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REVIEW

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Current advances in Hepatitis C diagnostics

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Abstract

Nearly 60 million people worldwide are infected with Hepatitis C Virus (HCV), a bloodborne pathogen which leads to liver cirrhosis and increases the risk of hepatocellular carcinoma. Those with limited access to healthcare resources, such as injection drug users and people in low- and middle-income countries, carry the highest burden. The current diagnostic algorithm for HCV is slow and costly, leading to a significant barrier in diagnosis and treatment for those most at risk from HCV. There remains no available vaccine for HCV, and infection is often asymptomatic until significant cirrhosis has occurred, which makes screening incredibly important to prevent liver damage and transmission. Recent investigation has sought to address these issues through improvements in various aspects of the diagnostic procedure, using methods such as isothermal amplification techniques for viral RNA amplification, the use of viral protein as an analyte, and the incorporation of streamlined, self-contained testing systems to reduce administrative skill requirements. This review provides a comprehensive overview of current commercial standards and novel improvements in HCV diagnostics, as well as a framework for future integration of these improvements to develop a one-step diagnostic that meets the needs of those most affected.

Keywords HCV, Diagnosis, Rapid, Microfluidics, Point of care

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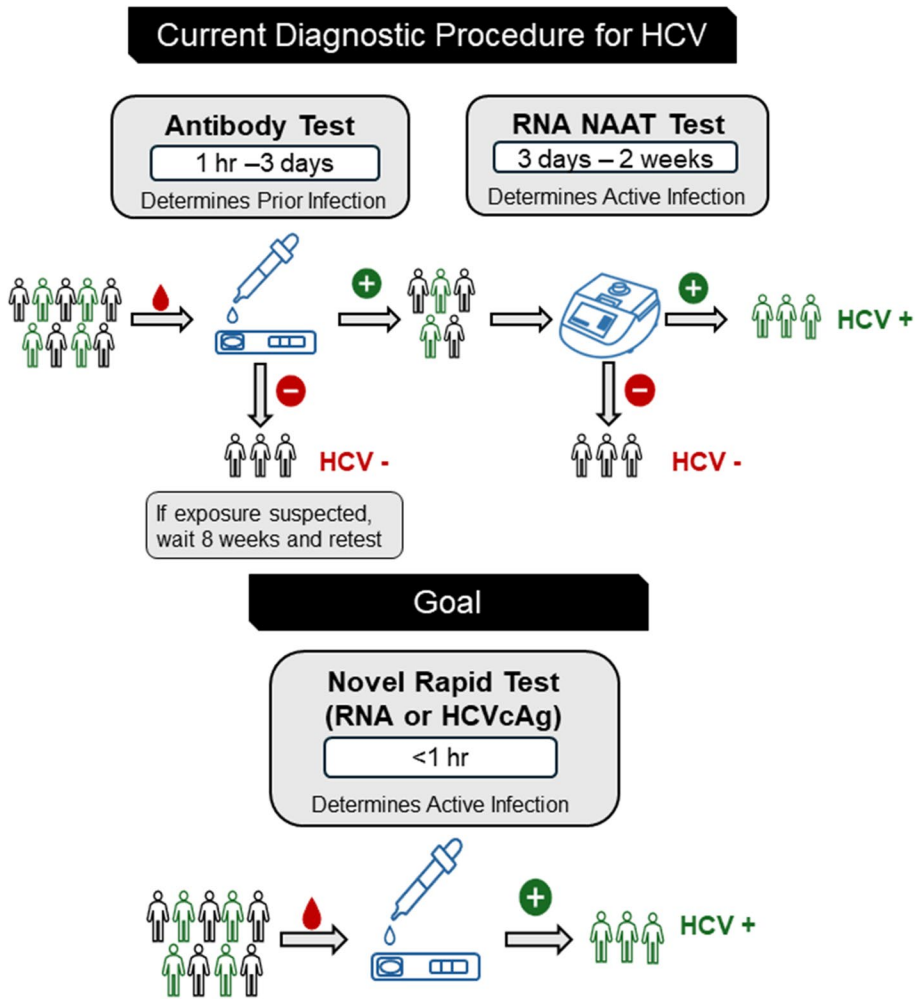
Graphical Abstract



Background

Hepatitis C Virus (HCV) is a blood-borne pathogen that causes inflammation of the liver. HCV is primarily transmitted through poor blood and organ donation screening procedures, and other poor sanitation practices [1]. Worldwide, 59 million people are living with HCV and an estimated 2.8 million people in the United States are currently infected [2–4]. HCV infection is separated into two designations, acute and chronic. Acute infection encompasses the first six months following exposure, and for around ~ 40% of those who contract HCV, the infection will resolve naturally. For the remaining 60% of people, the infection will become chronic [1]. Chronic HCV infections can lead to liver cirrhosis, cancer, and even death [1]. Unlike other hepatitis viruses, HCV currently has no available vaccine.

HCV is a ribonucleic acid (RNA) virus with seven core genotypes. Genotype 1 is the most prevalent and is responsible for about 46% of infections worldwide. It is also the primary genotype found in the United States [5]. HCV genotypes are generally regionally bound and have different disease courses and responses to treatment [5]. The structure of the virus is shown in Fig. 1. The virus itself is composed of two membrane layers around the RNA, one directly protecting the RNA that is referred to as the capsid and then an additional lipid membrane made of glycoproteins that surrounds the capsid [5]. The capsid is made of HCV core proteins that are relatively well conserved across genotypes and are the most common target for protein-based HCV detection methods. The lipid membrane is composed of two proteins, E1 and E2, which are also occasionally used as assay targets. The

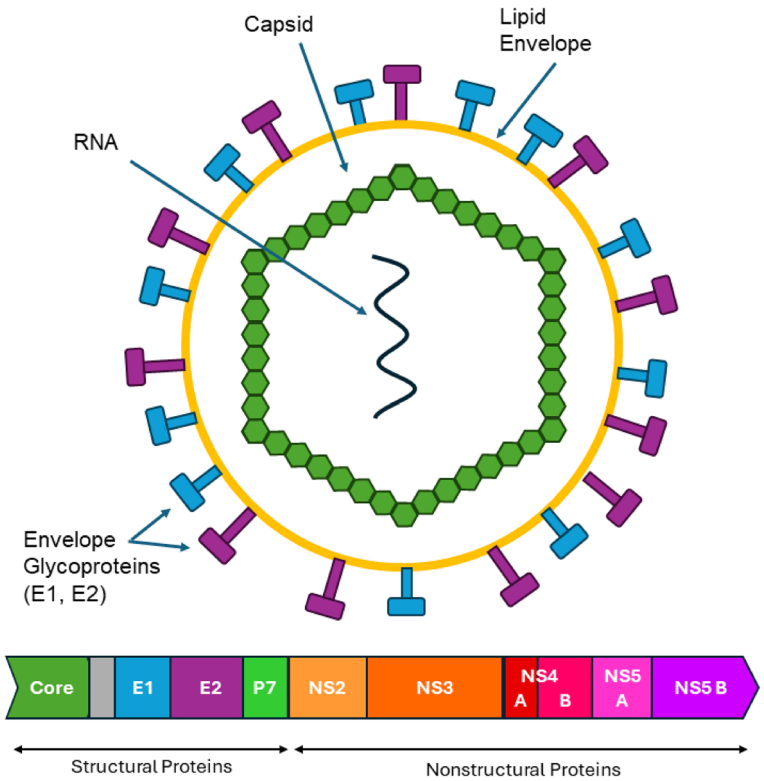


Fig. 1 Generalized structure and genome of HCV virus

HCV genome also encodes several non-structural proteins known as NS1-5(a/b). NS3-5 are commonly used together as antigens in immunoassays for the detection of anti-HCV in patient blood samples [5]. For these assays, the antigens are used to capture the patient's antibodies that have developed against the virus.

The effective treatment of HCV relies on early diagnosis and antiviral medication. This is essential to minimize liver damage and prevent transmission. In more affluent countries like the U.S., the focus is on screening high-risk groups such as dialysis patients and injection drug users [1]. However, in low and middle-income countries where disease prevalence is higher, testing is often conducted on the general population, despite limited access to testing. The traditional two-step testing process for HCV infection, illustrated in Fig. 2, can be

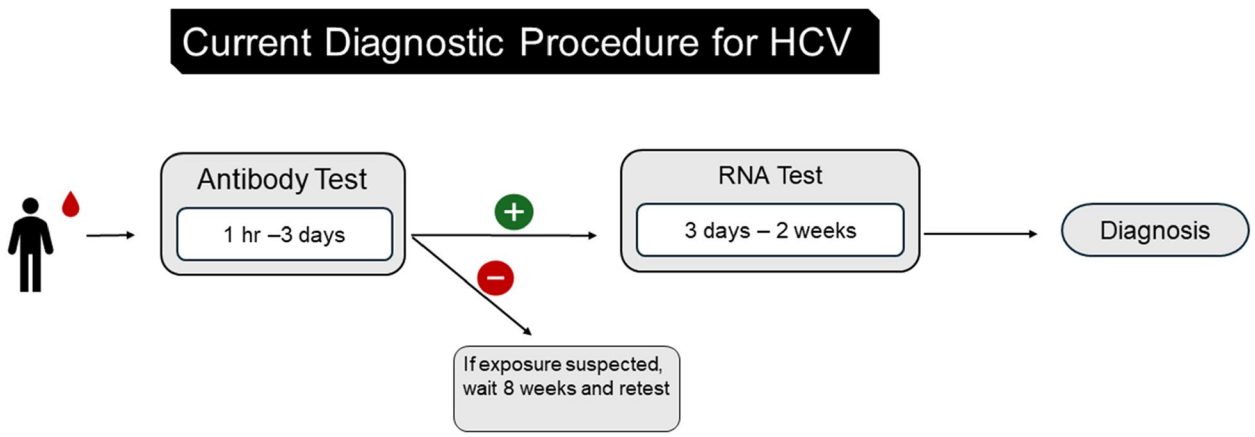


Fig. 2 Standard testing algorithm for HCV screening

time-consuming [1]. At-risk individuals are first tested to see if they have developed antibodies to the virus. If they are found to have developed antibodies, they are tested for viral RNA, which will confirm if they are currently infected. The initial tests for antibodies are standard for mitigating healthcare costs because they are less resource-intensive to conduct. However, they are not strictly necessary for diagnosis because the follow-up RNA tests determine HCV infection. Since HCV often does not present with clear symptoms until liver damage has already progressed, there is an especially high risk of accidental transmission. There is a clear and urgent need for the development of rapid, low-cost HCV diagnostics that can be used for at-risk populations, especially since HCV prevalence is higher among populations with limited access to healthcare. Both developed and developing countries require improved HCV diagnostics, but each setting has different constraints. Testing can be done in centralized laboratories with advanced equipment and technicians in communities with significant resources. The focus for these communities is ease of access and convenience for high-risk populations that may need more convincing to get tested. It is important to ensure that testing is affordable, and that sample collection can be introduced to widespread locations such as pharmacies. However, there are additional requirements in more remote settings where transport and equipment are limited. Low-resource communities require testing systems that do not need sensitive temperature controls or advanced centralized laboratories [6]. For treatment monitoring, the diagnostics must be highly sensitive to support the sustained virological response (SVR) standard for successful treatment, which equates to the virus being undetectable in the bloodstream [7]. Currently, treatment monitoring is carried out using quantitative RNA testing, but the SVR standard allows for qualitative tests using RNA or viral protein if they meet sensitivity standards.

