Laboratory and Molecular Diagnosis of Hepatitis C and Resistance Testing

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2.1 Introduction

Hepatitis C infection is a global epidemic. It is currently one of the major public health problems with a significant economic burden. According to World Health Organization (WHO) the most commonly affected regions are Eastern Mediterranean and Europe with a prevalence of 2.3% and 1.5%, respectively. In other regions of the WHO, the prevalence of infection ranges from 0.5 to 1.0%. It has been estimated that globally there are 23.7 new HCV infections per 100,000 people [1]. According to the CDC, estimated prevalence of chronic hepatitis C infection in the United States is 3.5 million [2].

Primary mode of transmission of hepatitis C infection includes intravenous drug use. Other common causes include exposure to contaminated blood and blood products in the healthcare setting such as transfusion before 1992, hemodialysis, occupational exposure to contaminated needles, and patient-to-patient transmission of HCV due to poor infection control policies. Vertical transmission from infected mother to infant at the time of delivery, tattoos from unlicensed parlors, and men who have sex with men (MSM) are relatively uncommon modes of hepatitis C transmission [3].

The natural history of the chronic hepatitis C infection is not completely defined. Acute hepatitis C infection is commonly unrecognized. Most of the patients with acute hepatitis C infection are asymptomatic or may have mild nonspecific flu-like symptoms such as fatigue, malaise, nausea, or jaundice. It has been shown from the

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available literature that approximately 18–34% of the patients are able to spontaneously clear infection. Several genetic factors such as the presence of DQB1*0301 allele of the MHC class II (major histocompatibility complex) and IL28B inheritance are found to be associated with spontaneous resolution of acute hepatitis C infection. Hepatitis C is a slowly progressing disease and approximately 80% of the acute HCV patients develop chronic hepatitis C infection. Progression from acute to chronic infection is usually subclinical. Persistent inflammation of the hepatocytes leads to the development of liver cirrhosis in about 15–20% of the patients with annual 1–4% risk of development of hepatocellular carcinoma and a 3–6% annual risk of hepatic decompensation. Decompensated liver cirrhosis and hepatocellular cancer due to chronic hepatitis C are the major causes of liver transplantation in the developed countries [4, 5].

Serological immunoassays and confirmatory virological tests are needed to diagnose, manage hepatitis C infection, and assess response to treatment and prevention of transmission. Rapid, inexpensive, sensitive, and specific tests are approved by FDA and include HCV antibody test, HCV viral load, genotyping, and less commonly liver biopsy. This chapter focuses on the laboratory molecular diagnosis of hepatitis C infection and resistance testing.

2.2 Indications for HCV Testing and Linkage to Care

According to World Health Organization (WHO), American Association for the Study of Liver Disease (AASLD), and European Association for the Study of the Liver (EASL) guidelines one-time screening test is needed in the following population [6–8].

2.2.1 Recommendations for One-Time HCV Testing

Persons born between 1945 and 1965, without prior ascertainment of risk (Grade 1b).

Other persons should be screened for risk factors for HCV infection, and one-time testing should be performed for all persons with behaviors, exposures, and conditions associated with an increased risk of HCV infection (Grade 1b).

1. *Risk behaviors*: Such as intravenous drug use and intranasal drug use.
2. *Risk exposures*: Which include people on long-term hemodialysis, percutaneous/parenteral exposures in an unregulated setting, people with occupation exposure in healthcare setting for example needlestick injury, children born to HCV-infected mother, recipient of blood transfusion before 1992 or clotting factor concentrate before 1987, solid-organ recipient, and people who were ever incarcerated.

3. *Other consideration includes*: People who are HIV positive, sexually active people about to start pre-exposure prophylaxis (PreP) for HIV, unexplained cirrhosis, or elevated liver enzymes in asymptomatic patients and solid-organ donors.

2.2.2 Recommendation for HCV Testing in Those with Ongoing Risk Factors (Grade IIa, C)

1. Periodic testing should be offered to other persons with ongoing risk factors for exposure to HCV. Annual HCV testing is recommended for persons who inject drugs and for HIV-seropositive men who have unprotected sex with men.

2.3 Laboratory and Molecular Diagnosis of Hepatitis C and Resistance Testing

Screening test: This includes serological test for the HCV antibody (Ab).

Confirmatory test: Positive anti-HCV test requires confirmation with the presence of HCV RNA (quantitative and qualitative test). Initial HCV-RNA testing is also recommended among people at risk of reinfection after previous spontaneous or treatment-related viral clearance, because an anti-HCV test is expected to be positive (Grade I, C) and prior to the initiation of antiviral therapy to document the baseline level of viremia (i.e., baseline viral load) (Grade I, A) [6].

Genotype: After confirmatory testing, specific genotype of the HCV and its subtype should be determined using genotype test.

Drug resistance: Mutations of some proteins in HCV can allow the virus to have resistance to direct-acting antivirals (DAAs), commonly referred to as resistance-associated variants (RAVs) or resistance-associated polymorphisms (RAPs).

2.4 Recommended Testing Sequence

The Centers for Disease Control and Prevention published the testing sequence for diagnosis of chronic HCV infection in May 2013 (Fig. 2.1). According to the new recommended sequence, the initial screening test for hepatitis C infection is HCV antibody (using either a rapid or laboratory-conducted assay), followed by HCV RNA testing for all positive HCV antibody tests. No further diagnostic tests are necessary if a person has a negative screening HCV antibody test and is considered as noninfected. However, high-risk individuals such as acute HCV infection, chronic hemodialysis, or an immunocompromised host need further evaluation to rule out false-negative result. On the other hand, individuals with a positive HCV antibody test and HCV RNA are considered to have current (active) HCV infection. An individual who has a positive HCV antibody test and a negative HCV RNA assay is considered not infected; further testing with different immunoassay is required to differentiate a false-positive result from infection [9].

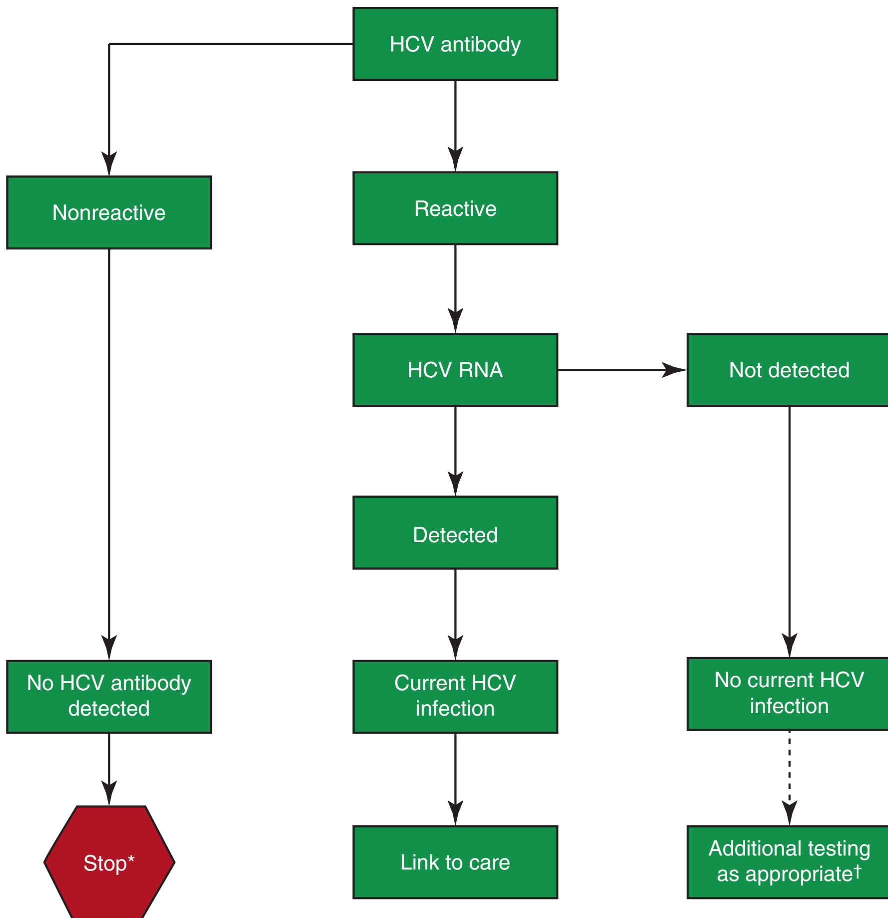

Fig. 2.1 HCV testing sequence for identifying current HCV infection (adopted from 2013 CDC guideline on the testing sequence of hepatitis C infection diagnosis)

