-PCR				
HCV subtypes	**Primer/probes**	**Sequence 5′-3′**	**Reported genotypes identified**	**Ref.**
1b	Upstream primer Downstream primer Probe	CTCGTAGACCGTGCACCATGA CAGATCGTTGGTGGAGTTTACT FAM GCACGAATCCTAAACCT MGB		
2a	Upstream primer Downstream primer Probe	CTCGTAGACCGTGCACCATGA CAGATCGTTGGCGGAGTATACT FAMGCACGAATCCTAAACCTMGB		
6a	Upstream primer Downstream primer Probe	CTCGTAGACCGTGCACCATGA CAGATCGTTGGCGGAGTTTACT JOEGCACTCTTCCAAAACCCMGB	1b, 2a, 3a, 3b and 6a	(50)
3a	Upstream primer Downstream primer Probe	CTCGTAGACCGTGCACCATGA CAGATCGTTGGTGGAGTATACG FAMACACCATCCGCCGCCCACAMGB		
3b	Upstream primer Downstream primer Probe	CTCGTAGACCGTGCACCATGA CAGATCGTTGGTGGAGTATATG JOEACACACCCCGTCGCCCACAMGB		
Primers for RT-LAMP				
Primer set/ genotype	**Primer ID**	**Sequence 5′-3′**	**Reported genotypes identified**	**Ref.**
DN1a	DN1-F3	CGGGAGAGCCATAGTGGT		
	DN1-R3	TGGAAGAGTGCTCATGATGCACC		
	DN1-FIP	TGAGCGGGTTTGATCCAATGTTTTTGCGGAACCGGTGAGTAC		
	DN1-RIP	CCGCAAGACTGCTAGCCGAGTTTTACCGTATCAGGCAGTACCAC		
	DN1-LF	TCGTCCTGGCAATTCCGG		
	DN1-LF	TAGCGTTGGGTTGCGAAAG		(53)
DN2b	DN2-F3	TCGAACAGCCTCCAGGACCC		
	DN2-R3	CACGGTCTACGAGACCTCC	1, 3, 4, 5 and 6	
	DN2-FIP	CGGGCATTGAGCGGGTTTATCCTTTTGGTGAGTACACCGGAATTGC		
	DN2-RIP	CGCGAGACTGCTAGCCGAGTTTTTAGCACCCTATCAGGCAGTAC		
	DN2-LF	AAAGGACCCGGTCATCCC		
	DN2-LR	GTCGCGAAAGGCCTTGTG		
DN3c	DN3-F3	GGCGACACTCCACCATGAAT		
	DN3-R3	CTATCAGGCAGTACCACAAGGC		
	DN3-FIP	CACTATGGCTCTCCCGGGAGTTTTCG-TCTAGCCATGGCGTTAG		
	DN3-RIP	GGAACCGGTGAGTACACCGGTTTTCC-CAAATCTCCAGGCATTGA		
	DN3-LF	AGGCTGCACGACACTCATA		
	DN3-LR	GACCGGGTCCTTTCTTGGA		
1b	F3	AGTACACCGGAATTGCCAG		
	B3	CGGTTGGTGTTACGTTTGGT		
	FIP (F1c-F2)	ACTCGGCTAGCAGTCTCGCG-TCTTGGATCAACCCGCTCA		(54)
	BIP(B1c-B2)	GTGGTACTGCCTGATAGGGTGC-GATTCGTGCTCATGGTGCA	1b and 2a	
2a	F3	AAACCCACTCTATGCCCGGCC		
	B3	TACTCCGCCAACGATCTGG		
	FIP(F1c-F2)	AGCACCCTATCAGGCAGTACCA-AAGACTGCTAGCCGAGCAGC		
	BIP(B1c-B2)	TGCACCATGAGCACGAATCCCA- CCGGGAACTTGACGTCTTGT		
	DN1M-F3	CGGGAGAGCCATAGTGGT		
DN1	DN1M-R3	WGGAWGTGTGCTCATGATGCACG		(51)
	DN1M-FIP	TGAGCGGGTTTDATCCAAGATTTTTGCGGAACCGGTGAGTAC	1a, 3a and 6	
	DN1M-RIP	CCGCRAGACYGCTAGCCGAGTT-TTACCCTATCAGGCAGTACCAC		
	DN1M-LF	TCGTCCYGGCRATTCCGG		
	DN1M-LR	TAGTGTTGGGTCGCGAAAG		

Commercial assays

There are a few commercial assays available for HCV detection and viral load measurement, all of which are frequently polymerase chain reaction technology (PCR) based assays. Recently, commercial molecular methods for HCV genotyping targeting various genomic regions are also available for laboratory and clinical settings. This includes line probe-assay (LiPA) that is based on reverse hybridization principles with specific probes for genotypes.