First level screening—testing for antibodies

Antibody tests are used as the initial test in HCV screening because they are less expensive and can provide rapid results. The initial antibody test can determine if the patient has ever been infected with HCV. There are two primary limitations to antibody tests. First, antibody tests rely on the body's response to infection. Since it could take 8—11 weeks for the body to develop HCV antibodies, accuracy of the tests is limited until over a month after initial exposure [8]. The second limitation with antibody tests is that they cannot discern between active and prior infections. This is because antibodies can remain present in the blood even after an infection has been resolved by the body. They are also mostly used for qualitative determination. Because of these limitations, antibody tests cannot serve as independent tools for diagnosis. Commercial antibody tests are available in two formats: enzyme-linked immunoassays (ELISAs) such as the BioRad Ortho HCV Test and rapid lateral flow assays (LFAs) such as the OraQuick test [1, 9]. The rapid LFA tests can return results within 20 min at a point of care level, whereas ELISA tests require more extensive work, must be performed by trained personnel, and can take 1–3 h to complete, with an average time to result being 1–2 days due to transport limitations [1]. There are also commercially available tools with similar methodologies and limitations as ELISA, such as the Electrochemiluminescence Assay (ECLIA) produced by Roche [10, 11].

Enzyme-linked immunosorbent assays (ELISAs)

ELISAs work by labeling the desired analyte with an antibody that has been conjugated with a reporter molecule. For ELISAs with antibodies as the target analyte, the sample is first exposed to a solution of antigens bound to a plastic well plate. The antibodies in the sample form a complex with the antigen, which binds them to the plate. The plate is then rinsed, following which a secondary antibody that has been tagged with an enzyme is introduced to the well, forming a complex with the desired antibody at the center. The plates are rinsed again, and finally, a substrate is added, which is catalyzed by the tagging enzyme, producing an evaluable result [12]. ELISAs can also be completed using beads as the base surface instead of a well plate.

ELISA tests can take a number of forms—direct, indirect, sandwich, and competitive, as shown in Fig. 3. Whether an ELISA is considered direct or indirect is determined by which antibody is conjugated with the signal molecule [13]. In direct ELISA tests, the antibody against the desired antigen (primary antibody) is directly tagged with a signal molecule that aids in fluorescent or colorimetric detection. In indirect ELISA tests, the antibody against the primary antibody is untagged, while a secondary antibody against the conserved region of the primary antibody is tagged [13]. This is primarily done to reduce cost since a tagged secondary antibody could be used in multiple ELISAs so long as the primary antibodies are from the same host. ELISA tests are also differentiated by what is affixed directly to the plate. The most basic ELISA adheres the antigen to the plate, and then an antibody is used to bind and detect that antigen. For Sandwich ELISAs, primary capture antibodies are attached to the plate, and additional primary detection antibodies are free in solution. Thus, the antibodies form a "sandwich" around the antigen. There are also competitive ELISAs that use a tagged version of the analyte and produce an observable signal that is inversely

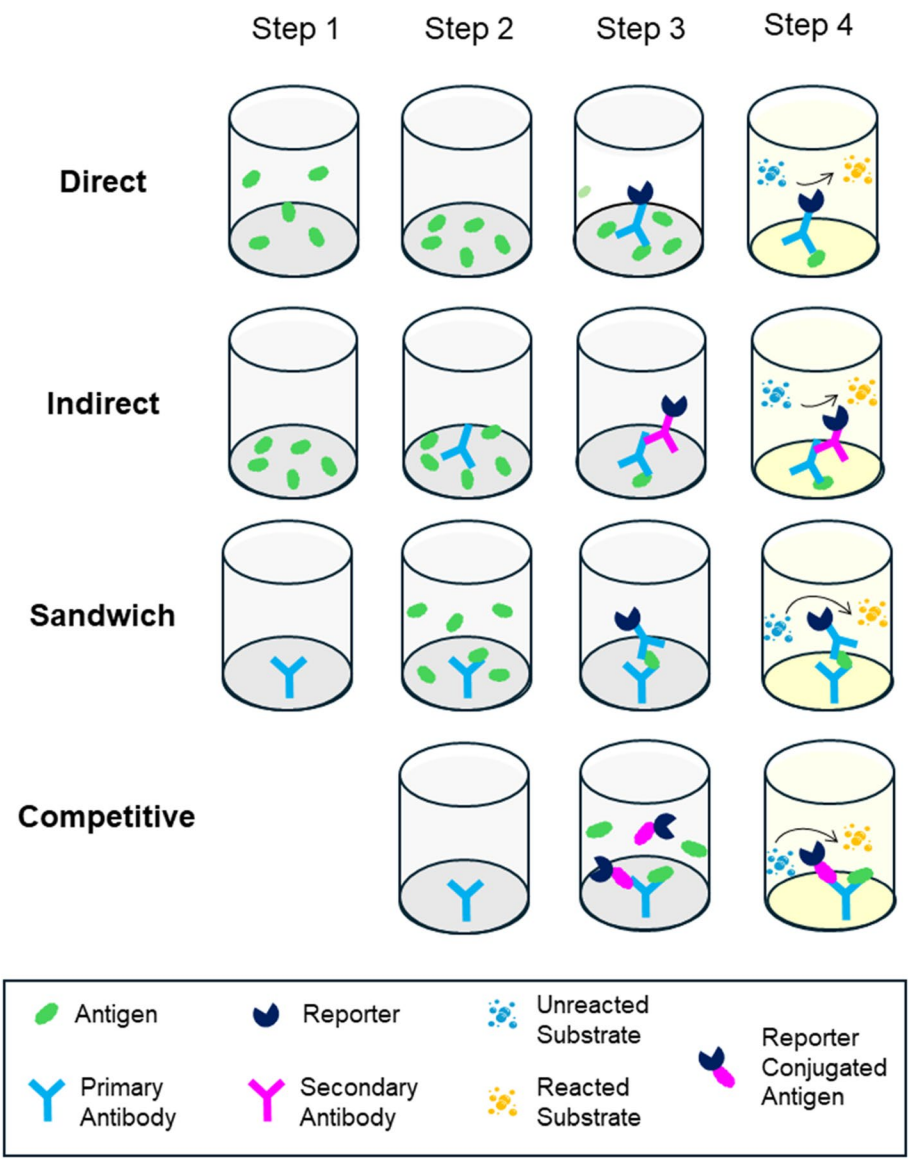


Fig. 3 Enzyme linked immunoassay forms: direct, indirect, sandwich and competitive

proportional to the amount of analyte in the sample. Since HCV ELISA tests traditionally detect anti-HCV, an HCV protein is used as the antigen affixed to the plate, and an anti-human antibody is used as the reporter in most tests [13].

Research-scale innovation for the detection of anti-HCV using ELISA has included two approaches. The first is the use of recombinant proteins from multiple parts of the HCV virus as antigens. Pedersen et al. developed a novel anti-HCV ELISA procedure using a customized recombinant protein composed of a maltose binding protein tag as well as HCV core, NS3, NS4, and NS5 proteins connected by flexible linkers. The protocol used an indirect ELISA with horseradish peroxidase (HRP) tagged anti-human as the detection antibody and 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) as the substrate for HRP. Using this novel protein, a diagnostic sensitivity of 98% and a specificity of 97% was achieved [14]. The second innovation was the development of diagnostics, which detected both viral proteins and anti-HCV in order to capture a greater range of the infection timeline. Since viral proteins are present earlier than anti-HCV in the blood detecting both reduces the chance of false negatives. In 2020, Kheirabad et al. evaluated multiple types of ELISA tests for anti-HCV and E2 and found that a combined approach that simultaneously detected

anti-HCV and E2, had the best diagnostic performance. For the detection of anti-HCV, an indirect model was used where a combination of HCV nonstructural proteins NS3, NS4 and NS5 were used as the antigens and HRP was used as the tag. To detect E2 an indirect sandwich design was used with anti-E2 as the primary antibody and a secondary HRP tagged antibody. Using this double detection assay a clinical sensitivity of 100% and a specificity of 99.6% was achieved [15].

Lateral flow assays

Lateral flow immunoassays (LFIAs) or more generally lateral flow assays (LFAs) are diagnostic methods based on the same principle as ELISAs but use capillary action and colorimetric detection to create a rapid platform that requires relatively little skill to use. Due to their ease of use, they are often used as P.O.C. diagnostics. As the sample moves through the LFA test strip by capillary action, the target analyte in the sample becomes bound to antibodies already present in the LFA which are tagged with reporter particles such as colloidal gold, magnetic nanoparticles, latex particles and quantum dots [16]. These complexes then proceed to the read-out line where capture bioreceptors are bound to the test strip. The conjugated antibody-analyte complexes then bind with the capture receptors. This binding orients the tagged domains on the reporter antibodies and condenses them which results in a visible readout [17]. A schematic of a basic LFA is shown in Fig. 4. The commercially available LFAs for HCV detection utilize a standard sandwich scheme. Gold nanoparticles decorated with antibodies against the conserved region of human antibodies are used in the conjugate pad as reporters and HCV antigens affixed to the test line serve as the capture receptor.

There have been numerous investigations into LFA's for anti-HCV detection. One of the main goals has been to improve the sensitivity of LFAs and provide quantitative data for physicians. Traditional colorimetric LFAs only