*For persons who might have been exposed to HCV within the past 6 months, testing for HCV RNA or followup testing for HCV antibody should be performed. For persons who are immunocompromised, testing for HCV RNA should be performed
†To differentiate past, resolved HCV infection from biologic false positivity for HCV antibody, testing with another HCV-antibody assay can be considered. Repeat HCV-RNA testing if the person tested is suspected to have had HCV exposure within the past 6 months or has clinical evidence of HCV disease, or if there is concern regarding the handling or storage of the test specimen

2.4.1 HCV Genome

First identified in 1989, hepatitis C virus is a single-stranded RNA, small (approximately 55–65 nm) in size, and an enveloped virus that belongs to the Hepacivirus genus in the family Flaviviridae. The HCV genome has structural (S) and nonstructural (NS) regions that encode 3011- to 3033-amino-acid polypeptides that translate

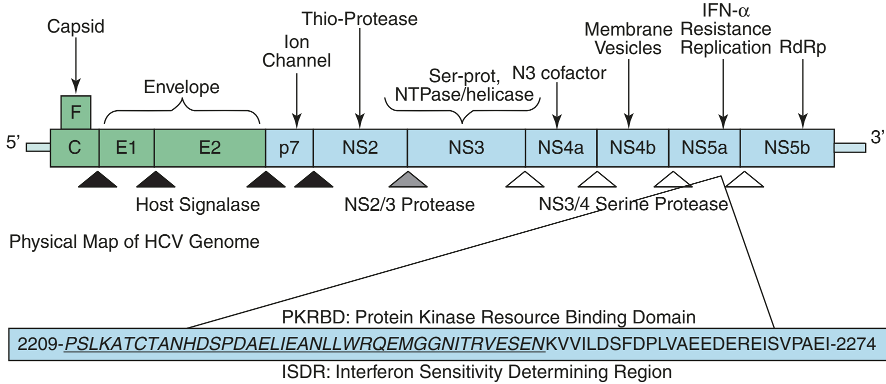

Fig. 2.2 Hepatitis C virus genomic map. The proteins are arranged as N terminal-core-envelope (E1)–E2–p7-nonstructural protein 2 (NS2)–NS3–NS4A–NS4B–NS5A–NS5B–C terminal

into ten structural and nonstructural proteins. The nonstructural (NS) proteins are classified as protease (NS2, NS3, and NS4A), RNA-dependent RNA polymerase (NS5B), and helicase (NS3). On the other hand, the structural region contains two envelope proteins (E1 and E2) and the core protein [10]. Genomic structure of the hepatitis C virus is illustrated in Fig. 2.2.

As shown in the figure the untranslated region (UTR) of hepatitis C virus mRNA consists of 5′ and 3′ ends which are important to the translation and replication of the viral RNA. At the 5′ untranslated region ribosome binds through the ribosome-binding site (IRES—internal ribosome entry site). HCV genome and protein form the basis of laboratory and molecular diagnostic tests for HCV and play a vital role in the emergence of resistant strain.

2.4.2 Hepatitis C Proteins

The hepatitis C genome can give rise to different proteins that are required for replication and these include [11–19]:

1. Structural proteins
   (a) Core protein: highly conserved and is part of the viral nucleocapsid. It consists of three domains and impacts functions of the host cell such as metabolism of lipids, transcription of genes, signaling pathways, and apoptosis.
   (b) Envelope proteins: are two types of highly glycosylated proteins called E1 and E2 and are key mediators in cell entry.
   (c) P7 protein: is a membrane-spanning polypeptide that consists of 63 amino acids and is found within the endoplasmic reticulum. It is required for assembly of the virus particle assembly and also mediates the release of these infectious particles by a process that is noted to be genotype specific.

2. Nonstructural proteins
   (a) NS2: is a transmembrane protein that is required for completion of the cycle of viral replication both in vivo and in vitro.
   (b) NS3: is a 63 kDa protein that serves numerous functions with its N-terminal having serine protease activity and the C-terminal carrying the NTPase/helicase activity for RNA replication.
   (c) NS4A: essentially serves as a cofactor for the NS3 protein.
   (d) NS4B: plays an important role of helping other viral proteins localize to a site in the endoplasmic reticulum referred to as the membranous web (formed by structural change in the endoplasmic reticulum induced by the NS4B protein itself). This site is where the replication complex is thought to form as all the viral proteins confine here.
   (e) NS5A: a hydrophilic phosphoprotein that is a crucial mediator of viral replication, affects signaling pathways and response to interferon.
   (f) NS5B: is a 65 kDa protein that is an RNA-dependent RNA polymerase and is involved in the formation of a new RNA genome.

2.5 Laboratory Diagnosis of HCV

2.5.1 Serological Immunoassays for HCV Detection

Serological assays for HCV detection are the preliminary tests to be performed when HCV infection is suspected and in select populations. Serological test aims at detecting anti-HCV antibodies after exposure to hepatitis C virus. A positive anti-HCV test indicates HCV infection at some point in time. However, it does not differentiate between current and resolved HCV infection and further confirmatory tests are required to make diagnosis of HCV and to guide appropriate treatment [20]. Below is the description of FDA-approved immunoassays.

2.5.1.1 Immunoassays

Enzyme Immunoassay (EIA)

Enzyme immunoassay was introduced in the 1970s and is among the first FDA-approved test which uses HCV recombinant antigens to identify the presence of anti-HCV antibodies. Enzyme immunoassay has been developed to detect antibodies to proteins expressed by structural (HC-34) and nonstructural (HC-31, c100-3) regions on the HCV genome. Solid-phase EIAs utilize immobilization of antigens or antibodies coated on a surface using solid support. After this step, the detection of antibodies or antigen is added, to form an antigen-antibody complex. The detection antibody can be covalently linked to an enzyme, or can itself be detected by a secondary antibody that is linked to an enzyme through bio-conjugation. The plate is usually washed with a mild detergent solution between each step to remove any antigens or antibodies that are not specifically bound to HCV proteins. The ultimate step during this process is the addition of an acid or an enzymatic substrate to

quantify the amount of antigen or antibody in the sample, which is read with a spectrophotometer [21].

According to available evidence single-antigen enzyme immunoassay for anti-HCV antibody detection is not as sensitive and specific as compared to multiple-antigen immunoassays. Hence, there is development of more sensitive and specific second- and third-generation assays [22].

- *First-generation EIA*: First-generation immunoassay was first developed in 1989 and involves identification of c100-3 epitope from the nonstructural NS4 regions of HCV genome. The major disadvantage of the test is low sensitivity, i.e., approximately 80%, leading to the high number of false negatives in the high-prevalence population. Also, the negative predictive value of the first-generation EIA is as low as 70% in the low-risk population such as in blood donors [23].
- *Second-generation EIA*: Developed in 1992 and included recombinant/synthetic antigens from the core and nonstructural NS3 and NS4 regions of the HCV genome. Second-generation EIAs have proved to be more effective as a screening test in individuals who are at high risk for chronic hepatitis C because of improved sensitivity and specificity. In acute HCV infection, second-generation EIAs can detect HCV antibodies in 20% more patients and 10% more patients with chronic HCV infection, i.e., increased sensitivity from 80% to 92–95% as compared to first generation and detect HCV antibodies 30–90 days earlier. The mean window of seroconversion was reduced from 16 weeks with EIAs 1.0 to 10 weeks with EIAs 2.0. The sensitivity of EIAs 2.0 in a high-prevalence population is approximately 95% (based on HCV RNA detection by PCR) [24].
- *Third-generation EIA*: FDA approved a third-generation EIA (EIA 3.0) in 1996 and currently Abbott HCV EIA (version 2.0; Abbott Laboratories, Abbott Park, IL) is used in the United States. The third-generation EIA detects antibodies that bind to recombinant antigens derived from four viral regions: core, NS3 (nonstructural 3), NS4 (nonstructural 4), and NS5 (nonstructural 5). The sensitivity of third generation of EIA is as high as 97% in a high-prevalence population and the mean time to seroconversion reduced from 10 weeks with second-generation EIAs to 5 weeks. The specificity of the anti-HCV assay (third generation) was found to be 99.5% for blood donor samples and 99.83% in volunteer blood donors and 99.79% in plasmapheresis donations based on an assumed zero prevalence of HCV antibody [25, 26].

Interpretation of the Test [27]

According to the ABBOTT HCV EIA 2.0 criteria the interpretation of the results is as follows:

1. Serum specimen with absorbance values ≥0.005 but less than the cutoff value is considered negative.
2. Specimen results having absorbance values less than 0.005 must be retested using the same product and test method. If the specimen still shows absorbance value <0.005 than the cutoff value, the specimen may be considered negative for anti-HCV antibodies.

3. Specimens with absorbance values greater than or equal to the cutoff value are considered initially positive. Original sample must be retested using the same product and test method. If the retested specimen still shows absorbance values greater than the cutoff value the test is interpreted as positive. However, if the retested specimen shows absorbance value less than cutoff value then the test is interpreted as negative.
4. Positive result, i.e., presence of HCV IgG antibodies in the patient serum, is not able to differentiate between current or past infection and exposures. To confirm the presence of viremia a positive antibody test result should be followed by confirmatory test such as HCV RNA.