Another method used for genotyping is genotype-specific real-time PCR and sequencing-based assays (55). The development of real-time PCR (qPCR)-based assays has enhanced viral load analysis and detection. The quantitative PCR (qPCR) is very sensitive as it could detect targets as low as 10-15 IU/mL, making it more sensitive and efficient than conventional PCR (48). The rapid growth in technology also made the sequencing-based assay by far a powerful tool in molecular studies. Sequencing technology is used to identify various HCV strains, which improves genotyping precision and increases sensitivity for determining all HCV subtypes (56). Unfortunately, the high level of sequence variation among the various kinds of genotypes and subtypes of this virus is a significant problem in HCV genotyping. Thus, sequencing analysis of a particular region of the HCV genome is used as the gold standard to rectify discrepancies since other approaches have a low agreement rate (47). An effective treatment plan in the management of hepatitis C requires a quick, easy, precise, and cost-effective HCV genotyping technology hence, opportunities abound for ongoing research and improvement in HCV genotyping and diagnosis technology.

Several commercially available qPCR assays have been utilised by different groups for genotype/subtype identification. In a study by Stezl and co-workers (57), the primer-specific real-time PCR (qPCR)-based cobas® HCV GT assay from Roche Molecular Systems, Pleasanton, California, USA, was used. This qPCR assay could determine HCV genotypes 1-6 and identify subtypes 1a and 1b. It comprises fluorescent dye-labelled oligonucleotide probes for differentiating HCV genotypes and subtypes as well as genotype- and subtype-specific primers for amplification of partials genome of the 5′-UTR, Core and NS5B regions of HCV. The genotype accuracy of this assay is 99.7% for detecting genotypes 1 to 6 and 100% for subtype 1a and 1b demonstrating its suitability in HCV diagnostics (58).

Another study evaluated the performance of Abbott m2000 RealTime genotype II (Abbott Molecular, Inc., Des Plaines, II., USA) to identify HCV genotypes (44). The Abbott RealTime HCV genotype II assay utilises the 5′-UTR and NS5B regions and can detect genotypes as validated by sequencing with accuracy for each genotype as follows: genotype 1 (99.6%) [subtyping accuracy; 1a (99.3%) and 1b (99.1%)], genotype 2 (99.1%), genotype 3 (100%), genotype 4 (98.75%), genotype 5 (100%), overall Abbott genotype accuracy (99.5%). From the study (44), this assay could detect genotype 1a, 1b, 2, 3, 4, 6, mixed genotypes [(1 and 4) and (1b and 2)] and subtypes of genotype 1 (except for subtype 1a and 1b). This real-time assay was, however, reported to be unable to identify subtypes from genotype 1 in 3.7%-15.9% of cases (32). Hence, further, improvement is needed to enhance the assay's performance.

One of the most popular commercial HCV genotyping assays is the VERSANT HCV genotype assay (LiPA). The VERSANT HCV LiPA 2.0 assay is based on a reverse hybridization principle. Biotinylated primers are utilized to amplify specific regions within the 5′UTR and core, followed by the hybridisation of PCR products to genotype-specific probes on a membrane. Amplicons that are not bound are removed. After washing, the hybridised products are detected using streptavidin labelled with alkaline-phosphatase. Subsequently, a colourimetric reaction occurs following the addition of the substrate nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate (BCIP) (44). Typically, BCIP produces purple or brown precipitates by the oxidation of nitroblue tetrazolium (NBT) (55). The HCV genotype is identified based on the banding pattern on the LiPA strips, and the genotyping results are interpreted according to an interpretation chart (59). A previous LiPA product known as INNO-LiPA HCV II assay had been utilised for HCV genotyping with 100% accuracy for genotypes 1, 2, 3, 4 and 6a. However, notably it was demonstrated that the assay miscategorised genotype 6 as 1. Additionally, the assay did not accurately discriminate between subtypes 1a and 1b. Due to these constraints, the VERSANT HCV Genotype (LiPA) 2.0 assay was then introduced. The core sequences were included to help identify genotype 6 and distinguish between 1a and 1b. The 5′-UTR sequence analysis was used to determine genotypes 2 to 5, as well as 6a/6b (55). The performance of the new LiPA 2.0 assay is much better at predicting the HCV genotype, with a sensitivity between 99.2% to 100%. For subtyping of 1a and 1b, this assay performs quite well. According to a comparison study, LiPA 2.0, Real-time HCV GT II, Trugene HCV 5′-UTR genotyping and INNO-LiPA test demonstrated an accuracy of 99.6%, 93.2%, 77.9% and 70.8%, respectively, for identifying subtypes 1a and 1b (55). Another study revealed that the overall agreement between VERSANT LiPA and sequencing assay exceeded 95% (32). However, there are still certain limitations with this commercial assay. For instance, it is unable to detect coinfection or recombination of HCV genotype in a sample.