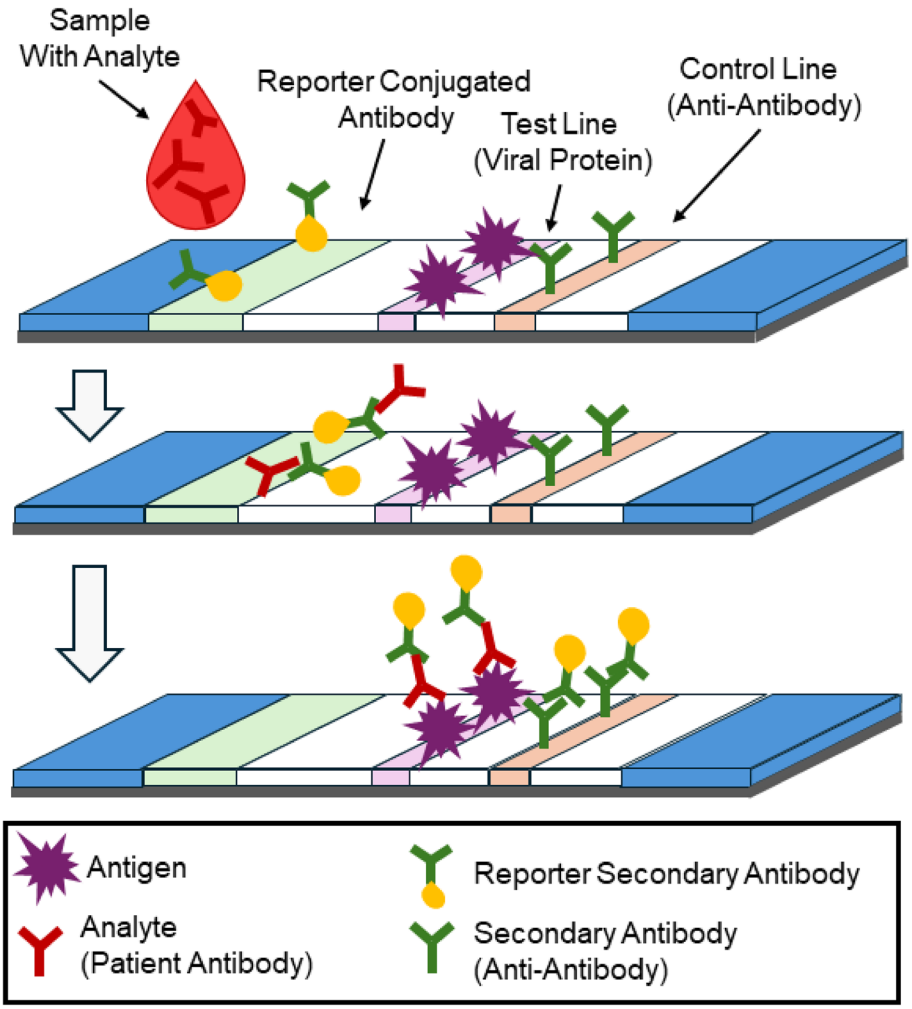


Fig. 4 Overview of lateral flow immunoassay mechanism

show a simple positive or negative result and are less sensitive compared to other methods like ELISA and PCR. To address this, Clarke et al. developed an LFA-based rapid vertical flow assay for Anti-HCV that can be evaluated visually and also provide a quantitative result using Surface-enhanced Raman spectroscopy (SERS). They used gold nanoparticles labeled with a Raman reporter molecule for this purpose. SERS is a spectroscopic technique that relies on the electromagnetic properties of surfaces and materials and can be influenced by topography and chemical changes. Using a benchtop Raman spectrometer, they demonstrated a limit of detection (LOD) of 53.1 μg/mL [18]. Using a benchtop Raman spectrometer, they were able to demonstrate a limit of detection (LOD) of 53.1 μg/mL [19]. While the benchtop spectrometer may not be applicable in lower resource point of care settings, the option of visual or spectroscopic readout contributes flexibility for the test to be used in different ways depending on the limitations of the use case.

The use of fluorescent labels has been investigated as a method for increasing sensitivity and allowing for quantitative determination of Anti-HCV in LFAs. This technology has been leveraged to create multiplexed diagnostics for HCV and other primarily bloodborne pathogens. Ryu et al. developed a multiplexed fluorescent LFA system that detected anti-HCV as well as two markers for Hepatitis B (HBV) and could be run on a small benchtop fluorescent reader. For the HCV LFA component a combination of NS3, NS4 and NS5 were used as the antigens on the test line and anti-human antibodies tagged with europium chelate were used as the fluorescent reporters [20]. The clinical sensitivity and specificity for the HCV component of the developed diagnostic was 98.8% and 99.1%, respectively. Overall, the whole test could be completed in 20 min. A LOD of the HCV component of the biosensor was not determined [20]. Another multiplexed fluorescent LFA to detect Anti-HCV, anti-Human Immunodeficiency virus (HIV), anti-treponema pallidum (TP) and HBV antigen, was developed by Rong et al. in 2021. A customized single cartridge multistrip device that channeled the sample from an initial well into four individual LFA strips using gravity applied by spinning centrifugal force was developed. As a label, nanobeads embedded with quantum dots were used. The label complexes were optically red and fluorescent when exposed to UV light. Quantum dots are semiconductor nanocrystals the exhibit unique electrical and optical properties due to their small size [21]. Using a customized fluorescence reader, a LOD of 0.14 NCU/mL, a clinical sensitivity of 93.59% and a sensitivity of 93% was achieved.

Similar to the anti-HCV ELISAs developed, recombinant proteins have also been used for anti-HCV LFAs. Zhou et al. developed an LFA for anti-HCV using recombinant HCV fusion protein composed of NS3 and HCV core domains as the antigen. An unconventional sandwich method was used for detection, with biotin conjugated HCV fusion protein used as the primary reporter and another recombinant protein made of streptavidin and maltose binding protein domains immobilized on gold nanoparticles as the secondary reporter. The resulting test was able to be completed in 10 min and displayed a clinical sensitivity of 95% and a specificity of 98% [22].

There has also been an investigation into the development of LFAs for joint detection of Anti-HCV and HCV-core antigen (HCVcAg) since the HCV core protein is often present in the bloodstream earlier but in lower amounts than anti-HCV, and anti-HCV alone cannot determine active infection. Patel et al. developed a multiline LFA to assess both HCVcAg and anti-HCV. As the receptor for HCVcAg, they used anti-HCV core antibodies were used, and a combination HCV protein made of NS3, NS4, and NS5 was used as the antigen for patient anti-HCV [23]. A direct sandwich format was used with gold nanoparticle-tagged anti-HCV core and anti-human antibodies to achieve colorimetric reporting [23]. Using the double-test LFA, they demonstrated a 100% clinical sensitivity and specificity, with inverse proportions of samples showing positive for both bands when tested on a seroconversion panel. That is, samples with weak anti-HCV results had a higher proportion of positive HCVcAg results and vice versa, which is in line with the delayed production of anti-HCV and resulting inhibition of the virus [23].

Electrochemical biosensors

Electrochemical biosensors have also been investigated for HCV diagnostics. Electrochemical biosensors work by employing a biorecognition element that, when bound to a desired analyte, causes a change in the electrochemical properties of the system, which can then be quantified [24]. A traditional electrochemical sensor is composed of three electrodes: the working electrode, the counter electrode, and the reference electrode. The working electrode serves as the active surface where a redox reaction will take place and, thus, is where the biorecognition elements are immobilized [24]. The counter electrode serves to complete the circuit, and the reference electrode allows for the comparison of the working electrode against an electrode with a known potential. In development, these electrodes are traditionally individual components, but in rapid testing applications, they are often combined into a single test strip or screen-printed electrode. Screen-printed electrodes essentially combine all the components of an electrochemical cell, the working, reference, and counter electrodes, into a single coated paper strip

with different cast lines of metal or carbon components to act as the necessary electrodes [25].

There are three main categories of measurements used in electrochemical biosensors: potentiometric, amperometric, and impedimetric. Potentiometric sensors measure the change in potential of the working electrodes occurring because of the recognition event on the electrode surface. A simple example of a potentiometric sensor is a pH meter or other ion-selective electrodes [26]. Amperometric sensors, the most common type of electrochemical sensors, work by measuring the current generated by the electrochemical reactions at the working electrode as a result of the recognition event. Home glucose meters are a common example of an amperometric electrochemical biosensor [27]. The final main category of electrochemical sensors is impedance sensors [27]. Impedance sensors work by measuring the amount of impedance across the electrode. A redox compound is used in a solution that naturally reacts at the electrode surface, and as the desired analyte becomes trapped on the surface by the bioreceptor, it begins to interfere with the redox compound, thus increasing the impedance across the electrode. In some cases, the analyte itself can act as the redox compound [27]. The three types of electrochemical sensors are shown in Fig. 5.

To create a more point-of-care (POC) applicable quantitative diagnostic for anti-HCV, expressed HCV core protein as the recognition molecule. Yeast cells with surface-expressed HCV core protein were placed on a gold screen-printed electrode (SPE) and used as the receptor for anti-HCV. In order to make the binding of the Anti-HCV to the yeast protein detectable, a secondary alkaline phosphatase (ALP) tagged anti-human antibody was used, which, when exposed to its substrate, catalyzes a reaction that is detectable using cyclic voltammetry(CV) [28]. Cyclic voltammetry is a potential sweep technique that aids in the determination of electrochemical signal strength (current) as a function of concentration. Using this method, a LOD of 2 nM was achieved as well as a detectable range of 4–250 nM for anti-HCV in buffer

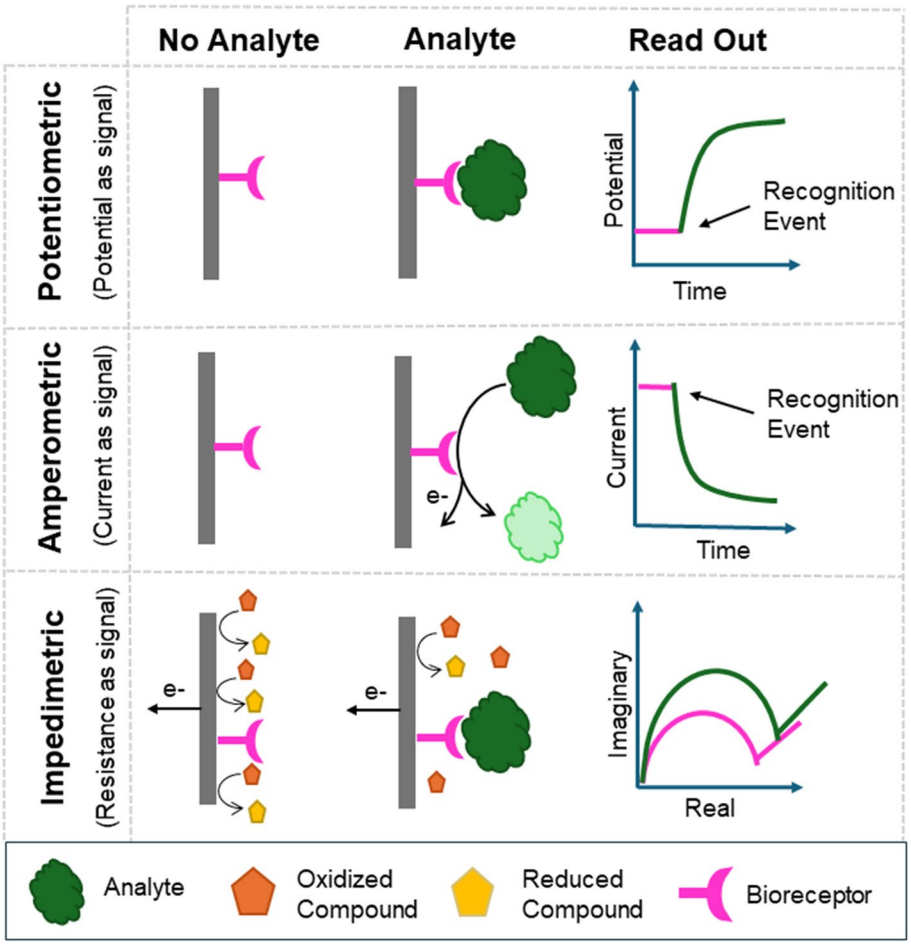


Fig. 5 Interactions on the working electrode for basic electrochemical methods. Counter and reference electrodes are not depicted but are consistent across method type. From top to bottom: Potentiometric, Amperometric and Impedimetric

solution with quantified readout on a smartphone [28]. Venkatesh et al. further investigated the electrochemical yeast model by looking at secreted and released versions of an HCVcAg – gold binding protein (GBP) fusion as the recognition molecule [29]. Using the same ALP reporter system as in Aronoff-Spencer, three strategies for immobilization of the protein on the electrode surface were investigated; protein displayed on yeast membrane, proteins chemically released from the yeast and proteins secreted from the yeast. The secreted protein was found to have the most consistent and stable results but displayed a lower LOD than the whole cell version at 32 nM. Using the secreted construct, a linear range of 32 nM to 3.2 µM as well as significant differences between healthy and infected serum samples was achieved [29].

