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ADVANCES IN MOLECULAR VIROLOGY AND DIAGNOSTICS OF HEPATITIS C VIRUS

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Abstract

The first and foremost step in the management of any infectious disease, viral or bacterial, is identifying the causative agent behind the disease, followed by determining the extent of incursion attained by it, so as to initiate the specific therapy against and control the exaggerated and self-damaging host reaction. The same applies to the Hepatitis C virus (HCV) infection which is still a global health burden. HCV infection is found worldwide with 71 million people affected globally exhibiting a greater risk of developing advanced-stage liver diseases like fibrosis, cirrhosis and hepatic carcinomas. However, this complex disease state is attained if only the infection is considerably prolonged and left untreated or mistreated. Thus, providing a means to intervene and stop its course, in favour of which an early and accurate diagnosis is required. The timely and correct diagnosis also helps to contain the spread of HCV infection. The basic assays prompting the development of diagnostics are the Enzyme-Linked Immunosorbent (ELISA) and Polymerase Chain Reaction (PCR). On the other hand, recent techniques like microarrays and next-generation sequencing have taken the field of diagnostics to a new phase. Here we present a compilation of all the approaches, recent and older, used in laboratory diagnosis of HCV infection. This review shall help the researchers decide the best method for HCV detection per their requirements and availability of resources.

INTRODUCTION

Hepatitis is a medical condition that arises due to inflammation in liver and it is caused either by infectious or non-infectious agents [1]. The infectious agents posing a threat are viruses, bacteria, fungi, and parasitic organisms whereas, the non-infectious agents include the use of alcohol, drugs, autoimmune and metabolic disorders. Autoimmune hepatitis is a condition that arise as a result of the action of body's own immune system [2]. Hepatitis can be self-limiting or it can advance into chronic conditions leading to Fibrosis, Cirrhosis, or Hepatocellular carcinoma (HCC) [3]. The hepatitis caused by a viral infection usually leads to chronic disease conditions. There are mainly five types of hepatotropic viruses, which are types A, B, C, D and E, and are of significant concern due to the overwhelming incidence of illness and death they cause. These five types possess the ability for causing outbreaks and epidemics [4]. Types B and C particularly, can give rise to chronic illness in millions of people and in conjunction, liver cirrhosis (LC) and HCC are most frequently caused by them [5]. Both are transmitted through parenteral routes especially via blood transfusions, invasive medical procedures, and from mother to infant. To prevent the Hepatitis B Virus (HBV) infection, safe and effective vaccines are available [6]. But no vaccine is available for HCV although trials are going on [7]. In contrast, Hepatitis D Virus (HDV) infections are commonly reported in people already infected with HBV. The co-

infection of both HDV and HBV further worsen the disease and have a fatal outcome [8]. Hepatitis B vaccines have been found to be effective against infections from HDV as well [9].

EPIDEMIOLOGY

The HCV infection is not uniformly distributed in the world and varies with respect to its occurrence and intensity. Liver disease caused by HCV could be both acute or chronic and it could lead to liver cancer as well. About 71 million people are suffering with chronic HCV infection with Eastern Mediterranean and Europe being the most affected regions. Countries like Kyrgyzstan, Kazakhstan, Turkmenistan, Tajikistan, and Uzbekistan from central Asia are perceived to have one of the highest HCV prevalence levels worldwide [10, 11]. In addition to central Asian countries, China, Pakistan, Nigeria, Egypt, India, and Russia is heavily affected by HCV and accounts for more than 50% of all infections in the world [12]. Within the general population, the incidence of HCV ranges between 0.5 to 6.5%. The prevalence of HCV in western countries and Australia ranges between 0.5 to 1.5% and south-east Asian countries and eastern Mediterranean regions the prevalence is about 2.3% [13]. In China it is 3.2%, in India it is 0.9%, in Indonesia 2.2% and in Pakistan it is 6.5% [12]. Different geographical regions have variable distribution of genotypes of HCV [14]. The most widespread genotype is genotype 1, which accounts for 46% of all infections globally [15]. In regions like South Asia, Australia, and some European countries, the second most common genotype is genotype 3 accounting for up to 30% infections globally [11].

