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Review

Current and Future Diagnostics for Hepatitis C Virus Infection

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Abstract: Hepatitis C virus (HCV), a member of the Flaviviridae family, is an RNA virus enclosed in an envelope that infects approximately 50 million people worldwide. Despite its significant burden on public health, no vaccine is currently available, and many individuals remain unaware of their infection due to the often asymptomatic nature of the disease. Early detection of HCV is critical for initiating curative treatments, which can prevent long-term complications such as cirrhosis, liver cancer, and decompensated liver disease. However, conventional diagnostic approaches available, such as enzyme immunoassays (EIAs) and polymerase chain reaction (PCR)-based methods, are often costly, time-intensive, and challenging to be implemented in resource-limited settings. This review provides an overview of HCV disease and the structural components of the virus, illustrating how different diagnostic methods target various parts of the viral structure. It examines current diagnostic tests and assays, highlighting their mechanisms, applications, and limitations, which necessitates the development of improved detection methods. Additionally, the paper explores emerging technologies in HCV detection that could offer affordable, accessible, and easy-to-use diagnostic solutions, particularly for deployment in low-resource and point-of-care settings. These advancements have the potential to contribute significantly to achieving the World Health Organization's (WHO) target of eliminating HCV as a public threat by 2030.

Keywords: hepatitis C; point-of-care (POC); molecular testing; HCV diagnostics; microfluidics; PCR

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1. Introduction

The hepatitis C virus (HCV) is an enveloped RNA virus belonging to the Flaviviridae family [1]. The virus is transmitted parenterally through multiple routes, including sharing needles and syringes, nosocomial transmission, and blood transfusions [2,3]. Men who engage in sexual activity with other men face an elevated risk of contracting the disease, especially those infected with HIV [2]. Moreover, the transmission of HCV can occur

vertically from the mother to the child during pregnancy [4]. HCV was first discovered in 1989 when Choo et al. found complementary DNA that was constructed from a patient who received a diagnosis of non-A, non-B viral hepatitis (NANBH) [5,6]. Prior to this discovery, the primary mode of viral transmission was the transfusion of contaminated blood products. Kuo et al. later created the first specific assay to detect HCV from circulating HCV antibodies [6]. At the time of the discovery of HCV, it constituted up to 90 percent of transfusion-associated hepatitis cases [6].

HCV prevalence is estimated to be around 50 million people [7]. The HCV virus has six genotypes: HCV 1, 2, 3, 4, 5, and 6 [8]. The most widespread genotype is 1, accounting for 59.7% of global infections [9]. Furthermore, genotypes 3, 2, and 4 comprise 25.7%, 8.6%, and 3.8% of HCV cases worldwide, respectively [9]. HCV genotypes 1 and 3 are the most common worldwide; however, genotypes 4 and 5 are more common in lower-income countries [9]. As per WHO, the Eastern Mediterranean region exhibits the highest prevalence globally, while the Americas have the lowest prevalence (Figure 1) [7]. WHO specifies the main hurdles in these developing countries as limited decentralization of testing, lack of local production of in vitro diagnostics (IVDs), and the inability to sustain the cost associated with expensive tests and employ human resources to process them [7]. A total of 1.3 million deaths were attributed to viral hepatitis in 2022, which was the second leading cause of death in communicable diseases after COVID-19 [7]. The majority of deaths were caused by hepatitis B (HBV-83%) and HCV (17%) [7]. The WHO estimates that out of the 36.7 million people with HIV, an estimated 2.3 million individuals, ranging from 1.3 to 4.4 million people, are co-infected with HCV [10]. This is primarily due to the shared transmission routes, such as injection drug use and unprotected sexual contact.