Most of the recent research into anti-HCV detection, as shown in Table 1, has been limited to assessing clinical efficiency or analytical specificity instead of a holistic approach that evaluates quantification abilities and clinical use. Some of this may be due to the movement away from anti-HCV as a diagnostic target due to its late expression in the infection and its inability to determine active infection on its own. Overall, anti-HCV detection methods have a greater capability for POC use than RNA-based methods but are limited by the need for follow-up testing and minimally reported quantification.

Second level screening—testing for active infections

Screening for HCV viral proteins

The detection of viral proteins represents another approach in HCV diagnosis, offering distinct advantages over RNA and anti-HCV as detectable analytes. Viral proteins serve as markers of active infection, being present only during ongoing HCV infection, in contrast to anti-HCV, which may persist for years following clearance of the infection. Additionally, viral proteins are detectable in the blood much earlier after exposure compared to HCV antibodies, which can take up to two months to reach detectable levels [1, 23]. Despite appearing earlier than antibodies, viral proteins are not detectable as early as viral RNA. In addition, viral proteins eliminate the need for amplification, reducing the equipment and temperature-dependent components required to determine active infection.

The HCVcAg core protein is currently the only viral protein developed for commercial diagnostic use. The Abbott Architect HCV test is the sole commercially available test tailored for HCVcAg, capable of detecting levels as low as 3 fmol/L of protein [35]. Roche has also introduced a joint test that detects both HCVcAg and anti-HCV. Both tests employ CLIAs, a subset of immunoassays similar to ELISAs, where the reporter is a chemiluminescent molecule [36]. Although not currently utilized for HCV, other traditional methods for viral protein detection include ELISAs and latex particle agglutination tests, in which the binding of the desired protein to antibody-coated particles results in agglutination [37].

Electrochemical methods

Many commercial tests require the integration and use of large and expensive diagnostic machines, which limits their application as a point-of-care or low-resource diagnostics. Much of the work into HCV protein detection has been done using electrochemical methods due to their relatively quick detection time and quantifiable results. In both commercial and research scale diagnostics HCVcAg is the most common HCV protein chosen as the analyte. This is largely due to the core protein's conserved nature across various genotypes [5]. Antibodies are a popular receptor for electrochemical sensors due to their high specificity and relative ease of generation. Valipour et al. developed an electrochemical biosensor for HCVcAg using thiolated graphene quantum dots layered on a glassy carbon electrode, with subsequent layers of silver nanoparticles and anti-HCV. Differential pulse voltammetry (DPV) was used to detect the HCVcAg. This method achieved a LOD of 3 fg/mL and a linear range of 0.05 pg/mL—60 ng/mL in spiked phosphate-buffered saline (PBS) solution [38]. Pusomjit et al. developed a similar immunosensor for HCVcAg using a SPE based with single walled carbon nanotubes (SWCNTs) and platinum nanoparticles as a conducive layer. Anti-HCV was used as the bioreceptor, and the signal analyzed using DPV. Using a phone adaptor potentiostat a LOD of 0.015 pg/mL in buffer. The sensor was also shown to be stable for 20 days at 4°C [39].

There has also been an investigation into sandwich-style approaches to increasing signal. Wang et al. used sandwich stacking of antibodies with additional tags to develop an HCV core antigen diagnostic. The initial setup of the electrode used anti-HCV fixed to a gold electrode with BSA as a blocking component. Following exposure to the sample, the electrodes were incubated with additional DNA tagged anti-HCV, forming a sandwich around the captured HCVcAg. To further increase the signal response, terminal deoxynucleotidyl transferase (TdT) was used to extend the length of the DNA tags without a template. The electrodes were then additionally incubated with methylene blue loaded DNA nanowires, which bound to the extended DNA tags, maximizing signal response. DPV in ferri/ferrocyanide solution was used to quantify the HCVcAg detection and resulted in a LOD of 32 fg/mL with a linear range of 0.1–312.5 pg/mL. The primary drawback to this method was the requirement for multiple temperature-sensitive

Table 1 Comparative analysis of anti-HCV diagnostic techniques

Stage of Development	Product/ Reference	Technique	Preprocessing Steps	Required Equipment	Receptor	LOD	Sensitivity %	Specificity %
Commercial	OraQuick [9]	Colorimetric LFA	Fingerpick	None	NR	N/A	NR	NR
Commercial	Abbot Bioline HCV [30]	Colorimetric LFA	Fingerpick	None	NR	N/A	99.3	98.1
Research	[20]	Fluorescent LFA	Blood Draw/ Plasma Separation	Automated Fluorescent Immunoassay System Machine, Centrifuge	NS3, NS4, NS5	N/A	98.8	99.1
Research	[31]	Fluorescent LFA	Blood Draw/ Plasma Separation	Custom Portable Strip Reader, Centrifuge	NR	0.14 NCU/mL	93.59	93.15
Research	[23][a]	Colorimetric LFA	Blood Draw/ Plasma Separation	Centrifuge	Combined HCV Antigen	N/A	100	100
Research	[22]	Colorimetric LFA	Blood Draw/ Plasma Separation	Centrifuge	HCV Antigen	N/A	NR	NR
Research	[19]	Colorimetric/ SERS RVF	Blood Draw/ Plasma Separation	Centrifuge, RAMAN Spectrometer	NR	53.1 µg/mL	NR	NR
Commercial	Acon Foresight [32]	Indirect Colorimetric EIA	Blood Draw/ Plasma Separation	Centrifuge, Plate Reader	Recombinant Core, NS3, NS4, NS5	N/A	99.9	99.8
Commercial	BioRad Ortho [33]	ELISA	Blood Draw/ Plasma Separation	Centrifuge, Plate Reader	c22-3, c200, and NS5	N/A	99.95	NR
Commercial	Liaison Murex [34]	Indirect Chemiluminescent ELISA	Blood Draw/ Plasma Separation	Liaison analyzer	Core & NS4	N/A	98.9	99.7
Commercial	Roche Elecsys Anti-HCV II [10]	Electro chemiluminescence (ECLIA)	Blood Draw/ Plasma Separation	Cobas analyzer, Centrifuge	Recombinant Antigens	N/A	99.67	99.8
Commercial	Roche Elecsys HCV Duo [11][a]	ECLIA	Blood Draw/ Plasma Separation	Cobas analyzer, centrifuge	Recombinant Antigens, Anti-HCV Core	N/A	99.87	99.94
Research	[14]	Indirect Colorimetric ELISA	Blood Draw/ Plasma Separation	Centrifuge, Incubator, Plate Reader	Recombinant multiepitope protein	N/A	98	97
Research	[15][a]	Indirect Sandwich Colorimetric ELISA	Blood Draw/ Plasma Separation	Centrifuge, Incubator, Plate Reader	Core, NS3, NS4, NS5	N/A	100	99.6
Research	[28]	Electrochemical	Blood Draw/ Plasma Separation	Centrifuge, Smartphone Add-on Potentiostat	HCV Core Protein	2 nM	NR	NR
Research	[29]	Electrochemical	Blood Draw/ Plasma Separation	Centrifuge, Potentiostat	HCV Core GBP fusion protein	32 nM	NR	NR

NR Not Reported, *N/A* Not Applicable

[a] Detects both anti-HCV and HCV protein

biologic compounds and the significant time consumed by repeat incubation steps [40].

Other bioreceptors, such as aptamers, have also been investigated for electrochemical detection of HCV proteins. Aptamers are strands of RNA or DNA that fold in a way that allows them to bind or interact with various compounds or biomolecules. While aptamers are often used as receptors and can have high selectivity, they are unable to be directly generated like antibodies. Instead, they must be filtered and modified from a large screening library [41]. The primary method of identifying aptamers with ideal binding behavior for a particular application is a process called Systematic Evolution of Ligands by EXponential Enrichment (SELEX) [41]. The SELEX process works by starting with a large library of oligonucleotides with randomized central sequences and uniform beginning and end regions to allow for efficient amplification. They then undergo repeat screening rounds for interaction with the target protein, where the qualifications for progression to the next cycle become increasingly rigorous, first filtering by the presence of any interaction over a longer incubation time to preferential quick binding in the presence of generalized competitor proteins [41].

Ghanbari et al. designed an impedimetric biosensor using a SELEX identified aptamer as the bioreceptor against HCV core protein. Graphene quantum dots were used on a glassy carbon electrode to increase conductivity and serve as a binding base for the aptamers. Using Electrochemical Impedance Spectroscopy (EIS) a clear concentration dependent increase in the charge transfer resistance was demonstrated and the LOD found to be 3.3 pg/mL [42]. Building on the previous sensor, Ghanbari et al. also developed an electrochemical sensor for HCV core antigen that combined aptamers and a molecularly imprinted polymer (MIP). MIPs are often referred to as "artificial antibodies" and work by forming a polymer matrix around a desired analyte and then removing that analyte to create a molded receptor pocket for the desired protein or compound. MIPs offer advantages over antibodies in that they are more reproducible and have better storage capabilities since they are abiotic. They are also often less specific than antibodies and must be custom generated instead of commercially purchased. The developed sensor used a combined aptamer-antigen complex bound to a multi-walled carbon nanotube-chitosan base layer as the template for the MIP. DPV with ferri/ferrocyanide as the redox compound was used to quantify HCVcAg binding. With this method, a LOD of 1.67 fg/mL was demonstrated which was a significant improvement from the aptamer alone structure [43]. MIPs have also been applied to other HCV proteins. Antipchik et al. created an MIP for E2 on a gold screen-printed electrode. Using ferri/ferrocyanide as the redox compound and DVP as the analysis method. A LOD of 4.6×10^{-4} ng/mL was demonstrated for HCV mimetic particles with E2 expressed on the surface [44].

Other detection methods

Other varied approaches to HCV protein detection have been employed such as the use of graphene field effect transistors (GFETs). GFETs are composed of a graphene channel connected to an electrode on either end. Modifications or binding in the channel effect the electric field channel resulting in changes in conductivity, essentially creating a modulable resistor [45]. Walters et al. developed a GFET to detect HCV core protein in small (5 µL) sample volumes using anti-HCV as the receptor. The final GFET device demonstrated a LOD of 100 pg/mL in spiked PBS solution [46].

Less conventional diagnostics have also been developed. Ozefe et al. created a smartphone-assisted magnetic detection device for the HCV protein NS3. Carboxylated polystyrene microbeads were functionalized with anti-NS3 and placed in a magnetic chamber within a mirror system stand that allowed a phone to be placed on top and used for visualization of the beads. The device operated on the principle of density-associated sinking so that as NS3 attachment to the beads increased, their position in the magnetic field dropped. The final device required 30 µl of sample and could provide a result in three minutes. It demonstrated a LOD of 50 µg/mL [47]. Additionally, Ozefe et al. designed a paper-based microfluidic colorimetric detector for NS3. The paper device had wax-walled channels that distributed the sample into a test well. A standard indirect sandwich immunoassay for colorimetric detection is used, with HRP-tagged anti-goat as the reporter and TMB as the substrate. Using this method, a LOD of 10 ng/mL was demonstrated [48].