HCV GENOME

HCV is a type of ribonucleic acid (RNA) virus which are enveloped, spherical, positive sense, single stranded (ss), belonging to *Flaviviridae* family and genus *Hepacivirus* having a diameter of about 50 nm [16]. The total length of viral RNA genomic sequence is approximately 9.6kb consisting of one open reading frame (ORF), 5' and 3' untranslated regions (UTRs) [17]. The genome of HCV has a conserved sequence at 5' untranslated region (5' UTR), which has proved to be helpful in evolutionary studies and genotyping [18]. At the 5' UTR, there is a presence of an internal ribosome entry site (IRES) that helps in translation initiation. The IRES covers most of the 5' UTR region and 24-40 nucleotides of core-coding region, spanning about 340 nucleotides. This region of HCV genome is highly prone to mutation and cause a range of effects on IRES activity, particularly the mutations within subdomain IIId of IRES hinder translation [19]. The genome encodes a polyprotein consisting of ten different proteins categorized into structural proteins namely Core (C), E1, E2 and non-structural proteins namely P7, NS2, NS3, NS4A, NS4B, NS5A and NS5B [20]. The major element of nucleocapsid is the core protein (21 kDa) which is made up of 191 amino acids. Apart from structural role, core protein regulates gene transcription, apoptosis, cell division and interference metabolism

bringing about oxidative stress and liver steatosis ultimately leading to carcinoma. It also interacts with mitochondria and results in increased ROS production in mitochondria and a reduction in threshold for Ca^{2+} and ROS-induced permeability transition. An important property of HCV infection is the mitochondrial dysfunction [21]. Cell entry is mainly brought about by two glycosylated HCV envelope proteins E1 and E2. The process of cell entry starts with the interaction of envelope glycoprotein with the protein on surface of target cell mediated by a number of host receptors and co-receptors. All these make a key contribution for the entry of HCV in cell. The envelope protein E1 does not directly interact with host receptor but instead depends upon E2 which directly binds with receptors. E1 mainly assists E2 by maintaining the E2 in functional conformation. It has also been reported that E2 alone cannot communicate with CLDN1 whereas E1E2 can [22]. . Moreover, binding of E1E2 to receptors SR-BI and CD81 was found to be modulated by the interaction between E1 and E2 proteins [24]. Assembly of virus and ion channel is aided by protein P7 [23]. The HCV P7 is a 63 amino acid long integral membrane protein whose gene is present between the junction of structural and non-structural genic regions. The maturation of P7 protein via cleavage from the polyprotein results in an E2-p7-NS2 precursor. *In vitro*, P7 has shown to conduct ions artificial membrane in cation selective manner [24]. The NS3 serine protease and NS5B play a major role in replication of virus and are targeted for development of antiviral drug [25]. The 5BSL3.1 and 5BSL3.3 domains of HCV genome act as translation managers [26].

VIRAL QUASI-SPECIES

Viral quasi-species of HCV are the population of virus having variant genome [27]. The genetic instability of HCV is because of the presence of RNA-dependent RNA polymerase NS5B. This polymerase has a very low fidelity due to absence of proofreading activity, particularly for guanine: uridine mismatches (10^{-3} incidences of error/site) [28]. This results in the genetic diversity and generation of quasi-species which was first recognized in the hypervariable region (HVR) of E2. The N-terminal of E2 protein contains HVR-1 [29]. The HVR-2 is positioned slightly downstream of HVR-1. A study conducted on HCV patients revealed that the acute phase has an elevated rate of amino acid substitution per site than the chronic phase [30]. The C-terminal of NS5A contains interferon (IFN)-sensitivity-determining region (ISDR) which is responsible for amino acid substitution. Mutations in the ISDR region of NS5A gene interferes with IFN therapy response in patients infected with HCV-1b [31]. Although drug resistant HCV mutants have arisen due to treatment with direct-acting antiviral agents (DAAs), the newly approved DAAs exhibit

better activity all across the genome, with a greater hinderance to viral resistance [32].