Recently, a new commercial assay known as the 6 HCV Genotyping 9G Test (Biometrix Technology Co., Ltd., Gangwon-do, Korea) was introduced. The 6 HCV Genotyping Test is a modern assay that can detect various HCV genotypes including 1a, 1b, 2, 3, 4 and

6 in patients by running PCR products on lateral flow assay which requires 9G technology (47, 48). This technology has 100% target-specific hybridisation at 25°C without needing any costly equipment. HCV genotypes are identified when PCR products bind with the immobilised probes on the 9G membranes (60). The assay's workflow entails viral RNA extraction, synthesis of complementary DNA (cDNA), PCR amplification and eventual detection. The differentiation of genotypes 1a, 1b, 2, 3, 4, 6a/6f and 6i/6n is achieved by targeting the HCV 5'-UTR region. This commercial test is a reliable HCV genotyping assay as it demonstrated a sensitivity greater than 92.6% and a specificity higher than 99%. Furthermore, it shows a good concordance with the commercial LiPA 2.0 assays in detecting HCV genotypes in 79/98 clinical samples with 80.6% agreement. In addition, the 6 HCV Genotyping 9G test also showed 100% agreement with sequencing results for genotype 1b, 3 and 6i identification (59). When genotyping was performed in 280 plasma samples against LiPA 2.0 test and sequencing method, Warkad and colleagues reported that the 6 HCV Genotyping 9G tests shows sensitivity, specificity, positive predictive (PPV) and negative predictive (NPV) values of 99.5%, 98.8%, 99.5%, and 98.8%, respectively (47). The 6 HCV Genotyping 9G test was also discovered to be superior to the LiPA 2.0 assay (47). As a result, the 6 HCV Genotyping 9G test can deliver vital data to healthcare professionals and expedite proper hepatitis C treatment. Table IV shows various HCV commercial assays/kits that are available for diagnostic use.

Table IV: Summary of selected HCV commercial assays/kits

Assay/kits	Accuracy	Genotype	Ref.
Abbott m2000 Real Time genotype II	99.5% 98.28% 100%	1a, 1b, 2, 3, 4, 6, and mixed (1/4 and 1b/2) 1, 1a, 1b, 2, 3, 4 and 5 1a, 1b, 2a, 2b, 3, 4, 5 and 6	(44) (61) (62)
Versant LiPA 2.0	99% >95%	1a, 1b, 2, 3a, 3b and 6c-l/6m 1a, 1b, 2a/c, 2b, 3a, 3b, 3c, 4a/c/d,4b, 4f, 4h, 5a and 6a/b	(63) (32)
Cobas® HCV GT assay (Roche Molecular Diagnostics, CA)	100% 100%	1a, 1b, 2 and 3 1b and 6	(63) (64)
Real-time PCR-based melting curve analysis assay	100%	3a, 1b, 3b, 2a, 6a, 1a and mixed (3a/3b and 3a/1b)	(65)
Anatolia Bosphore HCV Genotyping Kit v3	-	1b, 1a, 3, 2 and 4	(66)
Sentosa® SQ HCV Genotyping Assay	100%	1a. 1b, 3a, 4, 5a, 6 and mixed (2k/1b)	(67)

Sequencing of the genomic nucleotide sequence

Sequencing technology has made a significant contribution to accurate HCV genotyping. Sanger sequencing-based genotyping techniques are more effective than the hybridization-based technique in that they enable concurrent identification of genotype, subtype and resistance-associated substitutions (RASs) (59). DNA sequencing and the subsequent phylogeny analysis of the core/E1 and/or NS5B regions are required for HCV genotyping since they correctly identify HCV subtypes, allowing for accurate epidemiological modelling of the circulating viral strains. The Basic Local Alignment Search Tool (BLAST) analysis is crucial to the evaluation of sequencing results. In addition, the extent of available HCV sequences in the database affects how accurately HCV may be genotyped using BLAST (38). However, despite being laborious with multiple experimental steps such as RNA extraction, reverse transcription, nested PCR, DNA sequencing and phylogenetic tree construction, the advancement of technology and the availability of trained staff make HCV genotyping by direct sequencing a method of choice for many laboratories.