Colorimetric detection of HCVcAg using an aptamer assay was developed by Vazquez et al. Three Enzyme Linked Oligonucleotide Assays (ELONA) were developed to confirm aptamer performance in HCVcAg detection. The first was an indirect ELONA wherein histamine tagged HCVcAg was immobilized on the plate, and a digoxigenin tagged aptamer was in solution and eventually bound to anti-DXN which was in turn tagged with HRP. The second ELONA system used a sandwich design using biotin-tagged aptamer bound to the plate and free HCVcAg in solution with the reporter complex mentioned in the indirect ELONA. The final assay was competitive ELONA, where free DXN-aptamer was allowed to bind to free HCVcAg in solution before being put in a well with the same components as the indirect assay allowing for assessment of release rate between the aptamer and HCV [49]. The aptamer showed good,

continued binding resulting in proportional decreases in HRP tagged complexes with an increase in initial solution HCVcAg concentration. For each assay multiple aptamer versions were tested to compare sensitivity and binding properties. Using these assays, a calculated LOD of 2 pM was achieved though the selected aptamer was not directly utilized in an HCV diagnostic format [49].

As shown in Table 2, novel electrochemical methods have already achieved initial LODs of less than 5 fg/mL. Unfortunately, there is little consensus on the conversion between g/mL and the IU/mL standard for HCVcAg diagnostics, making it difficult to determine how close these methods have come to clinical viability. Moving forward, the main challenge will be overcoming the practicalities of whole sample detection such as nonspecific binding and alterations to experimental fluid viscosity. None of the shown studies evaluated their sensor on clinical samples which could significantly reduce the sensitivity of the systems. Overall, HCV protein diagnostics have the advantage of being able to perform one-step diagnosis with detection earlier in infection that Anti-HCV, but most are still relatively early in their development and require significant further work before they can be clinically applied.

Screening for HCV viral RNA

For individuals who tested positive in the antibody test, an additional screening based on RNA, the nucleic acid amplification test (NAAT) is used to determine if the infection is currently active. RNA NAAT tests work by amplifying the viral nucleic acid found in the blood stream. Traditional methods of RNA quantification and detection require comparatively large amounts of RNA as they solely isolate and visualize the existing sample RNA. RNA NAAT testing is able to be much more sensitive because it amplifies the RNA signal. NAAT tests traditionally do this using the polymerase chain reaction (PCR) but can also use other techniques such as loop-mediated isothermal amplification (LAMP). RNA from the blood sample is first isolated and then exposed to an enzyme called reverse transcriptase which translates the RNA into a deoxyribonucleic acid (DNA) strand. This DNA strand then undergoes PCR which makes copies of the desired sequence. Specific primers are employed to only amplify the HCV RNA, and from there the amount of DNA generated by PCR after a given number of cycles can be used to estimate the original concentration of viral RNA in the sample [50, 51].

RT-PCR current standards and improvements

The current gold standard NAAT for diagnosis of active HCV infection is reverse transcription polymerase chain reaction (RT-PCR) due to its ability to detection infection soon after exposure and its high sensitivity allowing for detection of small amounts of HCV RNA found in the blood stream. For this reason, it is also the strategy used to monitor effectiveness of anti-viral medications [1]. Conventional RT-PCR for HCV detection has five

Table 2 Comparative analysis of HCV-Protein diagnostic techniques

Stage of Development	Product/ Reference	Approach	Receptor	Target	Pre-Processing Steps	Required Equipment	LOD
Commercial	Abbott Architect [35]	Chemiluminescent Microparticle Immunoassay	Anti-HCVcAg	HCV-Core Antigen	Blood Draw/ Plasma Separation	Centrifuge, Architect System	3 fmol/L
Research	[38]	Electrochemical	Anti-HCVcAg	HCV-Core Antigen	Blood Draw/ Plasma Separation	Centrifuge, Potentiostat	3 fg/mL
Research	[44]	Electrochemical	MIP	E2	Blood Draw/ Plasma Separation	Centrifuge, Potentiostat	0.46 pg/mL
Research	[42]	Electrochemical	Aptamer	HCV-Core Antigen	Blood Draw/ Plasma Separation	Centrifuge, Potentiostat	3.3 pg/mL
Research	[43]	Electrochemical	Aptamer MIP	HCV-Core Antigen	Blood Draw/ Plasma Separation	Centrifuge, Potentiostat	1.67 fg/mL
Research	[40]	Electrochemical	Anti-HCVcAg	HCV-Core Antigen	Blood Draw/ Plasma Separation	Centrifuge, Potentiostat	32 fg/mL
Research	[46]	GFET	Anti-HCVcAg	HCV-Core Antigen	Blood Draw/ Plasma Separation	Centrifuge, Resistance Monitor	100 pg/mL
Research	[47]	Magnetic Levitation	Anti-NS3	NS3	Blood Draw/ Plasma Separation	Centrifuge, Phone	50 µg/mL
Research	[48]	Immunoassay	Anti-NS3	NS3	Blood Draw/ Plasma Separation	Centrifuge	10 ng/mL
Research	[49]	ELONA	Aptamer	HCV-Core Antigen	Blood Draw/ Plasma Separation	Centrifuge, Plate Reader	44 pg/mL

main steps. The first step is blood collection and serum isolation. A blood sample is taken from the vein in the patient's arm by a trained phlebotomist, and then spun in a centrifuge to separate the red blood cells from the plasma and serum [52]. Once the serum or plasma has been isolated the RNA must then be extracted from the sample using a standardized microbiologic procedure by a trained professional, often completed using a commercial kit. The actual RT-PCR process begins once the RNA has been isolated. The RNA is incubated with reverse transcriptase, as well as with the necessary oligonucleotide primers to transcribe the desired RNA sequence. The process produces complementary DNA (cDNA) which then undergoes PCR. During the PCR process the cDNA is incubated with a thermostable polymerase and primers to initiate the desired sequence. The solution then undergoes thermocycling which heats the formed DNA to denature it and assure it becomes single stranded. It is then cooled enough to allow for the primers to bind in a step referred to as annealing [53]. From there the final step in the cycle, extension, begins when the polymerase binds to the primers and starts creating a complementary strand. The cycle is repeated multiple times to generate exponential copies of the original DNA. The final step in the overall RT-PCR detection process is quantification of the PCR process, determining how many copies were formed which can be used to calculate the amount of initial template and thus how much viral RNA was present in the sample [53]. The most common way to quantify PCR products is using real time quantitative PCR which uses a fluorescent oligonucleotide probe designed to anneal to a mid-section of the desired DNA strand. This probe has a fluorescent tag, as well as a quencher tag, which when in close proximity to the fluorescent tag, as it is on the probe, inhibits the fluorescence. When the DNA polymerase moves along the strand and encounters the probe it will degrade it allowing the fluorescent and quencher tags to separate from each other producing a fluorescent signal that can be detected by a sensor in thermocycler [54]. The conventional RT-PCR process has many points that require long periods of time, specialized equipment and trained personnel, all factors that can increase cost and limit access for resource low communities. Because of this, there have been several innovations focused on reducing the limitations of various steps in this process. An overview of the novel RNA detection methods discussed is shown in Table 3.

Many approaches for improving nucleic acid tests for HCV detection focus on streamlining or improving existing RT-PCR methodology. One avenue of innovation has been improvements to RNA extraction step. Vaghi et al. developed an on-chip microdevice platform that allowed for RNA extraction, reverse transcription, and PCR on the same chip. The initial well was functionalized to adsorb the RNA onto the well surface and retain it for reverse transcription. The cDNA product was then transferred to a secondary non-functionalized well for PCR. The chip still required a thermocycler to maintain necessary temperatures for the RT-PCR steps but did reduce some of the necessary equipment. Using capillary electrophoresis to quantify the PCR product, DNA from clinically infected samples was successfully amplified but the limit of detection as well as the clinical sensitivity and specificity were not evaluated [73].

There has also been an investigation into paper-based devices to reduce instrumentation necessary for reverse transcription. Shiju et al., developed a cellulose membrane using lyophilized reverse transcription reagents with a low operating temperature that allowed for room temperature reverse transcription of the RNA without the need for additional instrumentation. Using this paper device, a LOD of 100 copies/μL as well as 30-day storage stability at 20°C were demonstrated [76].

Other works have focused on making DNA quantification more accessible and eliminating the need for a specialized piece of equipment to quantify the results. In 2022, Kim et al. developed a method for PCR product visualization using primers tagged with biotin and digoxigenin, respectively. The resulting DNA then formed crosslinked aggregates when in solution of Anti-DXN coated and streptavidin coated particles which could be seen with the naked eye. Using this method, a LOD of 10 IU/mL with a 100% clinical sensitivity and specificity was demonstrated, though it should be noted that the alternate viruses that were tested against did not include any close relatives of HCV [74]. Teengam et al. developed a method of HCV cDNA visualization that eliminated the requirement for amplification. Pyrrolidinyl peptide nucleic acid (acpcPNA) was used as a probe for a paper-based device that allowed for visualization of the cDNA within 15 min. AcpcPNAs are synthetic nucleic acids where instead of the standard sugar-phosphate backbone they have a peptide like backbone that makes them more stable and resistant to various degradation enzymes and pHs. A single stranded DNA specific dye was then used to visualize the bound cDNA with an adapted iPhone under black light. Using this paper-based device a LOD of 5.1 pmol, a limit of quantification (LOQ) of 16.9 pmol and a linear region of 5–100 pmol were found [75].

Adaption of existing commercial diagnostics platforms to detect HCV has also been investigated as a way to limit the equipment burden for the RT-PCR process. Llibre et al. developed an adaptation of the standard RT-PCR process for HCV to operate on the Genedrive instrument, a commercial benchtop diagnostic platform. The RNA extraction, reverse transcription and PCR were

Table 3 Comparative analysis of anti-HCV diagnostic techniques

Stage of Development	Product/ Reference	Technique	Preprocessing Steps	Required Equipment	L.O.D	Sensitivity %	Specificity %
Commercial	Roche Cobas [55]	RT-PCR	Blood Draw/Plasma Separation	Cobas Analyzer, Centrifuge	9.2 IU/mL (400 µL), 15.3 IU/mL (200 µL)	NR	NR
Commercial	Abbott RealTime HCV [56]	RT-PCR	Blood Draw/Plasma Separation	RealTime Thermocycler, Centrifuge	12 IU/mL	NR	NR
Commercial	Hologic Aptima Quant Dx [57]	Real-time transcription-mediated amplification	Blood Draw/Plasma Separation	Centrifuge, Panther Analyzer	Plasma: 3.9 IU/mL, Serum: 3.4 IU/mL	NR	NR
Research	[58]	RT-LAMP	Blood Draw/Plasma Separation, RNA Extraction	Centrifuge, Vortexer, Gel Electrophoresis System, Heat Block	100 IU/mL	91.5	100
Research	[59]	RT-LAMP	Blood Draw/Plasma Separation	Centrifuge	500 virons/mL	NR	NR
Research	[60]	RT-LAMP	Blood Draw/Plasma Separation, RNA Extraction	Centrifuge, Vortexer, Heat Block	260 copies/rxn	96	91
Research	[61]	RT-LAMP	Blood Draw/Plasma Separation	Centrifuge, Custom Processor	NR	NR	NR
Research	[62]	RT-LAMP	Blood Draw/Plasma Separation, RNA Extraction	Centrifuge, Heat Block	20 copies/rxn	100	100
Research	[63]	RT-LAMP + Cas9	Blood Draw/Plasma Separation, RNA Extraction	Centrifuge, Heat Block	10 ng/µL	96	100
Research	[64]	RT-LAMP + Cas9	Blood Draw/Plasma Separation, RNA Extraction	Centrifuge, Heat Block, PCB Transducer	1 copy/rxn	NR	NR
Research	[65]	RT-RPA	Blood Draw/Plasma Separation, RNA Extraction	Centrifuge, Vortexer, Heat Block, Fluorescence Analyzer	500 copies/rxn	100	100
Research	[66]	RAA	Blood Draw/Plasma Separation	Centrifuge, Heat Block	10 copies/uL	100	100
Research	[67]	Catalytic Hairpin Assembly	Blood Draw/Plasma Separation, RNA Extraction	Centrifuge, Vortexer, Heat Block	10 fM	NR	NR
Research	[68]	Hybridization Chain Reaction	Blood Draw/Plasma Separation, RNA Extraction	Centrifuge, Vortexer, Heat Block, Fluorescence Analyzer	0.625 nM	NR	NR
Research	[69]	RCA	Blood Draw/Plasma Separation, RNA Extraction, Reverse Transcription	Centrifuge, Vortexer, Heat Block, Fluorescence Analyzer	48 fM	NR	NR
Research	[70]	Exo III Assisted Target Recycling	Blood Draw/Plasma Separation, RNA Extraction, Reverse Transcription	Centrifuge, Vortexer, Heat Block, Fluorescence Analyzer	10 pM	NR	NR
Research	[71]	PCR	RNA Extraction	Centrifuge, Vortexer, PCR Machine, Fluorescence Analyzer	27.1 IU/mL	60	100
Research	[72]	PCR Genedrive	Blood Draw/Plasma Separation	Centrifuge, GeneDrive Instrument	2362 IU/mL	98.6	100