ORTHO HCV Version 3.0 ELISA Test System

ORTHO HCV Version 3.0 is an enzyme-linked immunosorbent assay with the main purpose to detect antibody to hepatitis C (anti-HCV) in the human serum, plasma, and cadaveric specimen. Recombinant HCV-encoded antigens used in ORTHO HCV Version 3.0 ELISA Test system are c22-3, c200, and NS5 which cover 60% of the HCV genome. The test has high sensitivity and specificity, i.e., >99% [28, 29].

Principle of the Procedure

ORTHO HCV Version 3.0 ELISA Test System is a three-stage test, i.e., performed in a microwell coated with HCV recombinant antigens (c22-3, c200, and NS5).

- *Stage One*: In this stage human serum, plasma, or cadaveric specimen is diluted and then incubated in the test well for a specified length of time. Antigen-antibody complexes will be formed on the microwell surface if the sample has antibodies reactive to any of these above-mentioned antigens.
- *Stage Two*: During this stage, murine monoclonal antibody conjugated to horseradish peroxidase is added to the microwell which would subsequently bind to the human IgG portion of the antigen-antibody complexes.
- *Stage Three*: In this final stage, *o*-phenylenediamine (OPD) and hydrogen peroxide are added to the microwell. The OPD will be oxidized if antigen-antibody conjugate is present leading to a colored product which is detected by a spectrophotometer.

Interpretation of Results

Results of ORTHO ELISA are interpreted as follows [30]:

1. If the tested specimen is with absorbance values <−0.025, the specimen should be retested in the single microwell. If the retest absorbance value is less than the cutoff value, the specimen should be considered nonreactive.
2. Further testing is not required for specimens with absorbance values less than the cutoff value but greater than or equal to −0.025 and these specimens are considered nonreactive.
3. Specimens with absorbance values greater than the cutoff value are initially considered reactive and should be retested in duplicate before final interpretation. Retested specimen is considered repeatedly reactive, if either or both duplicate determination(s) have absorbance values greater than the cutoff value.

False-Negative and False-Positive HCV Ab Results

False-negative and -positive results with immunoassay are possible despite high sensitivity and specificity and HCV RNA should be ordered to confirm viremia in those situations.

False-Negative HCV Antibody

False-negative enzyme immunoassay should be suspected in the following situations [31]:

Serological Window Period: The average time for "serological window" period which is defined as the period after acute HCV infection but before seroconversion of negative to positive IgG HCV antibodies is 8 weeks in hepatitis C infection. It is the time during which EIA is often reported as false negative. It is appropriate to order HCV RNA if acute infection is suspected to happen within the past 8 weeks.

Immunocompromised patients: For example patients with diagnosis of HIV, recipients of organ transplantation, patients on long-term hemodialysis, patients with mixed cryoglobulinemia, and immunosuppressed patients. In these patients if HCV EIA is negative but there is strong suspicion of infection, HCV RNA testing should be performed.

False-Positive HCV Antibody

False-positive HCV antibody test may occur due to cross-reactivity of EIA HCV polyprotein with other viral antigens or secondary to the presence of certain autoimmune disease such as lupus arthritis or psoriasis [32].

Chemiluminescence Immunoassay (CLIA)

Chemiluminescence immunoassay (CIA) detects anti-HCV antibodies in human serum or plasma specimens by emitting light when a chemical reaction occurs between hepatitis C virus antigen and antibody. Like ELISA, antigens are initially immobilized during solid phase on monoclonal antibody-coated well. This step is followed by the addition of horseradish peroxidase (HRP)-labeled antibody or antigen [33]. After incubation for a shorter period, i.e., 1 h at 37 °C, unbound enzyme-labeled Ab is removed and chemiluminescence reagent added (Fig. 2.3).

The concentration of anti-HCV is expressed as a signal-to-cutoff (S/CO) ratio, and S/CO levels correspond to the antibody concentration. CLIA analyzers are fully automated, commercially available, and widely used, particularly in high-volume clinical settings. They are optimized for efficiency and throughput. Chemiluminescence immunoassays are reliable, reproducible, and technically simple to perform. For the diagnosis of chronic hepatitis C infection, the CLIA has much better sensitivity and specificity as compared to third-generation EIA. However, skilled personnel and suitable lab infrastructure are required to run these analyzers in the low-income countries [34]. The characteristics of the different CLIA analyzers are presented in Table 2.1.

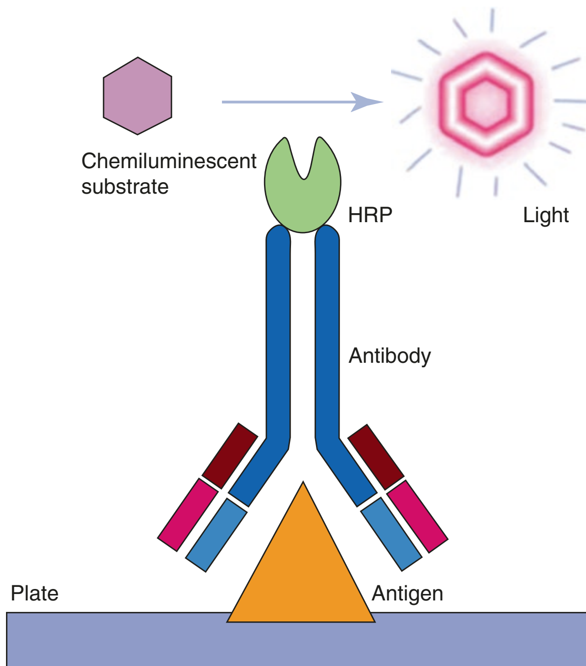

Fig. 2.3 Schematic presentation of CLIA for detection of anti-HCV antibodies

Table 2.1 Comparison of accuracy of commonly used point-of-care rapid diagnostic tests

Chemiluminescence immunoassay (CLIA)	Manufacturer	Method	Sensitivity	Specificity	Signal-to-cutoff ratio
ARCHITECT	Abbott	CMIA	90%	100%	≥5.0
Advia Centaur	Siemens	CIA	100%	99.9%	≥11.0
VITROS immunoanalyzers	Ortho	CIA	100%	99.7%	≥8.0
AxSYM anti-HCV	Abbott	MEIA	99.84%	98.9%	≥10.0

2.5.1.2 Point-of-Care Rapid Immunoassays/Rapid Diagnostic Tests (RDTs)

Though Centers for Disease Control and Prevention (CDC) recommends the use of enzyme immunoassay for the screening and recombinant immunoblot assay or HCV nucleic acid testing for RNA, these tests are expensive, require high-technology laboratories, and have long turnaround times. Rapid immunoassay tests detect HCV antigens from core, NS3, NS4, and NS5 regions of the hepatitis C virus [35]. RDTs are simple to perform, acceptable to patients, and cost-effective tests with an excellent turnaround time [36]. For the abovementioned reasons, this has the potential to substantially improve the access to HCV testing in hard-to-reach rural populations,

in limited resource settings, and can be used in outreach programs such as prison services, and substance use/treatment services. There is substantial evidence to suggest that rapid diagnostic tests improve linkage to care as the test can be performed by well-trained lay providers and hence reduce loss to follow-up [37]. According to the results of the study published by Morano et al., patients with HCV infection who had the rapid test were more likely to be linked to care as compared to those with conventional testing (93.8% compared with 18.2%). Patient and staff preference surveys showed strong support to use the rapid diagnostic tests at point of care [38].

Point-of-care rapid diagnostic tests can be run on the oral fluid, capillary, or venous whole-blood specimens and have high sensitivity (99%) and specificity (100%) in different populations and a wide range of settings based on the findings of the systematic review [39, 40]. An oral fluid-based point-of-care test has several advantages. They are noninvasive and simple to perform and do not require expert staff to carry out the test. However, the sensitivity of the oral RDTs is much lower than blood-based tests possibly due to lower concentration of anti-HCV antibodies in human saliva than blood specimens. According to the study published by Shivkumar et al. POCTs of blood (serum, plasma, or whole blood) have the highest accuracy, followed by RDTs of serum or plasma and POCTs of oral fluids [41].

Cost analysis showed that the average cost of RDTs for detection of HCV antibodies for capillary and whole-blood assay specimens ranges from $0.50 to $2.00 and $10 for oral fluid [42]. Although there are several rapid diagnostic tests available commercially, OraQuick is the only one approved by FDA from June 2010. The characteristics of the commercially available RDTs are compared in Table 2.2.