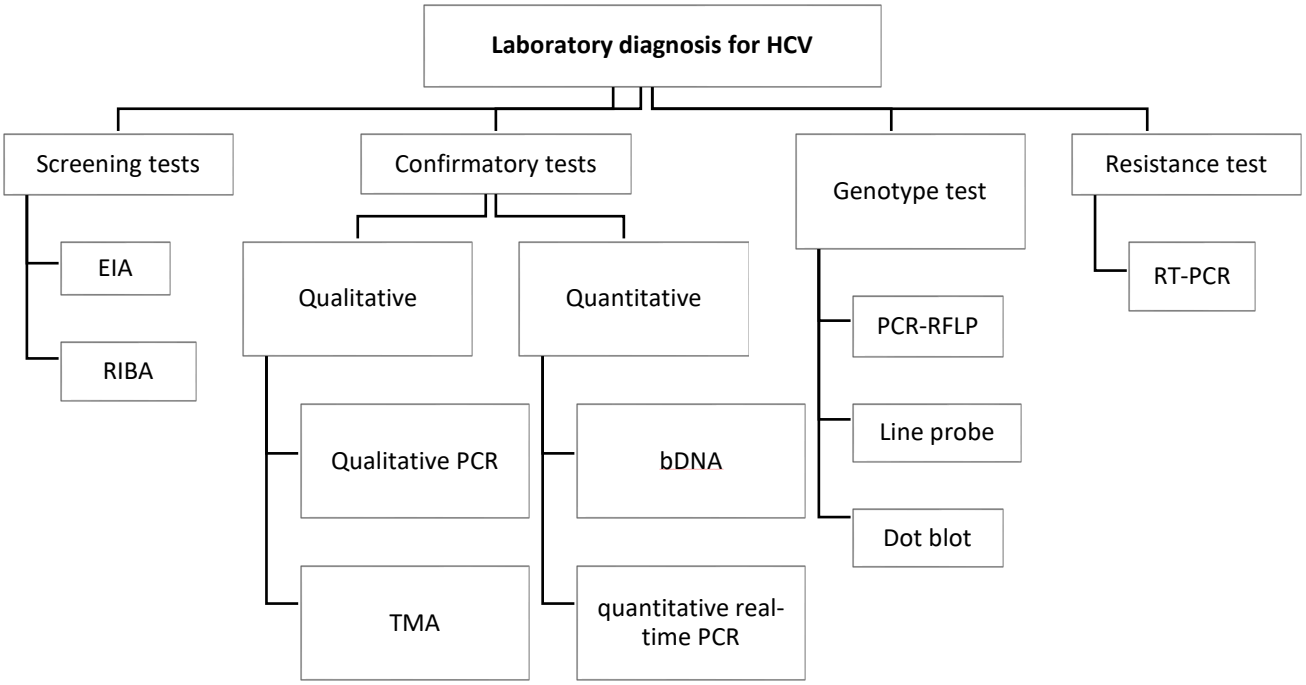
GENOTYPES AND THEIR DISTRIBUTION

HCV genotypes stand for the genetic variations that occur in the HCV genome [33]. There are seven recognized genotypes of HCV strains which are classified based on the phylogenetic and sequence analysis of the genome. The difference in genotypes is due to alterations at 30-35% of sequence regions [15]. HCV comprises of 7 genotypes and 67 subtypes [34]. Subtypes 1a, 1b, 2a and 3a account for a considerable share of HCV infection in developed countries [11]. Genotype 1 is distributed globally, subtype 1a or 1b have been reported to have a prevalence of 60 to 70 % in United States. Genotype 2 is predominantly found in Central and West Africa, genotype 3 in South Asia, genotype 4 in Central Africa and Middle East, genotype 5 in Southern Africa, genotype 6 in Southeast Asia whereas genotype 7 is reported in Central Africa (Congo) [35]. Various studies conducted in India have revealed that in Northern, Eastern, and Western India, genotype 3 is generally found [36]. whereas genotype 1 has a high occurrence in South India [37]. In a retrospective analysis from Hyderabad, India, among the patients with chronic HCV infection from Hepatology Department of the Asian Institute of Gastroenterology from January 2009 to December 2015, the distribution of genotype in relation to viral load is studied. It has also been found that the HCV genotypic distribution pattern changes from genotype 3 to 1 in a span of seven years. According to other national studies, HCV genotypes 3 and 1 are detected in Jammu region of Jammu and Kashmir (J&K) state [38]. The predominance of genotype 3 has also been reported in several studies from Pakistan and Southern Asia [39].