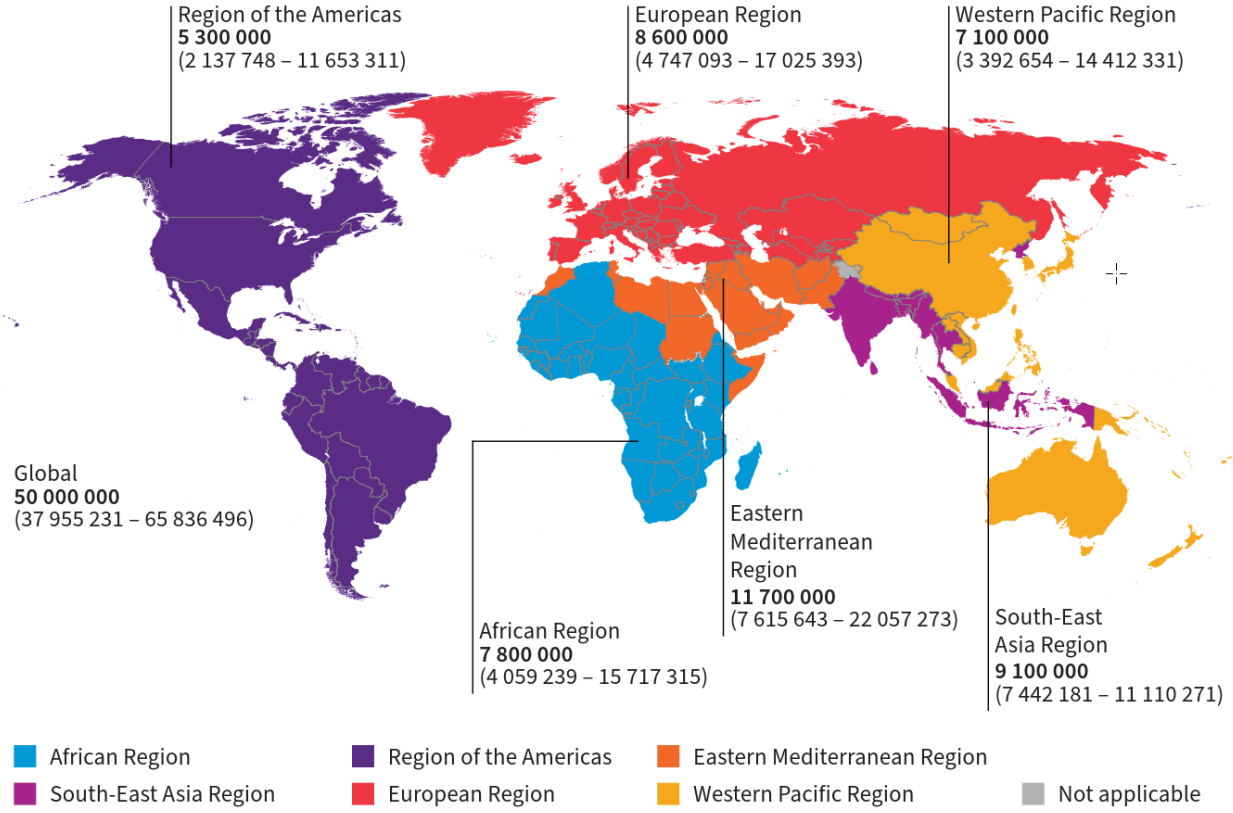

Figure 1. The world map shows the prevalence of chronic HCV globally in 2022. While HCV is prevalent throughout the world, the Eastern Mediterranean region exhibits the highest prevalence [7].

HCV can cause either acute or chronic hepatitis [11,12]. Acute HCV presents similarly to other acute hepatitis viruses clinically, with symptoms encompassing malaise, nausea,

jaundice, right-upper-quadrant pain (RUQ), and bilirubinuria [12]. The average HCV incubation period is 14–84 days [3]. A small percentage of acute HCV infections become fulminant hepatitis, also known as acute liver failure [13]. Acute HCV infections lead to chronic HCV infections in 75–85% of the infected people [11]. Patients with chronic hepatitis frequently experience a long-term, asymptomatic course. However, the infection can progress to hepatic failure and hepatocellular carcinoma [11].