OraQuick HCV Rapid Antibody Test

The OraQuick HCV Rapid Antibody Test is approved by FDA for the qualitative detection of anti-HCV in individuals 15 years or older. Basic principle of the OraQuick HCV RDA test is to detect antibodies to both structural and nonstructural HCV proteins by utilizing the synthetic peptides and recombinant antigens from the core, NS3, and NS4 regions of the HCV genome that are immobilized as a single test line on the assay strip [43]. Specimens with positive anti-HCV antibodies produce a visible line in the test zone and are visualized by colloidal gold labeled with protein A. The OraQuick is very accurate, reproducible with sensitivity and specificity performance similar to third-generation EIAs. Though rapid kits have been extensively used for surveillance purposes, OraQuick HCV test has not been approved for general screening of hepatitis C infection. Furthermore, it is not well

Table 2.2 Comparison of accuracy of most commonly used point-of-care rapid diagnostic tests

Test	Sample	Sensitivity	Specificity	Positive LR	Negative LR
OraQuick	Whole blood, plasma, and serum	99.5%	99.8%	445.84	0.004
TriDot	Serum	98.2	98.4	61.22	0.009
Chembio	Whole blood and serum	95.1	98.6	68.45	0.065
Spot	Whole blood and serum	75.4	95.2	14.38	0.072

suited for high-risk groups and immunocompromised patients for detection of HCV infection [44]. World Health Organization recommends rapid diagnostic assays in the low- and middle-income countries and where there is limited access to the high-technology laboratory and immunoassay testing such as EIA and CIA.

2.6 Confirmatory Test

2.6.1 Recombinant Immunoblot Assay (RIBA)

The hepatitis C recombinant immunoblot assay (RIBA) is an in vitro qualitative enzyme immunoblot assay for the detection of HCV antibodies. It is one of the confirmatory tests for the hepatitis C infection and performed on the specimens found to be repeatedly positive for anti-HCV antibodies using anti-HCV screening procedure.

2.6.1.1 CHIRON' RIBA' HCV 3.0 SIA

The CHIRON' RIBA' HCV 3.0 SIA is an in vitro qualitative assay based on strip immunoblot assay methodology. It utilizes recombinant HCV-encoded antigens (c33c and NSS) and synthetic HCV-encoded peptides (C 100p and 5- 1- 1 p) immobilized as individual bands onto test strips. Detection of anti-HCV antibodies is facilitated by the addition of calorimetric enzyme detection system composed of hydrogen peroxide and 4-chloro-1-naphthol. The visual band pattern is produced when antigen reacts with specimen antibodies on the strip.

Advantages of the RIBA include provision of additional information in humans who had positive HCV immunoassay and a negative HCV RNA test. In this circumstance, the RIBA could differentiate whether the patient had resolved HCV infection or had a false-positive EIA. One of the advantages of RIBA is its high specificity (99.5%). However, CHIRON' RIBA' HCV 3.0 SIA is no longer used in the United States because of low sensitivity, high cost of the test, long duration to perform procedure, and hands-on complexity [45].

2.6.1.2 Interpretation of RIBA Results

Test is interpreted as positive if two or more antigens are present in the specimens, indeterminate if 1 antigen is identified, and negative for no antigen [46].

True-positive RIBA results indicate the presence of anti-HCV and do not differentiate past infection with spontaneous clearance from active infection; in this situation testing for HCV RNA is required to confirm active hepatitis C infection.

Indeterminate results: In acute HCV infection, assays are usually indeterminate during the first week of infection and it takes 1–6 months for the test to be positive. Moreover, presence of cross-reacting antibodies in specimen may result in indeterminate RIBA assay.

False-negative RIBA: RIBA may be false negative in immunocompromised patients or individuals with resolving hepatitis C infection because of waning level of anti-HCV antibodies.

2.7 Molecular Diagnosis of HCV Infection

As mentioned above, serological assays detect whether the person is infected with HCV or not and do not differentiate between current and resolved infection. Molecular diagnostic tests are required to confirm infection and identify current (active) hepatitis C infection status after the initial serological assays positive for anti-HCV antibodies. Molecular diagnostic tests serve important function of detection of HCV RNA, its quantification, and determination of genotype. HCV RNA can be detected in approximately 1–2 weeks after initial HCV infection with nucleic acid amplification test [25]. Several FDA-approved HCV nucleic acid amplification tests (HCV-NAAT) are available commercially and vary based on their accuracy, efficiency, and cost per test.

For the detection of HCV viremia FDA has approved both qualitative and quantitative tests. Qualitative HCV RNA nucleic amplification test is reported as positive and negative based on the presence or absence of detectable HCV RNA. In contrast, quantitative NAAT detects the quantity of HCV RNA in serum as viral load and provides information regarding specific genotype. Results of quantitative tests are important in making decision regarding treatment and response to therapy [47].

2.7.1 Sample Preparation

It is the first step in nucleic acid amplification test and involves extraction of HCV RNA from plasma using silica-based solid-phase and probe-based capture for RNA extraction. Silica-based solid-phase extraction is used in Cobas AmpliPrep (Roche), m2000 (Abbott Molecular, Abbott Park, IL), and VERSANT kinetic polymerase chain reaction (kPCR) sample prep system (Siemens) and involves reversible adsorption of nucleic acids to silica-coated magnetic beads in the presence of chaotropic salts and organic solvents. Probe-based capture for RNA extraction is used by direct tube sampling (DST) system, TIGRIS, and PANTHER platforms (Hologic, Bedford, MA) [48].

While Hologic TIGRIS is a fully automated system, the Siemens VERSANT kPCR Sample Prep system, Abbott m2000sp platform, and Qiagen Qiasymphony SP/AS (Qiagen, Venlo, the Netherlands) use semiautomated extraction system and assay setup. Manual transfer of eluted DNA is required in the final step of HCV RNA extraction. The Hologic TIGRIS system is currently incorporated in Procleix Ultrio Assay and APTIMA HCV RNA assay [49].

2.7.2 Amplification and Detection

Amplification and detection after RNA extraction entail polymerase-based target amplification via reverse transcription PCR or isothermal methods, coupled with endpoint or real-time detection formats. Assays based on real-time detection of virus such as Cobas TaqMan RT-qPCR HCV Assay are favored over endpoint

detection because of low risk of contamination. Other RT-qPCR assays commercially available are the Abbott RealTime and Siemens VERSANT kPCR HCV viral load assays, which use a similar principle [50].

2.7.3 Use of Quantitative or Qualitative NAT Assays

According to 2016 World Health Organization guidelines, either qualitative or quantitative NAAT is acceptable for the detection of HCV. However, the preferred strategy depends on resources of the healthcare system and laboratory infrastructure. Qualitative assays are preferred in resource-limited settings because these assays have the potential to be cheaper and more accessible. The cost of quantitative NAAT is high, ranging from $30 to $200 [51, 52]. According to the results of a systematic review that was conducted to determine the accuracy of qualitative versus quantitative NAT methods, the sensitivity of quantitative assays was 87–100% with lower limit of detection of 10–15 IU/mL as compared to qualitative assays (600–1100 IU/mL). However, with the advent of new quantitative HCV RNA assay the lower limit of detection of HCV has improved to 15 IU/mL (range: 12–108 IU/mL). Since 95% of chronic infection has a viral load >10,000 IU/mL most of the qualitative or quantitative NAT assays can detect HCV infection [53].

2.8 HCV Genotyping Assays

HCV genotyping is necessary to determine appropriate treatment selection, response to treatment, duration of treatment, and dose of ribavirin. Genotype is determined by analyzing genotype-specific sequences of the viral genome. Methods by which HCV genotyping can be done are as follows:

1. Direct Sequencing
   Direct sequencing for HCV genotyping is mainly used in epidemiological studies and currently considered a gold standard test. It involves direct sequencing of specific regions in viral genome (NS5, core, E1, and 5′ UTR) followed by alignment and phylogenic analysis. Examples of direct sequencing tests are HCV DupliType assay which is offered by Quest Diagnostics (New York, NY) and TRUGENE HCV Genotyping Assay by Siemens. TRUGENE HCV Genotyping Assay is an FDA-approved test. However, it is used solely for research purpose. Basic principle of the test is to use 244 base pair fragments of the 5′ UTR of the HCV viral genome. This fragment of HCV genome is then amplified via real-time PCR and sequencing using bidirectional cross-linking immunoprecipitation [54].
2. RT-qPCR
   Real-time PCR (RT-PCR) is a widely used and commercially available FDA-approved test for the purpose of genotyping of hepatitis C virus. It uses RT-qPCR technology with Taqman probes to detect and differentiate the HCV genotypes.