Table 3 (continued)

Stage of Development	Product/ Reference	Technique	Preprocessing Steps	Required Equipment	L.O.D	Sensitivity %	Specificity %
Research	[73]	PCR	Blood Draw/Plasma Separation	Centrifuge, Thermocycler	NR	NR	NR
Research	[74]	PCR (Bead Aggregation)	Blood Draw/Plasma Separation, RNA Extraction, Reverse Transcription, PCR	Centrifuge, Vortexer, Thermocycler	10 IU/mL	100	100
Research	[75]	acpcPNA Tag	Blood Draw/Plasma Separation, RNA Extraction, Reverse Transcription	Centrifuge, Vortexer, Heat Block, Custom Phone Adapted- Fluorescence Analyzer	5.1 pmol	NR	NR
Research	[76]	Reverse Transcription	Blood Draw/Plasma Separation, RNA Extraction, PCR (post)	Centrifuge, Vortexer, PCR Machine with Fluorescence Reader	100 copies/µL	NR	NR
Research	[77]	AuNP Aggregation	Blood Draw/Plasma Separation, RNA Extraction	Centrifuge, Vortexer	4570 IU/ml	93.3	100
Research	[78]	AuNP Aggregation	Blood Draw/Plasma Separation, RNA Extraction	Centrifuge, Vortexer	100 IU/mL	NR	NR

NR Not reported

all completed in individual cartridges within the instrument allowing for a more streamlined process. Using this method, a LOD of 2362 IU/mL was achieved. A clinical sensitivity of 98.6% and clinical specificity of 100% was also demonstrated [72].

Isothermal amplification methods

One of the primary focuses in improving diagnostic PCR for HCV has been on the use of isothermal amplification methods to eliminate the need for the expensive and highly specific thermocycler. Schematics of the isothermal methods that have been applied to HCV diagnostics are shown in Fig. 6. The most common method of isothermal amplification is LAMP. LAMP was first published upon in 2000 and is a strand displacement-based method [79]. It utilizes four primers and a DNA polymerase prone to strand displacement. Initially the innermost primers bind to the template strand and polymerization is initiated [79]. The outermost primers then bind further back on the template and polymerization begins again. As the outer primer-initiated strand encounters the inner primer it pulls it up and separates it from the template, which begins the stand displacement process. The displaced strand then forms a loop at the end as the inner and outer primer regions interact. From there the inner primers continuously attach to the formed loop region initiating and displacing new stands eventually forming a zigzag like set of strands with loops at each turn which occasionally separate into smaller components and then continues the generation – replacement process [79].

HCV genotype can play a key role in determining the course of antivirals prescribed to a patient. Nyan and Swinson used reverse transcription LAMP (RT-LAMP) to detect HCV and differentiate HCV RNA by genotype. Custom primers were used to amplify multiple sequences in the non-coding region of the HCV genome, allowing for variance in amplification product that can be visualized in the band patterns of a gel. The commercial GelGreen UV-fluorescent DNA stain was used to quantify DNA production. A consistent LOD at 100 IU/mL was found but the test was able to demonstrate detection as low as 10 IU/rxn when evaluating on a gel for 40% of the tests. The method was also evaluated on clinical samples and demonstrated a 91.5% diagnostic sensitivity and 100% diagnostic specificity [58].

One of the most prominent challenges to conventional NAATs is the need for trained personnel and specialized equipment; because of this there have been multiple investigations into the use of microfluidic or cassette systems to streamline the process. Sharma et al. developed a multichambered microfluidic device to detect HCV RNA utilizing RT-LAMP. Magnetic beads were used to attract the RNA and direct it through the different chambers using an automated magnetic bar. The first chamber included a lysis buffer to allow the RNA in the sample to associate with the beads. To purify the RNA, the beads were then moved through a series of chambers with alternating oil-buffer solutions. The RT-LAMP reaction occurred in the final chamber with an integrated heater underneath to facilitate the process and the DNA dye SybrGreen was used to quantify the result. A chip detector was able to detect the HCV at a LOD of 500 virions/mL after 45 min of processing time [59]. Seok et al., expanded the use of RT-LAMP and microfluidics to develop a multiplexed cassette device that detected HCV, HBV, and HIV simultaneously. The device used a paired slider set up wherein the sample sat on a lower slider and was moved through the cassette in time with an upper slider which activated successive blister packs for each part of the process. A fluorescent green DNA dye was used to quantify the results which were able to be captured with a camera using an attached LED to excite the dye. The multiplex diagnostic demonstrated a clear result with 6.9×10^4 IU/μL of HCV but did not evaluate the analytical sensitivity with HCV.

RT-LAMP has also been used to detect HCV in paper-based applications. In 2021, McConnell et al. demonstrated a detection device that combined an RT-LAMP reaction chamber with a LFA strip. For the LFA, gold nanoparticles conjugated with antibodies which bind to a tag on the DNA were used as the reporter, the tag in this case was a biotin molecule covalently attached to the forward loop primer. On the test line antibodies for fluorescein isothiocyanate (FITC) were adhered to the strip and used to bind with the FITC tag on the backwards primer thus tying the gold nanoparticle to the strip resulting in a red color visible to the naked eye. The detection device was able to demonstrate a LOD of 260 copies/rxn and had a clinical sensitivity of 96% with a 91% specificity [60]. Shi et al. also combined RT-LAMP with an LFA to achieve a LOD of 20 copies/rxn [62].

Pairing isothermal amplification methods with LFA readout systems has been a popular method to overcome the relatively poor analytical sensitivity afforded by LFAs and the general need for advanced equipment for DNA quantification. Wang et al. utilized recombinase aided amplification (RAA) in combination with a LFA strip to create an HCV diagnostic. RAA operates by using a mix including recombinase which is natively part of the repair pathway, a displacing polymerase, primers for the desired sequence, crowding agents, and single stranded binding proteins. The crowding agents, recombinase and primers form a complex that looks for homologous regions in the DNA and then inserts the primer, thus opening the DNA. From there, polymerase comes in and extends the new strand. This then repeats

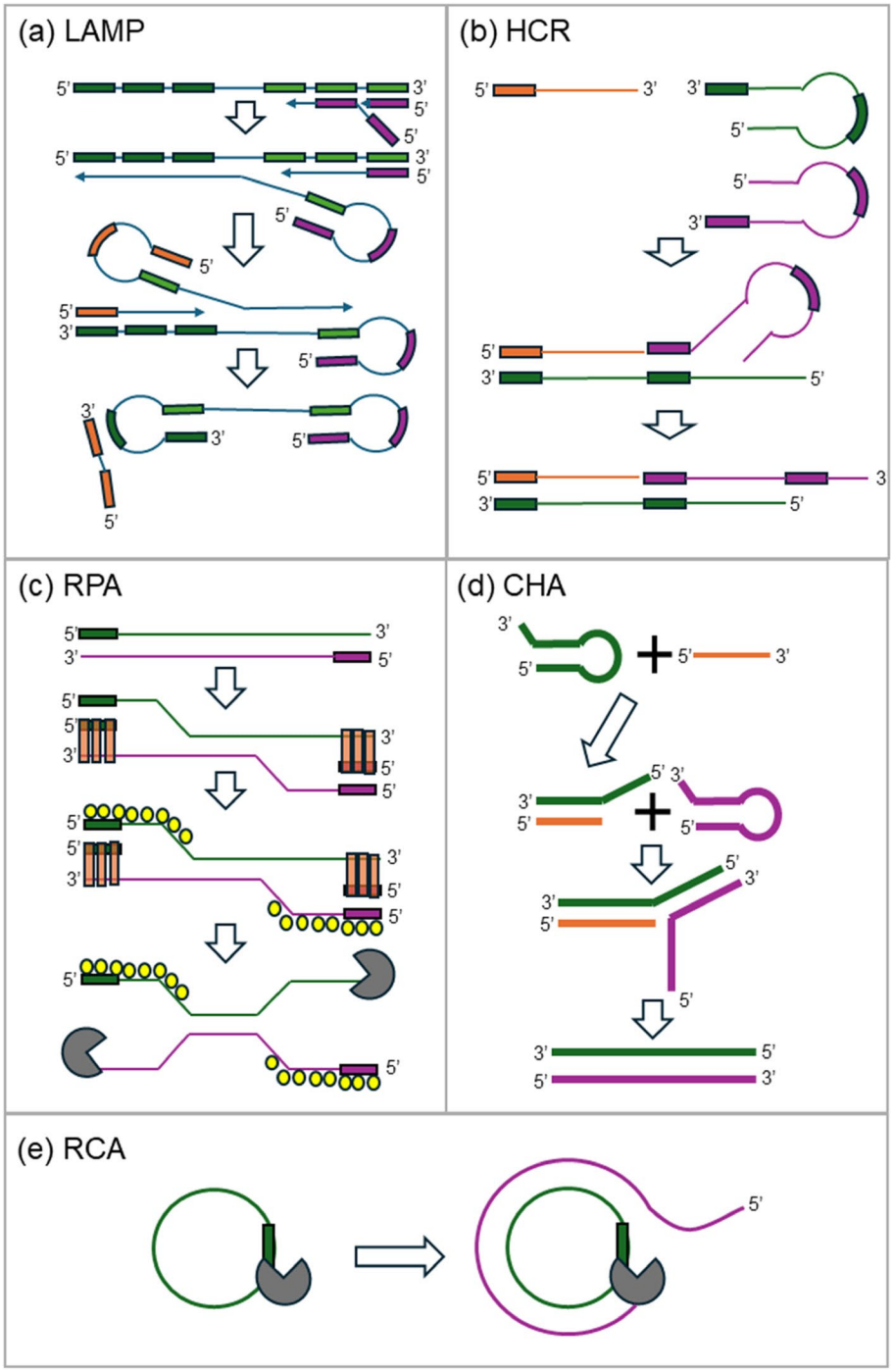


Fig. 6 Methods of isothermal nucleic acid amplification. **a** Loop mediated amplification (**b**) Hybridization chain reaction (**c**) recombinase polymerase amplification (**d**) catalytic hairpin assembly, (**e**) rolling circle amplification

to amplify the target DNA [80]. For this diagnostic, the RAA process was paired with an LFA that used a similar system to the LAMP-LFA mentioned previously and used biotin-FITC tagged primers as the label component. The system was found to have a 100% clinical specific and sensitivity with a detection limit of 10 copies/µl [66].