Since no vaccine currently exists for HCV, effective treatment depends upon early diagnosis and antiviral medication. Direct-acting antivirals (DAAs) for HCV are oral medications taken over an 8–12-week period and have been shown to significantly improve cure rates to achieve a sustained virologic response (SVR) in over 90% of patients, indicating no detectable levels of the virus [14]. Effective DAA regimens are specific to the HCV genotype, highlighting the importance of diagnostics in determining the appropriate therapy [15]. Therefore, addressing WHO's aim to eliminate HCV by 2030 requires developing robust diagnostics that allow healthcare professionals to identify undiagnosed patients so they can be linked to appropriate care [7,16].

Current guidelines of the Centers for Disease Control and Prevention (CDC) for HCV testing recommend first performing an HCV antibody (Ab) test. If nonreactive, no further testing is required. If the patient is Ab positive, reflex testing should be undertaken using an HCV RNA assay [17]. If HCV RNA is detected in a patient along with an anti-HCV-positive result, a diagnosis of chronic HCV infection is made, and the patient should be linked to care for assessment of liver disease and subsequent initiation of antiviral therapy [17]. However, it is difficult to differentiate between a prior resolved infection (spontaneous viral clearance) and a case of obtaining a false-positive anti-HCV test result along with a negative HCV RNA test. Additional testing should be pursued in these patients [17]. HCV testing is currently limited in large parts of the world, especially economically disadvantaged regions. Point-of-care (POC) technology is paramount to minimizing these disparities in care, and it may lower the cost associated with traditional testing that leverages reflex HCV RNA in patients who are HCV Ab-positive. This review will focus on current conventional technologies and future technologies that can be expanded to POC settings. To meet WHO's goal of eliminating HCV by 2030, advancements in diagnostic approaches are essential to enable early and accurate detection, facilitate prompt treatment initiation, and overcome challenges related to diverse HCV genotypes and viral strains [18].

2. Genome and Virion Structure

HCV is classified as an enveloped, positive-sense, single-stranded RNA (ssRNA). Upon entering hepatocytes, the virus facilitates the direct translation of its structural and non-structural proteins. Its genome is approximately 9.6 kb long. The virus includes a 5′ noncoding region (NCR), a site of internal ribosomal entry (IRES), an open reading frame for about 3000 base pairs, and a 3′ NCR. Internal ribosome entry is a crucial process for viral protein synthesis, highlighting the importance of the 5′ NCR in viral replication [19]. This makes the 5′ NCR region a highly conserved one in the HCV genome and, therefore, a critical target for diagnostic assays that target the viral RNA [20]. HCV RNA can be detected within 2 weeks of exposure, preceding antibody development [21]. For self-resolving cases, the RNA levels show a drop after 6 months [22]. Many assays utilize the quantitative measurement of the HCV RNA to detect active infection and monitor treatment efficiency. The initially transcribed polyprotein is modified co-translationally and post-translationally with the help of protease present in the host cell to produce ten mature proteins. Three mature proteins are structural, and seven are nonstructural among the ten mature proteins produced. Residing at the 5′ end of the open reading frame (ORF), the structural proteins of the virus consist of a core nucleocapsid protein (C) as well as two envelope glycoproteins

(E1 and E2). These envelope glycoproteins play a crucial role in facilitating the binding and entry of the virus into hepatocytes [23,24]. The seven nonstructural (NS) proteins are designated p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B [25]. The NS3, NS4, and NS5 proteins play an important role in viral replication, so various classes of DAAs directly target them [26]. Additionally, assessing antibodies against these nonstructural proteins helps to confirm HCV exposure and evaluate acute and chronic infections [27].

Ultrastructural analysis of the HCV particle reveals heterogeneous sizes ranging between 40 and 100 nm in diameter [24,28]. The genetic material is surrounded by a nucleocapsid consisting of the HCV C protein and E1/E2 heterodimers [24,28,29]. Anchored by these E1/E2 heterodimers is the viral lipid membrane derived from the host endoplasmic reticulum (ER) [24]. The lipid bilayer incorporates high amounts of cholesterol, resembling the composition of very-low-density lipoprotein (VLDL) and distinguishing it from human host cells and other viral envelopes [30–33]. The major targets of anti-HCV neutralizing antibodies (nAb)s are the structural envelope glycoproteins E1, E2, and their E1/E2 heterodimer (with a vast majority targeting E2 due to its direct role in host cell entry) [34,35].