LABORATORY DIAGNOSIS AND MOLECULAR ANALYSIS

The contemporary assays for diagnosis and management of HCV infection include serological tests, molecular tests, and genotyping techniques, as represented in flow chart (Figure 1).

The diagnosis of HCV positivity is done by either directly detecting the viral RNA or core antigen by PCR or immunoassay, respectively; or indirectly detecting the anti-HCV antibodies from the blood sample [40]. The HCV antibodies and core antigen detection in blood of the patient forms the serological testing, whereas, the detection and quantification of HCV RNA forms the molecular testing.



*EIA- Enzyme Immuno Assay, RIBA- Recombinant immunoblot assays, PCR- Polymerase Chain Reaction, TMA- Transcription Mediated Amplification, bDNA- Branched DNA, RFLP- Restriction Fragment Length Polymorphism.

Figure 1. Different phases in diagnosis of HCV

SEROLOGY ASSAYS

The serological assays are aimed at screening the blood samples for the presence of anti-HCV antibodies. The anti-HCV antibodies are generated inside the host as a result of the immunological response against the virus. Thus, the presence of antibodies indicates the occurrence of disease.

ENZYME IMMUNOASSAY

The presence of anti-HCV antibody in the blood comprises initial screening achieved by enzyme immunoassays (EIAs). This screening of anti-HCV antibody utilises the use of recombinant viral structural and/or non-structural proteins as the capture antibodies. The first-generation anti-HCV antibody assay used C100-3 peptide which is a fusion protein comprising a portion of the NS3 and NS4 regions of the HCV polyprotein sequence. All these assays successfully detected anti-HCV immunoglobulin G (IgG) in the patient's blood in almost 80% of the posttransfusion hepatitis cases and also able to decrease the incidence of transfusion-linked HCV contagions but the reactivity level and precision was not adequate [41]. On the contrary, recombinant proteins of HCV were used in second generation of immunoassays. These proteins and/or peptides were selected from either core regions (C22), non-structural protein 3 (NS3) regions (C33-C) or NS4 regions (5-1-1 and C100-3). Although second generation of immunoassays were initially successful and reduced the window period by an average of 5 weeks, it has been associated with false positive results [42]. Because of the limitations of the aforementioned immunoassays, third generation of anti-HCV antibody assays were later developed which utilized broader range of peptides from HCV genomes (core, NS3, NS4, NS5) which made them more sensitive, precise (99%) and anti-HCV antibody detection time was reduced to 10 weeks after initial introduction. In recent times fourth generation of immunoassays are developed which uses novel combinations of modified core, NS3, NS4 & NS5 peptides that could identify all the subtypes of HCV with a greater degree of precision. In the second and third generation assays, the window period is reduced by an average of 5 weeks as compared to the first generation. The sensitivity/specificity of third-generation EIAs is approximately 99% [43]. Despite the high sensitivity and specificity, the EIA tests sometimes give false-positive and false-negative results. The false-negative test result may be seen in a situation where the test is performed during the window period of infection, before any seroconversion has occurred. Additionally, it may also be observed in immunocompromised and haemodialysis patients. In such cases, the HCV RNA test would be highly beneficial. On the other hand, a false-positive HCV Ab result, can be seen in the case of immunologic disorders, such as lupus or rheumatoid arthritis or due to cross-reactivity with other viral antigens which again necessitates the HCV RNA testing. Recombinant immunoblot assays (RIBA) can be utilized to discriminate between true-positive and false-positive result. RIBA is more specific and utilizes a recombinant HCV antigen fixed to a solid substrate. The anti-HCV antibodies are captured on the solid support by submerging it in the patient's serum or plasma. The bound antibodies are further captured by Anti-human IgG antibodies conjugated to the enzyme alkaline phosphatase which produces a colour change on reaction with substrate [44].