Su et al. used a similar diagnostic structure of an amplification reaction followed by transfer to an LFA. Instead of RAA, catalytic hairpin assembly (CHA) was utilized.

CHA is an isothermal amplification method which is advantageous in that it only requires tagged DNA strands and no enzymes. This reduces the need for cold chain preservation of the components. Catalytic hairpin assembly works by having primers which will fold to form a hairpin with themselves as well as bind to each other when straightened out. The primer initially opens to bind to the target DNA which leaves a portion of the primer that was folded over is now exposed which then binds to the alternate primer and folds down to form a double stranded DNA and recycles the template strand [81]. The sample DNA acts as a trigger for the primers to anneal together. In the case of this HCV diagnostic the primers were tagged with biotin and digoxigenin (DXN) so that when the amplified DNA was put on the LFA strip the biotin bound to streptavidin coated gold nanoparticles and then the digoxigenin bound to antibodies on the test line. This test was able to achieve a LOD of 10 fM with a linear range of detection of 10 nM-10 pM using fluorescent quantification [67].

The use of a paired LAMP-Cas12 systems has also been investigated as a method of achieving high sensitivity reporting of LAMP products. CRISPR/Cas 12 is a bacteria nuclease employed for its combined target and non-target cleavage. In LAMP applications, it is used to identify and cleave the target DNA output from the amplification process as well as cleave reporter non-target ssDNA, which is tagged on each end with a quencher and fluorophore, respectively. This cleavage of the reporter then allows for a fluorescent signal, which can be used to quantify the amplification output. Kham-Kjiing et al. used this method paired with an LFA readout to develop a diagnostic with a 10 ng/μL LOD, 96% sensitivity, and 100% specificity [63]. The LAMP-Cas12 system has also been employed in combination with a field effect transistor sensor to achieve an LOD of one copy/rxn for synthetic HCV RNA [64]. While the application of CRISPR systems for HCV diagnostics has been limited, they have been applied to other viral diagnostics in many ways. CRISPR technology is commonly used for diagnostics in two main ways. The first is that it can be used as a reporting tool to create an amplified signal through sequence-specific cleavage, such as described above [82]. The second is that CRISPR systems can be used as a checkpoint for isothermal amplification methods [83, 84]. Isothermal methods are often prone to non-specific amplification and the sequence specificity of a CRISPR system can be used to ensure that only desired amplification is reported to the user [85, 86]. Since CRISPR systems can be operated isothermally their benefits can be utilized without compromising the POC use goals for many diagnostics.

Another isothermal amplification method that has been applied to HCV is rolling circle amplification (RCA). The primary mode of action is the use of a specially designed circular single-stranded DNA, which is copied on a constant loop. The polymerase binds to the primer, completes the circle, and then displaces the previously made strand creating a long strand with many repeats of the same region. Wu et al. developed a method for HCV RNA detection using RCA and Exonuclease III-assisted target recycling amplification. The target DNA was captured by a custom DNA strand with a padlock probe region and a strand complementary to the target. When the target bound, it signaled the exonuclease to cleave the complementary strand and release the target. The single-stranded DNA fragments from the exonuclease were then used to hybridize the padlock region forming a full circle and initiating the RCA. The probe sequence was designed to have cysteine-rich sequences, which could accumulate silver nanoparticles when incubated in a solution of AgNO3, resulting in a fluorescently detectable signal. This paired amplification method resulted in a LOD of 48 fM and a linear region of 10^2–10^7 fM [69]. Exo II-assisted target recycling has also been used on its own in HCV detection. Wu et al. developed a method that used FeOOH to reduce the inhibition of fluorescent signal allowing for more sensitive detection of HCV cDNA. Fluorescently tagged DNA probes are attracted to and inhibited by the FeOOH nanosheets. When in the presence of target DNA, the probe formed a double-stranded complex with the target, initiating the degradation of the probe by the exonuclease. This then caused the probe to be in such small fragments that it was no longer attracted to the nanosheets and became free floating in the solution which allowed for the tag to fluoresce. Using this method, a LOD of 10 pM was achieved [70].

Other isothermal amplification methods have also been explored for HCV detection. Chia et al. used reverse transcription recombinase polymerase amplification (RT-RPA), which allowed for a faster run time (10—15 min) and lower operating temperature (35—42 ^{0}C) than LAMP. RPA works in a very similar way to RAA. The primary difference is the source of recombinase; RPA uses a bacteriophage recombinase whereas RAA utilizes recombinase from bacteria or fungi [87]. For HCV detection, two strategies were investigated: one using a single step method, the other split into RT and RPA steps. The two-step assay was found to perform better on real samples and displayed a LOD of 25 copies/rxn for genotype 1, with higher LODs for the other genotypes, with the highest LOD being 500 copies/rxn for genotype 3. The test also demonstrated 100% clinical sensitivity and specificity using 78 samples [65].

Hybridization Chain Reaction (HCR) is another non-enzymatic isothermal amplification method. The primary difference between HCR and catalytic hairpin assembly is that it is structured to that the primers can add infinitely to a single strand [88]. Zhang et al. investigated the use of HCR in HCV RNA detection in an unconventional way, measuring the RNA as an inhibitor not as an initiator. An HCR system was used that included an initiator so that without target DNA the HCR would progress exponentially. If target DNA was present, it would take the place of one of the hairpin primers and prevent additional primers from being added on. The detection of the cycle inhibition was done by measuring fluorescence. Each hairpin had a fluorescent and a quencher tag and when the oligonucleotide was in hairpin shape the fluorescent and quencher were close enough to turn of the fluorescence but when stretched out in a double stranded DNA was allowed to fluoresce. A LOD of 0.625 nM was demonstrated though there was minimal quantification ability at higher concentrations [68].

Other novel reporter methods

Isothermal methods and traditional RT-PCR both rely on reverse transcription and a period of amplification to be effective. To streamline HCV RNA detection even further Shawky et al. developed a method for direct RNA detection without amplification using gold nanoparticle aggregation. Citrate capped gold nanoparticles (AuNPs) coated with HCV specific RNA probes were used, that when heated with the RNA, annealed the viral RNA to AuNPs. Cysteamine AuNPs were then added which when viral RNA was present adhered to the outer edges of the viral RNA staying relatively separated but when no RNA was present formed aggregates on the citrate capped particles. These aggregates affected the optical color turning the solution from a red to dark purple. The RNA was then quantified using absorbance at 530 nm. This method allowed for a LOD of 4570 IU/mL, a clinical sensitivity of 93.3% and a clinical specificity of 100% [77]. Mohammed et al. used a similar method to directly detect HCV RNA using citrate capped AuNPs but instead of using probes directly attached to the AuNPs, probe hybridization with the target was used to stabilize the AuNPs when subjected to salt induced aggregation conditions. This method resulted in a 30-min runtime and demonstrated a LOD of 100 IU/mL [78].

Limitations of current diagnostics

The current two step screening process for HCV has several limitations that inhibit its effectiveness, especially for high-risk, low-resource populations. One of the primary limitations is the time to result. Tests for Anti-HCV, though they may come in the rapid form of LFAs, cannot determine active infection. Thus, after Anti-HCV testing, which can take from 20 min to 3 days depending on the type of test used to return a result, an additional sample must then be taken for NAAT testing. Though individual NAAT tests can be completed in as little as three hours, given the need for highly specialized equipment and trained technicians, samples must generally be collected at POC or point of service (POS) facilities and be shipped to diagnostic labs. This adds more time to get a result and can take a few days up to a week [89]. This high resource demand screening is one of the primary reasons NAAT testing is not used as an independent diagnostic. The need for specialized equipment and personnel is another core limitation of the current testing scheme. This prevents POC diagnosis which can severely limit access for high-risk populations in both high and low- and middle-income countries (LMIC) since those most at risk often have limited ability to access centralized health care, because of cost, time, or travel necessities [90, 91]. High risk populations in low prevalence countries are often injection drug users and many times unhoused making patient retention for repeat assessments difficult [92]. In LMICs, health centers are often few and far between, making trips to them arduous and a significant investment for patients in addition to the general lack of trained personnel available [93].

There are also limitations to the research scale investigations completed so far for HCV diagnostics. All the Anti-HCV diagnostics that have been recently developed require pre-processing in the way of professional blood draw and plasma separation which increases necessary resources for the test. However, Anti-HCV detection methods overall have improved the need for test specific equipment as many of the LFA based diagnostics do not require any additional equipment or utilize a small benchtop device that could be used for detection of multiple diseases. Notably, there have also been reductions in the equipment necessities for electrochemical sensors through the use of small portable potentiostats that function as a smartphone add-on instead of having a benchtop potentiostat [28].

For RNA based diagnostics, there was a significant variance in reported detection limit even amongst those that use the same approach, RT-LAMP based methods alone demonstrated LODs from 100 – 11,818 IU/mL [58, 60]. Isothermal methods, though promising, are also prone to non-specific amplification, primer-dimers and carryover contamination which must be carefully controlled for [94, 95]. Additionally, many of the novel RNA diagnostics did not evaluate effectiveness on actual samples therefore their real-world detection capabilities may be poorer than what was reported. Overall, RNA based diagnostics have the advantage of being standalone tests capable of

early and sensitive detection but are still limited by the amount of time and equipment they require. For many of these methods, maintenance of cold chain materials is an additional concern since they largely rely on the use of oligonucleotides or enzymes that will degrade without careful storage.

Viral protein-based diagnostics offer an alternative to RNA for diagnosis of active infection, but no research scale development has gotten close to the level of analytical sensitivity established by the commercial architect test. Like the RNA diagnostics, investigations into HCV protein as an analyte lacked evaluation on clinical samples. They also did not establish stability longer than 20 days, even in refrigerated conditions which indicates a need for further improvement to make them practically applicable [38].

Though there have been significant improvement over commercial timelines, as shown in Fig. 2, testing time continues to be an important factor in developing the next generation of HCV diagnostics. When accounting for the time included in preprocessing steps RNA based methods on average are the slowest to return a result whereas Anti-HCV and protein-based methods have lower overall times. Non-RNA diagnostics still maintain an average testing time of over 30 min which is higher than the optimal testing time of 15 min established by the NIH endorsed target product profile discussed below [96]. In addition, as shown in Table 4, there has not been a demonstration of any method that could diagnosis active infection in under 45 min when accounting for sample preparation and readout times. This shows how far there is to go towards developing a diagnosis that could meet the optimal requirements established in the TPP.