Sample is analyzed using three different RT-qPCR reactions. During each reaction primer, internal control-specific and genotype-specific probes conjugated to different fluorophores are used to allow detection of HCV genotype. RT-PCR assay involves four different primer sets used for different purpose. First primer set amplifies a sequence in the 5′ UTR, and second and third primer sets amplify sequences in the NS5B regions present in genotypes 1a and 1b. Fourth primer set added to the assay has the function to amplify IAC in the form of an armored RNA. During the process of amplification, the target RNA is initially converted into complementary DNA (cDNA) by rTth DNA polymerase (thermostable). After the process of denaturation, i.e., raising the temperature of the process above the melting point of the double-stranded cDNA, RNA and double-stranded DNA product are formed. During each round of thermal cycling, annealing of the primer and extension is allowed by dissociation of amplification into single strand at high temperature. Real-time PCR is >99% accurate in identifying HCV genotypes 1, 2, and 4. The test is 100% accurate for genotypes 3, 5, and 6. Shortcomings of the RT-PCR are long run time of the test (120 min), risk of contamination of the specimen, and need of high-technology laboratory facility [55, 56].

3. Hybridization-based line probe assays
   The VERSANT HCV Genotype 2.0 test (Siemens) is an FDA-approved test to commercially use in the United States for the genotyping of HCV. In Europe, the approved tests are LINEAR ARRAY Hepatitis C Virus Genotyping Test (Roche) and VERSANT HCV Genotype 2.0 test (Siemens). Principle of VERSANT HCV Genotype 2.0 test involves the amplification of 119 sequences in the 5′ UTR and core regions of the HCV genome using biotinylated primers by RT-PCR methodology. The biotinylated amplicons are immobilized on strips of nitrocellulose membrane and hybridized to genotype-specific oligonucleotide probes. Then conjugate composed of streptavidin–alkaline phosphatase is added to the sample which binds to the biotinylated captured amplicons. The last step involves addition of chromogenic substrate (BCIP–nitro blue tetrazolium) which produces a colored product on the membrane. Results are interpreted on LiPA HCV Scan software [57, 58].

2.9 Hepatitis C Ag Testing

The HCV core protein protects HCV by forming a capsid shell. HCV core protein can serve as a diagnostic marker for the diagnosis of viral hepatitis due to its antigenic characteristics. The HCV core antigen test was developed as an alternative to NAT, as access to and affordability of HCV confirmatory RNA NAT assays remain a challenge in resource-limited settings. Levels of hepatitis C virus (HCV) core antigen (Ag) can serve as a test to diagnose infection and to monitor response to treatment [59].

The ARCHITECT HCV Ag assay is a twostep chemiluminescent microparticle immunoassay (CMIA) technology based on flexible assay protocols referred to as

Chemiflex, for the quantitative determination of core antigen of hepatitis C virus. It's a fully automated test in which antigen-antibody reaction takes place between microparticle-coated monoclonal antibody and the HCV core antigen which is later detected by chemiluminescence technology [60]. The Architect HCV Antigen assay is now commercially available in Europe but not approved for use by FDA in the United States.

The sensitivity and specificity of ARCHITECT HCV antigen assay were found to be 97.2% and 100%, respectively. The sensitivity for detection of genotype 1 is found to be slightly better than genotype 3. The HCV Ag assay showed good correlation and excellent linearity with HCV RNA level. The HCV core antigen assay is an immunoassay that does not require sample processing as in molecular assay sampler, and a positive result confirms active infection. Hence it proved to be more cost effective, has better turnaround time, and does not require sophisticated laboratory structure. The lower limit of detection of HCV viremia with HCV antigen test is approximately 1000 IU/mL resulting in lower sensitivity as compared to NAAT [61].

2.9.1 Interpretation of Results

Results of ARCHITECT HCV Ag assay are interpreted as follows [62]:

1. Specimens with concentration values <3.00 fmol/L are considered nonreactive for HCV Ag.
2. Specimens with concentration values ≥3.00 fmol/L are considered reactive for HCV Ag.
3. Specimens with concentration values ≥3.00 fmol/L to <10.00 fmol/L should be retested in duplicate.
4. If both retest values are nonreactive, the specimen must be considered nonreactive for HCV Ag.
5. If one or both of the duplicates is (are) ≥3.00 fmol/L, the specimen must be considered repeatedly reactive for HCV Ag, and the initial value is used as the final reported value.

2.10 Resistance Testing

With the development of newer drugs, there has been the simultaneous emergence of drug-resistant viral variants of hepatitis C over the years. It is extremely important to be cognizant of the fact that the hepatitis C virus has the ability to develop resistance to antiviral therapies. Therapy for chronic hepatitis C has advanced significantly over the years from the combination treatment regimen of pegylated interferon and ribavirin combination to the powerful direct-acting antiviral agents (DAAs) [33]. These DAAs are categorized according to their mechanism of action and their molecular target and they impact different phases of the HCV life cycle

[63]. There are predominantly four main groups of DAAs and include the NS5A inhibitors, NS3/4A protease inhibitors, nucleotide analogue inhibitors of NS5B RNA-dependent RNA polymerase (RdRp), and non-nucleoside inhibitors of RdRp [33]. Treatment with DAAs is highly effective and results in high rates of sustained virological response (SVR). However approximately 10–15% of cases result in failure of therapy and this is due to the emergence of resistant viral variants [64]. These arise secondary to mutational changes that occur due to alterations in the amino acid sequences in the target protein of the virus, hence decreasing the susceptibility to the antiviral agent [64]. As the genomic sequences of HCV are so variable the resistance-associated variants (RAVs) can exist (at a low quantity) even prior to the initiation of therapy with DAAs [64]. Of note, the genotype 3 virus has been shown to have decreased rates of SVR to DAAs as compared to the other genotypes [65].

The hepatitis C virus is a 9.5 kb RNA virus that has the ability to replicate quickly by the action of an enzyme that can lead to several transcription errors per cycle [66]. The resultant transcription errors can lead to modification of coding regions causing the antivirals to become ineffective, hence leading to development of resistance to therapy [66]. The RNA-dependent RNA polymerase (RdRp) is deficient in the ability to proofread and this combined with the fact that the hepatitis C virus is highly replicative makes it prone to develop resistance to the direct-acting antiviral agents [67]. Due to the process of natural selection, there is formation of a "quasispecies" within an individual infected with HCV that consists of genetically distinct viral isolates [67]. The production of these variants tends to occur when levels of antiviral drugs are below the therapeutic level [66]. The resistant viruses then continue to proliferate as the drugs are no longer effective against them. The minor variants with polymorphisms that render them drug resistant can become the dominant isolates resulting in a virological breakthrough causing treatment failure or relapse after completion of therapy [68]. It is possible to detect known resistant variants prior to selection of antiviral therapy and this is especially valuable in cases of treatment regimens that include NS5A inhibitors as this can lead to an unfavorable outcome with therapy [66]. These alterations in the virus structure that attribute to resistance are known as baseline resistance-associated substitutions (RASs) [66]. Another form of resistant variants arises due to treatment failure with direct-acting antivirals and are known as treatment-emergent or treatment-selected RASs [66]. NS5A and NS3 resistance-associated substitutions are known to commonly emerge when there is treatment failure with regimens that include NS5A or NS3 inhibitors [66]. This is opposed to NS5B nucleotide resistance-associated substitutions that seldom emerge from treatment failure [69]. This phenomenon is referred to as a high barrier to resistance as the area to which the nucleotides attach is extremely conserved, hence minimal chances of producing resistant substitutions [66]. It is also believed that if such a substitution were to occur then it would also impede replication of the virus [66]. Another dilemma that is associated with the NS5A RASs is that they can continue to replicate even without the stress of selection from drugs in contrast to NS3 protease or NS5B nucleotide polymerase inhibitor RASs [66].

The clinical effect of resistance-associated substitutions (baseline and selected) on the results of therapy depends not only on the RASs but also on the choice of the antiviral regimen and patient-related attributes as well [66]. Hence merely testing for resistance-associated substitutions only will not help to guide the choice of antiviral therapy as other factors also need to be accounted for and an antiviral with known decrease in efficacy for a particular RAS can be utilized in certain scenarios [66]. Therefore, resistance testing needs to be performed but other factors also help to choose the appropriate antiviral regimen.

2.10.1 Polymorphisms (Also Called Substitutions)

For each genotype of the hepatitis C virus there is a consensus or reference nucleotide (an amino acid sequence) that exists [66]. A polymorphism (also referred to as substitution) is a change in the amino acid sequence that occurs at a particular location of the HCV protein resulting in a modification of the protein in the patient as compared to the reference protein [66]. Though substitution is the term that is more widely employed by most, the US Food and Drug Administration utilizes the term polymorphism to describe this alteration [66].

To delineate a substitution, one has to first describe the HCV genotype, the subtype, the HCV protein, as well as the location of the amino acid in the sequence [66]. Substitutions are defined as letter-number-letter [66]. The letter at the beginning denotes the amino acid that is normally present in that location in the reference protein [66]. The numerical value defines the position of the amino acid and the last letter describes the amino acid that is present in the patient's HCV protein [66]. For example, NS5A Y93M denotes that the amino acid at location 93 of the NS5A protein is typically tyrosine and the amino acid in the patient is methionine at this location [66]. It is also possible to have numerous variants and hence various amino acids can be seen at a particular location [66].