3. Biomarkers and Diagnostic Testing

The key biomarkers that the diagnostic assay targets include the HCV RNA, HCV core antigen, and anti-HCV antibodies. The initial two weeks after infection are characterized by the previremic phase in which the HCV RNA levels gradually increase in blood, which can only be targeted by highly sensitive diagnostic assays [21]. Detection during the acute stage is unreliable, given that the RNA levels may fluctuate [21,36,37]. Nucleic acid testing (NAT) offers the gold standard approach for detection during this stage; however, it is limited by its high cost and high turnaround time [38]. The RNA levels peak at a million copies per mL within 6–10 weeks [21]. The RNA levels can drop to undetectable levels for self-resolving cases and patients undergoing DAA therapy after 6 months [22]. For such cases, viral load monitoring tests become necessary to evaluate the condition of the disease. The expression of the HCV core antigen is similar to that of the HCV-RNA but with a slight delay. Therefore, HCV core antigen has been shown to be an alternative to RNA testing [39]. The expression of anti-HCV antibodies, however, significantly lags. The serologic window period characterized by the initial infection until the seroconversion can take up to 8 weeks [21,36]. Regardless of acute or chronic infections, the anti-HCV antibody levels may remain detectable after this stage. These factors can cause problems with diagnosis, as anti-HCV-based tests can miss the patients who are acute HCV and cannot differentiate between patients who are recovered or have a chronic infection [22,39].

The traditional approach to establishing an HCV diagnosis is an initial Ab test to document exposure, followed by a reflex polymerase chain reaction (PCR) HCV RNA test [40]. Serological exams are widely used to detect HCV exposure due to their cost-effectiveness, availability in the POC setting, and simplicity (e.g., storage and handling) compared to molecular tests. The presence of HCV Abs warrants screening for HCV RNA and a basic assessment for any signs of liver disease. Additional testing for other virus infections, including HBV and HIV, is also recommended. Practically, however, that is not always the case. The CDC notes that one-third of patients have incomplete testing [41]. Incomplete testing is characterized by a reactive HCV antibody test, which is not followed by an HCV RNA test. To combat this, the CDC has updated guidelines to ensure all screening sites do single-visit sample collection, allowing automatic RNA testing on all samples that are reactive to the HCV antibody [41]. WHO has recommended adopting point-of-care HCV viral load testing as an alternative to centralized lab testing to make HCV diagnosis more accessible. This is important to reach marginalized populations with

whom it may be difficult to follow up [42]. To achieve this, an ideal test should work with capillary blood, have integrated sample preparation, have a low limit of detection (<3000 IU/mL) with a clinical sensitivity of >95%, have a low turnaround time of under 30 min, and be under USD 10 per test [43].

4. Current Detection Methods

4.1. Serological Assays

4.1.1. Enzyme Immunoassay (EIA)

The duration of acute HCV infection averages 8 to 11 weeks but has been reported to be from 2 weeks to 6 months [44,45]. Four generations of immunoassays have been developed [46]. The first generation of viral antigen-targeting enzyme immunoassays detected NS4 with a window period of 4–6 months [46]. The second generation detected core, NS3, and NS4 with a window period of 10–24 weeks [46]. The third generation detected the previously mentioned viral proteins utilized in the second-generation test with the addition of NS5, improving its window period to 7–8 weeks [46]. Finally, the fourth-generation test utilized the same viral targets but improved the window period to as early as 26 days [46].

The first antibody assay was developed by Kuo et al. in 1989 when they first discovered HCV [6]. They created a recombinant yeast clone that expressed the polypeptide c100-3 of NS4, which was utilized to capture anti-NS4 Abs, which were subsequently measured qualitatively [6,46]. Their c100-3 bound Ab was detected using a radioactive secondary antibody [6]. To test the sensitivity, they had seven samples of HCV serum from chimpanzees, and they found that all but one had a high signal for the anti-HCV antibodies [6]. However, a major limitation of the test developed by Kuo et al. was that it could not detect Abs in the acute phase of an infection competently [47].