The serological screening of anti-HCV anti-body in the blood cannot differentiate between past and present infection. The anti-HCV anti-bodies are generated inside the individual in response to the intruding virus and it remain detectable afterwards. It is present in the blood during active infection as well as resolved infection. Therefore, in a healthcare setup, the anti-body testing is usually accompanied by HCV RNA test to confirm the active infection [45]. However, the testing of HCV RNA is complex and costly. It requires skilled staffs, sophisticated instruments and costly chemicals. In this situation the HCV core antigen testing forms a better alternative. It not only delineates the active HCV infection but also an indicative of HCV RNA replication providing a basis for anti-viral treatment. Moreover, the HCV core antigen testing can be performed using same instruments as that of anti-HCV anti-body [46, 47]. Combining both the test is convenient and economical when both sousveillance and incidence of active infection is to be enquired. Despite, the confirmatory nucleic acid testing (NAT) is still the method of choice in majority of health care setups to confirm active viremia [48, 49] which may be due to the shortfalls in the sensitivity of HCV core antigen test [50]. Additionally, for research purposes one may need to isolate the HCV RNA in favour of which the PCR mediated detection and quantitation becomes more relevant. It is also known that the presence of HCV RNA in the serum is the earliest detectable marker of acute infection that appears before the anti-HCV antibody [51].

CONFIRMATORY TESTS

The serological HCV screening is usually followed by a confirmatory test where the presence of HCV RNA is detected and quantified. In addition to backing and cross verifying the serological test report, the confirmatory tests are helpful in elucidating the chronicity of HCV infection which will support in taking important treatment decisions.

HCV RNA DETECTION AND QUANTITATION

In recent times molecular detection and quantification of HCV RNA has become much more relevant than any other methods available. Molecular techniques facilitate various aspects of the HCV infections and its treatments such as rapid assessments of the present condition of the disease,

initiating the treatment procedure and others. Molecular techniques include nucleic acid tests which can identify the occurrence of HCV RNA directly and have greater sensitivity and specificity. Such molecular techniques are categorized into qualitative tests and quantitative tests. The former includes transcription-mediated amplification (TMA) and qualitative polymerase chain reaction (PCR), while the latter includes branched-chain DNA (bDNA) amplification, reverse transcription PCR and real time PCR [52, 53].

Qualitative HCV RNA Detection Assay

The most widely utilized qualitative technique to detect HCV RNA is reverse transcription PCR which mostly consist of 3 widely used tests including two kits, AMPLICOR 2.0 and Ampliscreen 2.0 and third is a reference testing known as UltraQual. The qualitative PCR for HCV RNA detection involves five major steps i.e., RNA extraction from sample, reverse transcription of target RNA to generate complementary DNA (cDNA), PCR amplification of cDNA using HCV specific primer, hybridization of amplified products to target specific probes and colorimetric detection of probe bound product. The extraction of RNA from serum/plasma is attained manually and HCV internal control is also introduced into the sample to monitor extraction and amplification process. The reverse transcription produces a cDNA copy of the HCV target and the HCV Internal Control. From this cDNA copy a double-stranded DNA of the HCV target and HCV Internal Control is produced which are then denatured to form single-stranded DNA. The oligonucleotide probe coated magnetic particles are used to capture the biotinylated HCV target and HCV Internal Control amplicon which is detected by a colour change. This test offers three outcomes i.e., HCV RNA detected, HCV RNA not detected, and gray intermediate zone. The samples falling under gray zone (absorbance at 660 nm range of 0.15 to 1.0) needs to be repeated.[54, 55].

The sensitivity of qualitative HCV RNA assay is >96% and specificities are >99%. Their limit of detection is also very low i.e., < fifty IU/mL.