Naturally occurring polymorphisms can exist in treatment-naïve patients and they can confer resistance to NS5A, NS5B, and NS3/4A NS5B inhibitors [70]. Their occurrence is variable according to the genotype and subtype of HCV [70]. These can be then selected during direct-acting antiviral therapy and become the predominant variant leading to failure of therapy [70–72]. An example of such a substitution is the presence of the NS3/4A Q80K which predominantly occurs in those inflicted with the HCV genotype 1a leading to reduced rates of SVR in patients that receive treatment with the combination of simeprevir (protease inhibitor) and PEG-INF/RBV in contrast to those lacking the Q80K substitution [73, 74].

2.10.2 Resistance-Associated Substitutions (RASs)

This refers to any changes in the amino acid from the reference sequence at a location that is related to decreased susceptibility of the virus to a single or even multiple antiviral agent [66]. They can be categorized as:

1. Drug-Class RASs
   These are amino acid polymorphisms that decrease the vulnerability of a virus to any antiviral in a particular class (at least to one drug in the class) [66]. Though the act on a specific drug class this does not imply that all drugs within that class have emergence of resistance [66].
2. Drug-Specific RASs
   These are amino acid polymorphisms that decrease the vulnerability of a virus to a particular drug [66]. This is the category of RASs that needs to be addressed when evaluating the effect of a resistance-associated substitution on a treatment regimen [66]. In a treatment-naïve population that is inflicted with HCV infection, the drug-specific resistance-associated substitutions will be much less as compared to the class resistance-associated substitutions [66].

2.11 Methods of Resistance Testing

The methods for resistance testing can be categorized into genotypic and phenotypic analytical methods. The genotypic assays that are used to detect the resistance-associated substitutions are the population sequencing (also referred to as Sanger sequencing) and deep sequencing (also known as next-generation sequencing [NGS]) [66]. Both of these are based on the process of sequencing the RNA of the hepatitis C virus followed by deciphering of the sequence of amino acids and then analyzing as to whether there are any resistance-associated substitutions present [66]. Both of these vary in their sensitivity towards spotting the presence of RASs [66]. Either of these methods can be used and will be considered comparable if a cutoff of ≥15% is employed for detection of RASs by the method of next-generation sequencing [66]. Some of the latest research reveals that when next-generation sequencing is at 1% sensitivity then this leads to the detection of extra RASs that do not lead to treatment failure and may not necessarily carry clinical relevance [75–77]. Clonal sequencing is another form of genotypic analysis that is not commonly employed these days [67]. Phenotypic methods assess the degree of resistance to antivirals along with the replicative ability that is conferred as a result of amino acid substitutions [66].

2.11.1 Genotypic Methods

1. Population-Based Sequencing (or Sanger sequencing)
   This form of sequencing can be carried out on the targeted coding region using reverse transcription polymerase chain reaction (PCR) as well as by sequencing of the entire PCR product [66]. This method has a sensitivity rate that ranges from 15 to 25% for detecting resistance-associated substitutions [66]. The detected substitutions are then evaluated in comparison to a genotype-specific wild strain [66]. It reveals the predominant viral variants that are present within a quasispecies [67]. PCR primers that are genotype specific have to be utilized to allow for effective amplification of a particular gene given the vast diversity of the genotype

and subtypes of the virus [67]. With this approach however there is a potential for missing some potential minority RASs that have a clinical bearing [78].

2. Clonal Sequencing
   This involves the isolation of individual variants by the method of genetic cloning or by a process known as end-point limiting dilution [67]. The viral variants are introduced into a plasmid vector and then introduced into a bacterial host following which Sanger sequencing is performed on the individual clones [67]. Every clone signifies a variant within the quasispecies [67]. This method is extremely cumbersome as well as costly and with a restricted quantity of clones that can be examined [67]. This is not commonly used nowadays since the availability of next-generation sequencing which is preferred instead [67].
3. Deep Sequencing or Next-Generation Sequencing (NGS)
   Next-generation sequencing techniques (or deep sequencing) employ one of the two techniques: quasispecies assessment of a target gene or sequencing of the whole genome [67]. It leads to increased rates of sensitivity for detecting less frequently occurring substitutions [56]. Once the targeted HCV coding regions are sequenced using the PCR approach, the sequences are then analyzed and sorted to isolate the substitutions detected at a particular level. This threshold can be variable and can be set to >1% but the level is usually fixed at ≥10% so that the results from NGS are comparable to those attained from population sequencing [66]. Variants that account for <0.5% of the total are typically not counted due to the high changes of false-positive results that can occur during the process of amplification and sequencing [67].

2.11.2 Phenotypic Methods

These methods analyze the extent of drug resistance that occurs when an amino acid substitution or polymorphism occurs [66]. These techniques also analyze the ability of an individual RAS to replicate in the presence of a consensus strain [66]. To analyze the degree of resistance, the RASs are inserted as point mutations into a HCV genome that is present in a cell culture or even an enzyme-based system [66]. The variants that have the RASs are subjected to antivirals at increasing amounts and monitored for decline in replicative activity or decrease in enzymatic activity [66]. This is analyzed in reference to the wild strain [66]. Fitness can be assessed when the replicative capacity of the variants is compared to the wild strain without the presence of the drug [66]. However these methods are not commonly used clinically and more often employed for research purposes [66].

2.11.3 Clinical Correlation

As progress is being made to develop new direct-acting antiviral agents, the hepatitis C virus itself is making advances and is evolving to give rise to resistant variants. Being aware of drug resistance is important in order to help choose appropriate

treatment regimens containing the DAAs and to prevent failure of therapy and to optimize the success rates [68]. Resistance testing for antiviral agents can be carried out by either the population sequencing or the next-generation sequencing to evaluate for resistance-associated substitutions in NS5A, NS3, and NS5B [66]. Clinically when either the population sequencing or the deep sequencing methods are utilized the resistance-associated substitutions should be prevalent at a minimum of 15% and this is the advised threshold [66]. Below this threshold the RASs are not believed to have a substantial impact [66]. Apart from that when evaluating the clinical impact of resistance-associated substitutions, one should look at the drug-specific ones [66]. Hence the drug-specific resistance-associated substitutions need to be prevalent in at least 15% of a patient's virions to negatively impact the rates of sustained virological response [79].

Research studies and clinical trials reveal that resistance-associated substitutions are not always detected when failure of therapy occurs [66]. Some viral variants are less fit as compared to others; hence their life span is limited. Those that exhibit resistance to NS3/4A protease inhibitors are less resilient (can survive for only weeks or months) as compared to the viruses that are resistant to NS5A inhibitors (survive for years) and this carries clinical significance for treatment [66].

The RASs that are under close clinical scrutiny and carry substantial importance are those predominantly in relation to genotypes 1 and 3 [66, 78]. The RASs are less relevant for genotype 2 due to the restricted clinical impact [78]. The impact of a RAS depends on the antiviral drug class, effect on replicative capacity due to the RAS, genotype of the virus, and patient-related factors (whether patient is treatment naïve or not and if cirrhosis is present) [78]. For cases of HCV genotype 1, the subtype has a pivotal role in determining the presence of baseline (prior to antiviral exposure) nonstructural protein 5A (NS5A) resistance-associated substitutions along with their significance [78].

2.11.3.1 Key RASs by HCV Genotype and DAA Regimen (AASLD/IDSA, HCV Guidance: Recommendations for Testing, Managing, and Treating Hepatitis C)

Elbasvir/grazoprevir

- Genotype 1a: M28A/T, Y93C/H/N, L31M/V, and Q30H/R
- Genotype 1b: Y93H
- Genotype 3: None

Ledipasvir/sofosbuvir

- Genotype 1a: Y93C/H/N, Q30H/R, and L31M/V
- Genotype 1b: L31V, Y93H
- Genotype 3: None

Sofosbuvir/velpatasvir

- Genotype 1a: None
- Genotype 1b: None
- Genotype 3: Y93H

Paritaprevir/ritonavir/ombitasvir with dasabuvir ± ribavirin

- Genotype 1a: None
- Genotype 1b: None
- Genotype 3: None

The resistance-associated substitutions that affect the NS5A inhibitors carry the most clinical importance [78]. The RASs that affect NS5B nucleotide inhibitor sofosbuvir are not present in treatment-naïve individuals and only occur rarely (about 1%) or in those with treatment failure [69, 80]. The NS5B mutation known as S282T gives some resistance to the variant but is not beneficial for replication [69]. Hence this mutation does not carry a clinical significance as it does not alter the overall effect of sofosbuvir and therefore NS5B resistance testing is not recommended routinely for patients that are treatment naïve or exposed to treatment in the past [78].