The ORTHO HCV second-generation ELISA test, by Ortho Diagnostic Systems, was created in 1992 and expanded on its first-generation counterpart by detecting both structural (HCV core) and non-structural proteins (NS3/NS4) [47]. In the second-generation test, non-structural antigens c100-3 and c33c, along with a recombinant antigen c22-3, were employed. However, as mentioned earlier, the first-generation test solely relied on c100-3 [47]. The second-generation ELISA platform exhibited higher sensitivity than the first-generation version, achieving 80% detection in acute HCV cases and 88% in chronic HCV cases [47]. First-generation testing only achieved a sensitivity of 60% in acute HCV and 72% in chronic HCV cases [47]. Both tests showed 99% specificity [47]. During another study involving Saudi Arabian blood donors, the application of the second-generation anti-HCV test revealed a 38% higher positivity rate compared to the first-generation test [48].

The third-generation tests, like the ORTHO EIA-3.0 and the Chiron RIBA-3 prototype, expanded on the second-generation tests by adding non-structural regions, including NS3, NS4, and NS5 [49]. Furthermore, these third-generation tests were able to reduce the false positive results that arose from c100-3 and c22-3 reactions [49]. Vrielink et al. conducted a test that compared the sensitivities and specificities of three different third-generation tests: Abbott HCV EIA 3.0, Murex anti-HCV VK47, and ORTHO EIA-3.0 [50]. The study showed that Abbott and Ortho demonstrated 100% sensitivity, while Murex had a sensitivity of 99.7% [50]. The specificities of Abbott, Murex, and Ortho were 99.7, 99.3, and 99.9 percent, respectively [50]. A major limitation of the third-generation tests was that these tests provided low positive predictive values in populations with a low HCV prevalence [46].

HCV antigen and antibody were simultaneously detected using the fourth-generation HCV tests, which functioned as an antigen-antibody combo assay. This generation of testing proved to be more convenient as it allowed the identification of two HCV markers using a single test. Consequently, it became highly suitable for resource-limited settings [46].

The ability to detect an antigen and antibody combo reduced the window period to 26 days [46]. An example of such a fourth-generation enzyme immunoassay is the Murex Anti-HCV Version 4.0, which has mostly replaced the 3.0 version. This assay has shown robust performance and high throughput, making it a valuable tool for early HCV detection and blood donor screening [51].

In conclusion, enzyme immunoassays adequately detect HCV infection. Each generation has improved upon previous versions, and now, the fourth-generation tests have a window period as low as 26 days [46]. The tests are highly sensitive and specific, which can ensure appropriate diagnosis. A huge drawback is that it takes approximately 24 h to complete EIA, and trained personnel are needed. The availability of trained technicians may be limited in certain regions, which can diminish the utility of this test, which is further limited by the fact that it is not a POC diagnostic tool.

4.1.2. Rapid Immunoassays

Rapid testing is defined as having a turnaround of fewer than 30 min and a test that does not need specialized equipment [46,52,53]. Rapid immunoassays can be used in POC settings and can reduce time and costs for obtaining rigorous and clinically informative information.

The OraQuick HCV rapid antibody test (OraSure Technologies Inc., Bethlehem, PA, USA) detects antibodies against the recombinant HCV C protein using an indirect lateral immunoassay [54]. Furthermore, OraQuick can also detect NS3 and NS4 [54]. To administer the test, a finger-stick blood sample is taken [55]. The test has two lines, a T line and a C line [55]. The test is considered reactive if the line appears at both the C and T zones. It is not reactive if there is a line in the C zone but no line in the T zone [55]. An invalid test occurs with a line only at the C zone or partial lines in the C or T zone [55]. Results are available for interpretation after 20 to 40 min, which renders this OraQuick test fully amenable for use in the POC setting. The sensitivity of OraQuick in field studies has ranged from 88.3 percent to 99.3 percent in sera, while the specificity is usually 100 percent [54]. Recently, the WHO also prequalified the OraQuick self-test for HCV [56]. This test is an extension of the OraQuick HCV rapid antibody test, which means that it offers similar testing performance in terms of sensitivity and specificity [57]. Although this is a great step in increasing access to diagnostics, the drawbacks of antigen testing, coupled with problems associated with user interpretation errors and limited data available on real-world effectiveness, necessitate ongoing evaluation to ensure its reliability across diverse populations and settings [58].