The TMA test, on the other hand is even more sensitive (>98%) [46]. It involves transcription-mediated amplification of regions within the HCV 5' untranslated region. The target HCV RNA is captured on oligonucleotide coated magnetic beads. The coated oligonucleotide is complementary to the 5’ untranslated region of HCV genome and thus hybridizes with it. An internal control RNA sample is also added to screen the target capture and amplification. The amplification of target RNA is achieved by addition of primers, reverse transcriptase, and T7 RNA polymerase. It is an autocatalytic and isothermal process that involves the amplification of RNA by reverse transcriptase followed by generation of transcript by RNA polymerase which is detected by hybridization with added probe via a chemiluminescent signal read as relative light unit. Additionally, a signal-to-cutoff ratio is also used to report the data. The sample is considered reactive when signal-to-cutoff is more than 1 and non-reactive when it is less than 1. There is no intermediate outcome [56, 57]. The TMA is more sensitive than qualitative PCR. Through TMA, the HCV RNA was detected in patients who had no detectable RNA through PCR [58].

Quantitative HCV RNA Detection Assay

For the detection of HCV RNA quantitatively three major techniques are available namely, bDNA method, quantitative reverse transcription polymerase chain reaction (RT PCR) and real-time PCR. In bDNA method, as opposed to the reverse transcription–PCR, the detection signal is amplified in place of target RNA. The HCV RNA is extracted by chemical lysis and captured directly by hybridization with oligonucleotide capture probe present in pre-coated wells. It is followed by a sequential addition of target probe to wells for chemiluminescent signal amplification which results in a sandwich structure. The capture probe and target probes bind to the 5’ untranslated region (5, UTR) and Core of the HCV RNA. The target probe further binds to preamplifier probes which in turn bind to amplifier probes, to build a branched DNA complex. Finally, the alkaline phosphatase labelled probes are added which hybridize to the DNA complex. On incubation with a chemiluminescent substrate, a strong signal is detected [59].

On contrary to the signal amplification assay, the target amplification assay has gained more popularity. It can increase the sensitivity and a large number of viral genome copies can be produced. The classical RT PCR and real -time polymerase chain reaction are the two target amplification methods used for HCV RNA detection. Both methods involve a reverse transcription of the HCV RNA into cDNA followed by synthesis of multiple copies of double-stranded DNA from the cDNA matrix and finally the detection of amplification products by colorimetry. The detection step differentiates between the two said assays. In the real-time PCR the detection of amplification is done simultaneously during the exponential phase of the amplification step. The amount of amplification achieved is proportional to the initial quantity of HCV present [60, 61].

Real-time PCR methods for HCV RNA quantification is critically important. Its sensitivity is comparable with that of qualitative tests, which is achieved by using TaqMan technology. With respect to the accuracy of this test, it is extended over 10 IU/mL to 10^8 IU/mL. The real-time PCR can be used for both quantitative and qualitative testing, being faster and more cost- effective. Despite, these tests have been restricted to the inhouse testing, losing to the higher sensitivities of commercially available qualitative assays [62–64].

ASSESSMENT OF VIRAL LOAD

The number of viral particles present in an organism comprises its viral load. In order to check the viral load, the quantitative HCV RNA test is administered that reads the number of viral particles in international units per millilitre. A "high" viral load is usually assigned to a value which is more than $8*10^5$ IU/ml and a "low" viral load is assigned to less than $8*10^5$ IU/ml respectively [65]. The knowledge of the patient's viral load is useful in deciding the suitable treatment.