RASs to NS3 protease inhibitors that would bear importance are uncommon in those that are treatment naïve to this antiviral class [78]. For HCV genotype 1a the testing of Q80K polymorphism is recommended only in cases of cirrhotic patients who are treatment experienced and not recommended routinely anymore when combination therapy of simeprevir and sofosbuvir is administered for the appropriate duration [81]. There is no effect of the Q80K polymorphism on the other NS3 inhibitors such as grazoprevir- or the ritonavir-boosted paritaprevir [78]. NS3 RASs occur in about 50% cases of treatment failure of a regimen that contains a protease inhibitor [82, 83]. Of the three main RASs, the R155K variant is seen in genotype 1a HCV and has no effect on the efficacy of grazoprevir [84]. However the D168 and A156 variants are seen in infections with both genotypes 1a and 1b [78]. They both occur more commonly and have an effect on the efficacy of all the protease inhibitors that are used in the treatment of HCV [78]. On the other hand they have meager replicative activity and hence disappear quickly once the antiviral agent is withdrawn (along with the stimulus to promote their selection) [82, 83]. Once variants disappear and are undetectable, it is unclear as to whether they will affect the follow-up treatments [78].

The most crucial resistance-associated substitutions are those that are in the nonstructural protein 5A (NS5A) [78]. Preexisting NS5A RASs are common with a prevalence rate of 13% in genotype 1a and 18% in genotype 1b [77]. The prevalence in genotype 3 infection is 12–17% [85, 86]. The effect of preexisting NS5A RASs depends on the genotype and subtype of the hepatitis C virus and has the most effect on the genotypes 1a and 3 [78]. Patient-related factors also play a role and increase the effect of NS5A RASs and these include patients inflicted with cirrhosis and treatment-experienced patients (not with an NS5A inhibitor) [78]. Once therapeutic failure occurs with a regimen containing NS5A inhibitor, the outcome is that most patients will then be infected with these NS5A RASs (noted from 75 to 90%) [80, 82, 83]. These NS5A RASs are very resilient and have significant amount of replicative fitness. In the majority of cases once they emerge in an individual they are detectable for over 2 years [82, 87]. There is also a significant degree of cross resistance that occurs among the NS5A inhibitors [78]. In genotype

1a, particularly the RASs at certain positions [80, 82, 83, 85] lead to cross resistance among the early-generation NS5A inhibitors [78]. By extending the duration of treatment and by combining ribavirin to the treatment regimen, one can dampen the effect of NS5A RAS [76].

2.11.3.2 Recommendations as per AASLD/IDSA Guidelines on Indications of RAS Testing According to Treatment Regimens

Antiviral combinations in which NS5A RAS testing is recommended (AASLD/IDSA, HCV guidance: recommendations for testing, managing, and treating hepatitis C).

Elbasvir/grazoprevir

1. Perform testing for genotype 1a: both treatment-naïve and -experienced individuals.
2. If RAS present: add ribavirin (weight based) and lengthen duration of therapy to 16 weeks.

Sofosbuvir/velpatasvir

1. Perform testing for genotype 3 cases that are treatment experienced, with or without the presence of cirrhosis.
2. Perform testing for genotype 3 treatment-naïve cases in the setting of cirrhosis if treatment for 12 weeks' duration is planned.
3. If Y93H detected: add ribavirin (weight based).

Ledipasvir/sofosbuvir

1. Perform testing for genotype 1a cases that are not cirrhotic and are treatment-experienced patients.
   (a) >100-fold resistance detected: add ribavirin (weight based) + duration of therapy should be 12 weeks (or alternative therapy).
2. Perform testing for genotype 1a cases that are cirrhotic and treatment experienced.
   (a) >100-fold resistance detected: add ribavirin (weight based) + duration of therapy should be 24 weeks (or alternative therapy).

Daclatasvir plus sofosbuvir

1. Perform testing for genotype 3 cases that are not cirrhotic and are treatment experienced in whom 12 weeks of treatment is planned.
   (a) If Y93H detected: add ribavirin (weight based).
2. Perform testing for genotype 3 cirrhotic cases that are treatment naïve in whom 24 weeks of treatment is planned.
   (a) If Y93H detected: add ribavirin (weight based) or alternative therapy.

Antiviral combinations in which NS5A RAS testing is not recommended (AASLD/IDSA, HCV guidance: recommendations for testing, managing, and treating hepatitis C).

Elbasvir/grazoprevir
Do not test in cases of genotype 1b.

Ledipasvir/sofosbuvir
NS5A RAS testing is not indicated in the following cases:

1. Genotype 1b cases.
2. Genotype 1a cases that are treatment naïve.
3. Genotype 1a or 1b non-cirrhotic cases that are treatment naive that have a viral load <6 million IU/mL in whom 8 weeks of therapy is planned.

Sofosbuvir/velpatasvir
Do not test in cases of genotype 1, 2, 4, 5, or 6 infections in whom 12 weeks of therapy is planned.

Glecaprevir/pibrentasvir
Do not test in cases of genotype 1, 2, 3, 4, 5, or 6 infections in whom 8, 12, or 16 weeks of therapy is planned.

Sofosbuvir/velpatasvir/voxilaprevir
Do not test in cases of genotype 1, 2, 3, 4, 5, or 6 infections in whom 12 weeks of therapy is planned.

Paritaprevir/ritonavir/ombitasvir with dasabuvir ± weight-based ribavirin, or paritaprevir/ritonavir/ombitasvir + weight-based ribavirin
Do not test in cases of genotype 1 or 4 that are treatment naive or treatment experienced in whom either therapeutic regimen will be used.

2.12 Tackling Resistance

The therapy for hepatitis C continues to make advancements. However, until more therapeutic options become available through clinical trials, there are some factors that can be modified to achieve better treatment outcomes in the setting of resistance to antivirals. These factors include those that are patient related and virus related and those in relation to the antiviral therapy itself [68].

1. Patient-related factors
   It is important to be aware whether the patient has fibrotic changes in the liver and if the patient is treatment naïve or treatment experienced [68]. These attributes will help guide therapy choices.
2. Virus-related factors
   The characteristics of the HCV virus that play a role in therapy are the genotype and subtype as well as the presence of any preexisting resistance-associated substitutions [66].

3. Therapy-related factors
   Duration of therapy can significantly impact the probability of an occurrence of a relapse [66]. Though shorter courses are more commonly linked to more replaces, allocating the appropriate patient population for them can reduce expenses and decreases chances of relapse [66]. However extended treatment with the combination of ribavirin can be used in certain cases as well to increase the rates of sustained virological response [66]. This tactic can especially be used in patients who had therapeutic failure with prior antiviral therapy even if there are preexisting RASs [66, 88, 89]. Combining ribavirin to the DAA regimens aids in improving the rates of SVR in patient populations that are more prone to failure of therapy, in cases of preexisting NS5A RASs and with previous failure of therapy with DAAs [66].

2.13 Emerging Technologies for HCV Detection

There have been recent promising advances in the molecular diagnostic technologies for the improved assays to diagnose hepatitis C infection. Few of the technologies are described here.

1. Isothermal amplification
   Isothermal amplification assay has the ability to develop into a point-of-care (PoC) HCV RNA detection and potentially can be used at laboratory or in the field. This technique has been successfully used to diagnose various infectious organisms such as mycobacterium tuberculosis and trypanosoma [90].
   (a) Loop-mediated isothermal amplification of DNA (LAMP)
       LAMP is developed recently and based on autocycling of DNA and its synthesis using DNA polymerase under isothermal conditions (63–65 °C). Large amount of pyrophosphate ion produced during LAMP reaction reacts with magnesium ions to form magnesium pyrophosphate which allows rapid visualization of amplified DNA. LAMP is a highly sensitive and specific DNA amplification technique suitable for diagnosis of an infectious disease. Though LAMP reaction is efficient as it synthesizes 10–20 μg of target DNA within 30–60 min and cost effective further evaluation is required for these novel modalities [90].
   (b) Simultaneous Amplification Test (SAT)
       Simultaneous amplification and testing (SAT) assay is also based on isothermal amplification of RNA followed by real-time fluorescence detection of amplified DNA. SAT assays have the potential to detect as few as ten copies of HCV RNA transcripts in 60 min. The sensitivity and specificity of SAT-HCV assay were 99.6% and 100%, respectively, when compared to the results from real-time PCR [91]. SAT assays are faster because T7 RNA polymerase which is used in SAT assays can produce 10–103 copies of RNA per copy of DNA template and for this reason, e.g., results of SAT assays are available in 60 min as compared to 120 min for real-time PCR with low risk

of contamination. Moreover, the SAT assays are cost effective as it requires only isothermal incubator and does not need PCR thermal cycler [92].