Potential approaches for one step diagnosis

Target product profile

In 2017 a target product profile for the creation of new HCV diagnostics was developed by gathering census from stakeholders in industry, government, and public advocacy [96]. This TPP was adopted by the National Institute of Health (NIH) as part of their call for research around HCV [97]. The developed profile described requirements for tests that could stand alone or serve as the second step in a two-step system. Stakeholders did not unanimously agree that single step systems were the best policy [96]. While one-test methods offer convenience and may increase successful diagnosis the restrictions provided by a two-step system also serve as a method of screening patients by likelihood to be complaint with a treatment regime. This is of particular concern given the risk of developing drug resistance due to inconsistent adherence to treatment. The profile outlined many minimum and ideal requirements for new diagnostics that could determine active infection, focusing on RNA and HCVcAg as analytes. Key requirements are shown in Table 5. The most basic requirements were those for sensitivity and specificity. The optimal analytical sensitivity was determined to be 200 IU/mL, and the minimum sensitivity was 1,000 to 3,000 IU/mL. The optimal diagnostic specificity was 99%, with 98% being the minimum acceptable specificity. Sample type was also a

Table 4 Duration of diagnosis in relation to recent advancements in HCV testing methods

Analyte	Shortest	Longest	Median	Reference
Anti-HCV	30 min [22]	230 min [28, 29]	80 min	[14, 15, 20, 22, 23, 28, 29, 31]
Viral RNA	50 min [66]	240 min [73]	110 min	[59–78]
Viral Protein	23 min [47]	170 min [40]	55 min	[38, 40, 42–44, 46]

Preprocessing steps accounted for using assumed values based on standard protocol when not thoroughly described in the investigational report. The assumed values are as follows: Blood Draw—5 min, Plasma Isolation – 15 min, RNA extraction – 30 min, Reverse Transcription—30 min and Gel Electrophoresis – 50 min. Shortest, longest, and median time determined from listed references

Table 5 Key target product profile metrics for new diagnostics of active infection [96]

Metric	Optimal	Minimum
Time to Result	> 15 min	> 1 h
Analytical Sensitivity	200 IU/mL	3000 IU/mL
Specificity	99%	98%
Specimen Type	Capillary whole blood	Venous whole blood or plasma
Instrumentation	Instrument free	Separate sample preparation device allowed
Specimen Preparation	Integrated sample preparation (no precision time or volume required)	≤ 2 Steps (no precision time or volume required)

primary metric for developing HCV diagnostics. Capillary blood was considered to be the optimal sample because of its minimal invasiveness and because, unlike venous blood that must be collected by a phlebotomist, capillary blood can be collected by someone with minimal training. Another key requirement defined in the TPP was time; optimal tests would return a result within 15 min, with the minimum requirement being able to provide a result within an hour. Even the minimum time requirement established is considerably shorter than current commercial methods for active infection diagnosis. The TPP also outlined more directly practical parameters, such as the necessary experience of the operator and the necessity for the test materials to be able to be transported without cold-chain requirements [96].

Future innovation

Research-scale diagnostics for HCV have mainly focused on improving two key metrics, time, and equipment requirements. Going forward there are two key technologies that offer a path towards rapid POC one-step HCV diagnosis: isothermal amplification of RNA and electrochemical detection of HCVcAg. The use of isothermal amplification methods for viral RNA detection significantly reduces equipment requirements by replacing the need for an expensive thermocycler with a simple heat block. Isothermal methods are often also quicker than traditional PCR with time to result including preprocessing steps around an hour. HCVcAg based diagnostics also offer improvements in time and equipment requirements. HCVcAg testing requires less pre-processing before analysis due to the lack of amplification steps and generally only requires a single readout instrument such as a potentiostat. However, RNA remains the most preferable analyte because it is capable of determining viral genotype and because as HCV mutates the core protein may become less recognizable to a developed sensor. The use of incorporated cassettes that mix components and transfer the sample through reaction chambers as necessary also go a long way towards reducing the technical competence necessary to complete the tests [59, 61].

So far, much of the research on HCV diagnostics has been focused on individual detection steps. The next step in moving towards practically functional detection methods is the integration of each of these steps into a single test. Diagnostic tests can be generally separated into three steps: sample collection, processing, and readout. Sample collection for most methods includes blood collection followed by centrifugation to isolate plasma or serum. For RNA amplification methods, this can also include RNA isolation and reverse transcription. The processing step then includes the primary test. For RNA methods this is the amplification step, be that through isothermal methods or standard PCR. For protein methods, this is the addition of the necessary buffers, redox components, and substrates for the readout. Finally, the readout is the component that delivers the result of the test. This can be an LFA, electrochemical signal, or other fluorescent or colorimetric output.

For each step there are innovations that can be leveraged to produce integrated diagnostics that could meet the target goals. While significant investigation has gone into reducing the equipment and components of HCV tests, relatively little work has been done to integrate these novel testing methods with simple sample preparation and collection. Most proposed methods require relatively large samples of purified plasma/serum (> 50 μL) which creates the requirement for both a trained practitioner to collect venous blood since it is greater than the volume produced by a finger prick as well as require a centrifuge to separate the plasma from red blood cells [98]. For sample collection, the integration of a plasma or serum isolation method such as microfluidics or membrane filtration could be used to isolate plasma or serum from whole blood samples and eliminate centrifuge requirements. For both methods, ensuring that additional specialized equipment is not necessary is key. Membrane filtration approaches rarely require additional instrumentation since capillary action is the major driver of flow through the system, but they are also at risk of filtering out desired analytes. For microfluid approaches, simple pressure systems such as the use of a manual plunger or finger pump may be successful at providing fluid flow through the system without the need for excess instrumentation [99, 100]. Integration of elution buffers for dried blood spots could also be used to improve the viability of the diagnostics in low-resource settings. Blood spots are often a preferred method of sample collection for methods which require centralized equipment since they can be collected by the patient themselves and are more robust in transport than liquid samples [101] For RNA diagnostics it will be necessary to incorporate RNA isolation into the sample processing component such as in Sharma et al. [59]. Overall, there also needs to be further optimization to develop systems and methods that can utilize the small sample volumes obtained from capillary blood collection. The ideal system would likely operate similarly to at-home glucose monitors where a small sample of the capillary can be collected by minimally trained personnel and deliver a result at the POC.

For the processing step, the main innovations are dependent on the analyte. For RNA amplification diagnostics, the use of isothermal methods could reduce equipment and time requirements. However, additional investigation into isothermal methods needs to be completed in search of components to allow room

temperature reactions so that a heat block or external running device is not necessary. Some work has been done into this such as the RT system developed by Shiju which was able to operate at room temperature [76]. Practical charting of run-time efficiencies as they relate to ambient conditions should also be completed. Effective diagnostics will need to be operatable in conditions that may have variable temperature and humidity and knowing the effect of these conditions on how to run the tests will be necessary. Combining these isothermal methods with blister-pack or otherwise simple fluid control systems such as in Seok and Sharma could also serve to improve the expertise requirements of diagnostics, allowing for disposable tests to be completed with minimal training [59, 61]. The use of cassette fluidic systems could also be applied in antigen diagnostics to automate buffer application and reduce the necessary user expertise. In this vein, designs that utilize disposable or single use components will be the way forward since most point of care facilities will lack the infrastructure for sterilization and system reloading would require technical expertise. Additionally, for processes and systems with higher inherent equipment burden, tailoring the diagnostic to fit into an extensively used high investment piece of equipment may allow for higher accuracy without the need for additional equipment purchases. For example, the GeneXpert system by Cepheid, has many commercial diagnostic tests for several likely comorbidities and key diseases increasing the likelihood such a system would be already present at a core medical facility [102].

These process improvements then must be paired with accessible readout methods. LFAs are the most traditional low-tech readout display and can produce naked eye colorimetric qualitative results. They are not as inherently sensitive as other methods, and further work into high efficacy reporting tags and capillary systems will be necessary to make them truly effective as a readout method. Combining LFAs with amplified RNA systems could overcome sensitivity hurdles by simply using the amplification steps to produce excess signal that could be easily displayed even from a low initial starting condition. For electrochemical methods, the use of portable battery powered potentiostats could reduce equipment burdens while still allowing for the sensitivity and quantification advantages inherent to those systems. The use of phone-adaptor components such as add-ons for fluorescent capture could also serve to make readout more feasible and reduce the resource burden.

There are several additional improvements that could further increase the utility of developed HCV diagnostics. The first is the development of multiplexed tests that can detect multiple diseases, particularly those such as HIV infection, which can increase susceptibility and severity of HCV infection. Multiplexed testing for severity markers such as ALP, which is upregulated in hepatocellular carcinoma, could provide additional insight for physicians when establishing treatment and monitoring plans. The second is the development of genotype-specific readouts. Direct-acting antiviral (DAA) regimens are informed by the HCV genotype, and knowing the genotype the patient is infected with will change which drugs are most effective. Lastly, further investigation and development into component storage methods are needed in order to develop shelf-stable diagnostics that can achieve the non-cold chain transport requirements established in the TPP. Many of the proposed diagnostics involve multiple biologics which must be temperature controlled and did not effectively evaluate storage stability. When reaching at-risk populations, it is essential that these diagnostics be stable long enough to both get to the point of care and be able to sit on the shelf without deteriorating immediately.

Overall, there has been considerable progress made toward improving HCV diagnostics from their current commercial standards. Many novel approaches to HCV diagnostics have yet to fully integrated from sample collection to readout which especially for more molecularly complicated methods results in diagnostic time not accounted for by the length of the test itself but that nonetheless would be important in clinical use.

Abbreviations

ABTS	2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
acpcPNA	Pyrrolidinyl Peptide Nucleic Acid
ALP	Alkaline Phosphatase
AuNP	Gold Nanoparticle
BSA	Bovine Serum Albumin
cDNA	Complementary Deoxyribonucleic Acid
CHA	Catalytic Hairpin Assembly
CV	Cyclic Voltammetry
DAA	Direct Acting Antiviral
DNA	Deoxyribonucleic Acid
DPV	Differential Pulse Voltammetry
DXN	Digoxigenin
ECLIA	Electrochemiluminescence Assay
EIS	Electrochemical Impedance Spectroscopy
ELISA	Enzyme Linked Immunoassay
ELONA	Enzyme linked Oligonucleotide Assay
FITC	Fluorescein Isothiocyanate
GBP	Gold Binding Protein
GFET	Graphene Field Effect Transistor
HBV	Hepatitis B Virus
HCR	Hybridization Chain Reaction
HCV	Hepatitis C Virus
HCVcAg	HCV Core Antigen
HIV	Human Immunodeficiency Virus
HRP	Horseradish Peroxidase
IU	International Units
LAMP	Loop-mediated Isothermal Amplification
LFA	Lateral Flow Assay
LFIA	Lateral Flow Immunoassay
LMIC	Low- and Middle-Income Countries
LOD	Limit of Detection
MIP	Molecularly Imprinted Polymer
NAAT	Nucleic Acid Amplification Test

NCU	China National Clinical Units
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
POC	Point of Care
POS	Point of Service
RAA	Recombinase Aided Amplification
RNA	Ribonucleic Acid
RPA	Recombinase Polymerase Amplification
RT-LAMP	Reverse Transcription Loop-mediated Isothermal Amplification
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RVF	Rapid Vertical Flow Assay
SELEX	Systematic Evolution of Ligands by Exponential Enrichment
SERS	Surface Enhanced Raman Spectroscopy
SPE	Screen Printed Electrode
SVR	Sustained Virological Response
SWCNT	Single Walled Carbon Nanotube
TdT	Terminal Deoxynucleotidyl Transferase
TMB	3,3′, 5,5′ Tetramethylbenzidine dihydrochloride
TP	Treponema Pallidum
TPP	Target Product Profile

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