HCV GENOTYPE DETERMINATION

After the confirmatory test is achieved, the researchers and doctors may be interested in knowing the specific genotype and subtype of virus present in the sample which will help in electing type specific therapies and prediction of treatment response. Additionally, genotype determination is important for the epidemiological studies where the prevalence and occurrence of genotypes need to be reported. There are about 7 genotypes and 67 subtypes of HCV. Most of the genotype testing relies upon PCR amplification of HCV RNA followed by type specific assays like restriction digestion and line probe reverse hybridization, or sequence analysis, alignments, and phylogenetic analysis. For the PCR amplification of HCV genome, the 5' UTR is usually targeted as it is the most conserved region in the genome of HCV. Additionally, the core, E1, NS4, and NS5 viral proteins are also targeted for genotyping assays. However, sequencing, alignment and phylogenetic analysis as a method for HCV genotyping are utilized in research laboratories more than in clinical laboratories as they are time consuming and require costly equipment. The clinical laboratories count on to the commercially available kits using PCR amplification and hybridization with type-specific probes. Nevertheless, direct sequencing can state the novel variability in viral genome and the occurrence of quasi-species can also be determined.

NEXT GENERATION BASED APPROACHES

The available tests for assigning HCV genotype based on the 5' UTR region may cause wrong classification of HCV genotypes due to spurious mutations. About 4 out of 50, 1a genotype isolates have been misclassified as 2a genotype, via commonly used line probe assay, severely impacting the treatment decisions and outcomes [66]. In view of such errors, whole genome sequencing becomes more desirable. To attain the whole-genome sequencing of HCV two approaches are adopted i.e., next generation sequencing (NGS) and population sequencing. The population sanger sequencing lags in its applicability in routine practice as it is too labour intensive and time-consuming. The NGS, however, is implemented in several ways from preamplifications with specific primers to direct sequencing of RNA extracts and generation of HCV replicon transcripts [65, 67]. These strategies also have some important limitations with regard to the cost involved and technicality. They are also very laborious and, in some instances, give false results too [68]. Whereas, some of the studies comparing the ability of these approaches have revealed a surprisingly accurate results from more than 90% of samples with different genotypes or subtypes of HCV with different viral loads [69].

However, the standard NGS technologies suffer one big drawback of short read lengths that impede the recognition of the physical linkages of individual single nucleotide variants. A third-generation sequencing technology seems to overcome this drawback and it has been successfully piloted for HCV. The PacBio RS II platform from Pacific Biosciences (Menlo Park, CA), provided very long, single-molecule reads so that the HCV molecule can be continuously sequenced as a single contiguous pass [68]. Using the same platform, Bull *et al.* analysed the near-full-length HCV quasi-species with a single read amplicon [70]. The Bergfors *et al.*, on the other hand, detected low levels of NS5A-resistant variants of HCV in patients infected with genotypes 1a and 3a [71]. Peng *et al.* enhanced the sensitivity of NGS by HCV probe-based capture sequencing [72]. . This could be a potential tool for detecting HCV in early stages of infection. Phylogenetic approaches for whole genome sequencing and partial gene sequencing by making use of wet laboratory methods together with bioinformatics tools have turned out to be a reliable technique for generating high-resolution data by reducing the error rates of NGS [73–75]. A straightforward and dependable approach for genotyping the subtypes of HCV is by use of Abbott's genotype plus RUO combined with Abbott Real-Time genotype II [76]. Sentosa SQ HCV Genotyping Assay which is a new technique based on deep sequencing, could be a preferred method for detection of HCV genotypes [77].

CONCLUSION

Diagnosis of HCV at an early stage is utmost important for its eradication and management, in favour of which various diagnostic techniques are available and each one carries its own significance. The serological testing establishes primary diagnosis based on which the next diagnostic practise may be carried out. Usually, after a positive serological test, HCV RNA detection is carried out to check the viral load. In some cases, the genotyping analysis is also directed to check the genotype and subtype of virus. Both the viral load and genotype information is crucial in managing the disease burden. The HCV core antigen testing has the potential to replace the NAT in viral diagnosis, however, in a research operation the extraction and PCR amplification of HCV RNA forms an irreplaceable technique. The qualitative confirmatory testing is dwindled in a diagnostic set-up but can be opted in a scenario where an earliest detection is required even before the appearance of ani-HCV antibodies in the blood. If one can overlook the cost and technicality

associated with NGS based approaches, it can form a new benchmark in the field of diagnosis as well as research.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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