The rapid development of the high-throughput deep-sequencing, next-generation sequencing (NGS) technology which is more advance than the Sanger sequencing is expected to improve accuracy. However, this technology requires specialised technical validation method and highly qualified professionals with bioinformatics understanding. The time-consuming process of NGS is made simpler by the genotypic interpretation services, such as the geno2pheno (68). In a recent study reported by Manso and co-workers, the establishment and technical verification of an HCV next-generation sequencing (NGS) experiment using sequence capture to ascertain viral genotype and antiviral resistance profile was described (69). The result showed a high concordance of the NGS assay with Sanger sequencing with the specificity of 92.3% and 96.1% for antiviral resistance and genotyping, respectively. For inclusivity and specificity, samples with known resistance markers and subtypes were first identified using Sanger sequencing, real-time PCR or probe-based methods (69). In the study, HCV subtypes 1a, 1b or 3a were detected in 72.5% (n=87/120) of the samples. NGS was found to be superior to the "gold standard" methods, as it was correctly assigned 3 subtypes and NGS affirmed that these were novel subtypes. An evaluation of the NGS assay's analytical performance parameters revealed equivalent, and usually a better result when compared to the present "gold standard" techniques. However, the key challenge in employing NGS in clinical settings is the cost and affordability.

EMERGING TECHNOLOGIES

A breakthrough in newly developed technology will be significant as it helps to make genotyping tests simpler, more rapid and more precise. Numerous methods have been developed to detect specific DNA sequences including electrochemistry, fluorescence, radiochemical tests, quartz crystal microbalance and surface plasmon resonance spectroscopy (70). Electrochemical detection

is indeed a useful technique for genetic testing as it is rapid, affordable, sensitive, and selective. An amperometric biosensor has been developed to detect and genotype HCV (70). In the assay, streptavidin was enclosed in thin films of siloxane–poly (propylene oxide) hybrids generated by the sol–gel method and which were then dip-coated onto the surface of a graphite electrode. Streptavidin was used to immobilise biotinylated 18-mer probes. Using this technique, genotypes 1, 2a/c, 2b and 3 were successfully detected (38, 59). However, this assay requires further improvement so that more HCV genotypes can be detected.

The study of electrochemical DNA biosensors has generated a significant amount of interest since it simultaneously offers both identification of target DNA and a simplified detection platform. Donmez and colleagues developed an affordable disposable Poly(L-glutamic acid) (PGA)-modified nucleic acid sensor that can genotype HCV 1a (70). The biosensor involved the immobilisation of HCV1a-related 20-mer probes on the surface of the PGA film with square wave voltammetry used to monitor hybridisation events arising from the oxidation signal of guanine. The nucleic acid biosensor displayed a limit of detection of 40.6 nM. Although a novel biosensor, considering the range of HCV genotypes and subtypes available, this nucleic acid sensor cannot be applied for broad HCV genotyping.

Another electrochemical sensor is the eSensor® HCV Genotyping Test designed for the detection of HCV 1a, 1b, 2a/c, 2b, 3, 4, 5, and 6a/b (72). The target DNA and the signal probe solution are combined in a cartridge and electrochemically detected by the device. Following PCR, the amplified DNA was digested and mixed with signal probes that were ferrocene-labelled and specific to the various subtypes. The amplified sample and signal buffer mixture was then loaded into a cartridge comprising single-stranded oligonucleotide capture probes linked to gold-plated electrodes. Subsequent reactions occurred when the cartridge was put into the XT-8 system. the single-stranded oligonucleotide bound to the appropriate signal probe and subsequently to the capture probe's complementary bases. The voltammetry, which produces distinct electric signals from the ferrocene-labelled signal probe, detects the presence of each target. This assay is suitable for HCV genotyping in a clinical setting for a targeted therapy due to its specificity, accuracy, and precision (72). The detection limit was rather low, less than 175 IU/mL for all subtypes except 6ab (72).

An isothermal amplification technology known as transcription-mediated amplification (TMA) has been used for the rapid amplification of target RNA or DNA. This assay has high sensitivity due to its low detection limits compared with the PCR technique (47). TMA assay has been successfully developed to identify HCV RNA in patients with sustained virologic response (73). The advantages of TMA include requiring only a low amount of nucleic acid and needing not more than four hours to obtain the results. Additionally, as it is performed in a single-tube reaction, the risk of cross-contamination is low in TMA-based assay (47).

CONCLUSION

Management and treatment of hepatitis is a challenge due to the high number of patients with undiagnosed HCV infection. A proper selection of drugs based on the infecting HCV genotypes/subtypes is crucial for the successful management of hepatitis C. Therefore, determining the HCV genotype in clinical samples is crucial for successful viral clearance. Nucleic acid-based detection methods remain a gold standard for diagnosing HCV infection and genotyping. Nonetheless, this assay is expensive and requires expertise, sophisticated facilities, and tools. Hence, there is an immediate need to have a simple, cost-effective, rapid and sensitive assay for HCV detection and genotyping. Simplifying the existing assays while significantly improving sensitivity and specificity would improve worldwide hepatitis C healthcare systems.

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