2. Aptamers

   One of the promising novel technologies to provide efficient, accurate, and cost-effective diagnostic modality of HCV infection is the use of aptamers as capture molecules. Aptamers are single-stranded oligonucleotides that bind to specific target molecule and in case of HCV detection they bind to hepatitis core protein. Aptamers are isolated using selective evolution of ligands by the exponential enrichment approach (SELEX) approach and displays high display and high affinity and specificity for HCV core protein. One of the major advantages of aptamers over serological test for anti-HCV antibodies is their convenient synthesis, easy modification with dependability, accuracy, and lack of immunogenicity. Furthermore, different HCV genotypes can be detected by core-specific aptamers [93].

3. Prototype nanoparticle-based diagnostic assays

   They have been developed for the detection of biomarkers in hepatitis C infection and are based on the principle of immunosensors. Immunosensors are solid-state affinity ligand-based biosensing devices that couple immunochemical reactions to appropriate transducers and have been studied extensively for clinical diagnosis of various infectious disease including HCV infection [94]. Immunosensors usually consist of sensing element which is formed by the immobilization of antigens or antibodies and transducers which measure signal produced by this binding event.

   Commonly used nanoparticles include quantum dots (QDs) and gold nanoparticles:

   (a) *Quantum dots*: These are the nanoparticles used in semiconductor material which emits light when antigen-antibody reaction takes place. These assays are found to be highly sensitive and specific.
   (b) *Gold nanoparticles*: These are small-size molecules (2–50 nM) and successfully used to detect anti-HCV and HCV RNA.

2.14 Technological Advances and Innovations to Improve Access to Hepatitis C Testing

Early diagnosis and management are needed to prevent complications associated with chronic infection such as chronic liver disease, decompensated liver disease, and hepatocellular carcinoma (HCC). Recent data suggests that current burden of HCV infection is just tip of the iceberg. It has been estimated that approximately 40–85% of the HCV-infected patients are unaware of their HCV status globally [95]. According to the data published by NHANES from 2001 to 2008, approximately 50.3% of the persons infected by HCV are unaware of their infection status in the United States. The situation is worse in middle- and low-income countries with only small fraction of people with HCV having access to screening and diagnostic tests for viral hepatitis [96]. Key barriers to HCV screening and diagnostic tests are listed in Table 2.3.

Table 2.3 Key barriers for the screening and diagnostic testing of HCV in low- and middle-income countries (LMIC)

Lack of awareness and understanding among the general population and healthcare workers about hepatitis C infection, its disease progression, and treatment.
High levels of stigma, discrimination, and social marginalization of those with or at risk of viral hepatitis, especially among persons who inject drugs (PWID), men who have sex with men (MSM), prisoners, and sex workers.
Few facilities and limited healthcare infrastructure for viral hepatitis testing.
Weak hepatitis surveillance programs and therefore limited data on the epidemiological situation to guide country-specific viral hepatitis testing approaches.
Lack of testing guidance for LMICs, and limited evidence base to guide hepatitis testing approaches. There have been few large studies or randomized controlled trials that have evaluated the impact or cost-effectiveness of different testing approaches to support the development of guideline recommendations.
Few LMICs have national viral hepatitis strategies or plans, and even fewer have designated units and budgets within their health ministries to lead, guide, and coordinate their hepatitis responses.
Poor laboratory capacity and infrastructure with inconsistent supplies of test kits and reagents due to poor logistics, shortages of trained staff, and poor-quality assurance and management systems.
Limited access to reliable and low-cost HCV diagnostics, including rapid serological tests and molecular viral load tests that are quality-assured by stringent regulatory authorities, leading to the use of poor-quality test kits and reagents.

Diagnostic innovations and interventions are needed to improve the access and increase the uptake of hepatitis C screening tests especially in low- and middle-income countries. In 2016–2017 World Health Organization proposed diagnostic innovations and future developments for the chronic hepatitis C infection which are as follows:

1. Simplification of testing algorithms
   As mentioned above, current diagnostic algorithm for hepatitis C infection includes screening test with serological test followed by confirmatory tests for viremia which can lead to loss to follow-up and increased cost on healthcare system. Rationale for simplification of testing algorithms is to increase affordability and so in turn uptake of testing. It includes considerations for one-step diagnostic approaches using either HCV core antigen or HCV RNA assays especially in high-risk population. This can be achieved with starting patients on pangenotypic direct antiretroviral drugs (DAA), which eliminates the need for costly genotyping which is largely unavailable in resource-limited settings. Also, on treatment monitoring with HCV viral load is not required while patient is on DAA [97].
2. Dried blood spots (DBS)
   DBS sampling approach has been successfully used in expanding access and care for HIV in many low-resource settings due to its low cost, simplicity of sample collection, more acceptability to patients, ease of DBS sample transportation, stability over time, and ability to withstand extremes of environmental condition with easier storage options. The 2017 WHO Guidelines Development

Group recognized the high diagnostic accuracy and impact of DBS for serological and nucleic amplification testing (NAT) for HCV. Though implementation of DBS sampling methodology can simplify the diagnostic algorithm for HCV diagnosis (serological and NAT testing can be done on single DBS sample) further validation studies are required to assess their performance in different populations and settings [98, 99].

3. Oral fluid RDTs

   Oral fluid rapid diagnostic assays are acceptable to the patients for HCV testing because of their simplicity and flexibility. The use of oral fluid RDTs has already been established in the field of HIV because of its potential to reach community and increasing access for hard-to-reach population and is especially successful in men who have sex with men (MSM). As discussed above, OraQuick® HCV Rapid Antibody Test is approved by FDA for HCV testing (oral fluid, venous blood, finger-stick capillary blood, serum, and plasma).

   Recently point-of-care NAT assays to detect HCV RNA are commercially available, and are expected to improve access to diagnosis, treatment, and linkage to care with health system by reducing the loss to follow-ups. Instrument to perform point-of-care NAT does not need electricity and uses reagents that are stable at 2–30 °C. Moreover, phlebotomy or precision pipetting is also not required to run the test and results are usually available within 2 h. HCVcAg portable point-of-care device is still under development and when available would have the potential to significantly improve the access and limit the cost of healthcare system by same-day diagnosis of infection [100, 101]. Characteristics of commonly used RDTs are as follows (Table 2.4).

4. Multiplex and multi-analyte testing

   It involves diagnosis of hepatitis C infection along with other infections of public health concerns such as HIV, syphilis, and hepatitis B and can be done by testing of one specimen in the same test device or reagent cartridge at the same time for multiple infections. As most of the countries are facing the overlapping epidemics of HIV-HBV and HIV-HCV coinfection, implementation of multiplex testing would improve the efficiency of screening program. Multiplex and multi-analyte testing is found to be cost effective (by lowering cost per pathogen) and efficient (less time required per pathogen per visit). Also, it is more acceptable to patients and providers as fewer needlesticks are required and less specimen volume is needed to run the test [102]. Despite all these advantages further data is required on their diagnostic accuracy. In 2014, Fisher et al. compared the accuracy of different rapid point-of-care test in terms of sensitivity, specificity, true positivity, and false negativity [103]. The MedMira rapid human immunodeficiency virus (HIV)/HCV antibody test, MedMira hepatitis B (HBV)/HIV/HCV antibody test,

Table 2.4 Comparison of accuracy of most commonly used oral fluid RDTs

Test	Sample	Sensitivity	Specificity	Positive LR	Negative LR
OraQuick	Oral fluid	95.9	99.4	147.98	0.037
Chembio	Oral fluid	88	94	14.93	0.141

Chembio HCV Screen Assay used with both whole blood and oral specimens, Chembio HIV-HCV Assay also used with both whole blood and oral specimens, Chembio HIV-HCV-Syphilis Assay, and OraSure HCV Rapid Antibody Test used with whole blood were compared. The results of the study showed that OraSure had the highest sensitivity and specificity at 92.7% and 99.8%, respectively, followed closely by Chembio. The sensitivities of MedMira HIV/HCV and MedMira HIV/HCV/HBV tests were the lowest, at 79.1% (95% CI = 72.6–85.5%) and 81.5% (95% CI = 75.2–87.8%), respectively [104].

5. Self-testing

   As the name indicates it's a test which is performed and interpreted in private by the individual, who wants to know his or her hepatitis C infection status. However, further confirmatory tests are required if the individual is tested positive for viral hepatitis. Currently, self-testing is an active area of research in HIV diagnosis and access to management. Several studies have been conducted to evaluate the effectiveness of self-testing in HIV care (HIVST). Results of these studies showed that HIV self-testing is acceptable in different high-risk population in different settings [105]. From the available studies, self-testing is a promising methodology for diagnosis of HIV but it's still not clear whether self-testing can promote the uptake of the viral hepatitis testing among marginalized population. One of the several advantages of self-testing is the simplicity and flexibility to perform test conveniently in private setting [106].

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