Another rapid immunoassay awaiting FDA approval that can be used to detect antibodies against HCV (anti-HCV), hepatitis B, and HIV 1 and 2 is MedMira's Miriad HBc/HIV/HCV rapid test, which does not require complex laboratory equipment or highly trained personnel, making it suited for resource-limited settings [59]. The ARCHITECT HCV Ag assay is also a fourth-generation chemiluminescent microparticle immunoassay (CMIA) that enables earlier detection of HCV infections due to its dual detection of HCV core antigens as well as anti-HCV antibodies, making it more cost-effective and potentially suitable for point-of-care settings [60].

Rapid Ab tests can be both cost-effective and time-effective in diagnosing someone with exposure to HCV. If a person tests Ab positive, per CDC guidelines, they are then given an HCV RNA test. A drawback of rapid tests is that they are not as sensitive as their enzyme immunoassay counterparts, which are also lower in cost per assay and can be used in settings where high-throughput testing is needed. Rapid tests, not conducted by well-trained technicians, can also be incorrectly performed or interpreted, giving someone a false positive or negative test result.

4.2. Molecular and Nucleic Acid-Based Detection

Nucleic acid amplification tests (NAATs) are a family of molecular diagnostics that detect viral RNA/DNA. These assays can be categorized as either qualitative or quantitative. NAATs exhibit high levels of sensitivity and specificity, enabling the detection of the virus during the acute phase of the infection [61,62]. To ensure accuracy and comparability, all HCV assays are calibrated utilizing international units (IU). According to the European Association for the Study of the Liver (EASL) guidelines as of April 2019, it is recommended to utilize assays that have a lower limit of detection (LLOD) of under 15 IU/mL, despite the vast majority of patients with HCV having viral RNA serum/plasma levels over 50,000 IU/mL [63].

A significant limitation of molecular diagnostics for HCV diagnosis is the expense of the test and their reliance on specialized laboratories [64]. This can pose a barrier for populations without access to capable healthcare facilities, such as developing nations, prisoners, sex workers, and migrants [65]. For certain assays and regions of the world, confirmatory HCV RNA assays can be more costly than the antiviral treatment itself [64]. This precludes the use of molecular testing in the initial diagnostic screening for the virus.

4.2.1. Polymerase Chain Reaction (PCR) Based Tests

PCR, developed in the 1980s, is a method of exponentially multiplying a target DNA strand through thermocycling, giving 2^n copies (where n is the number of cycles performed) [66]. PCR methods can be employed to amplify RNA samples by initially generating complementary DNA (cDNA) through reverse transcription (RT-PCR). The use of RT-PCR offers many benefits in the diagnosis and treatment of HCV. RT-PCR relies on the presence of HCV RNA in peripheral blood and not on the formation of anti-HCV Abs, making it the preferred method for both the acute and chronic phases of infection [67]. On the other hand, anti-HCV antibodies signify past or present infection, and the presence of serum HCV RNA is specific to ongoing viral replication [67]. In 1998, Krarup et al. developed the first successful assay for quantitative RT-PCR for HCV detection, which has remained the gold standard for confirmation of acute infection and viral load [68–70]. Real-time RT-PCR employs the same principles as traditional PCR, with the added ability to quantify the sample DNA/cDNA, as the process takes place in real-time using fluorescent reporter dyes or probes (Figure 2) [71,72]. In this assay, a fluorescent signal is emitted during every replication cycle and measured proportionally to the amount of DNA/cDNA produced at that moment [72]. Multiple probes can target multiple genes simultaneously (multiplex real-time PCR) [72].

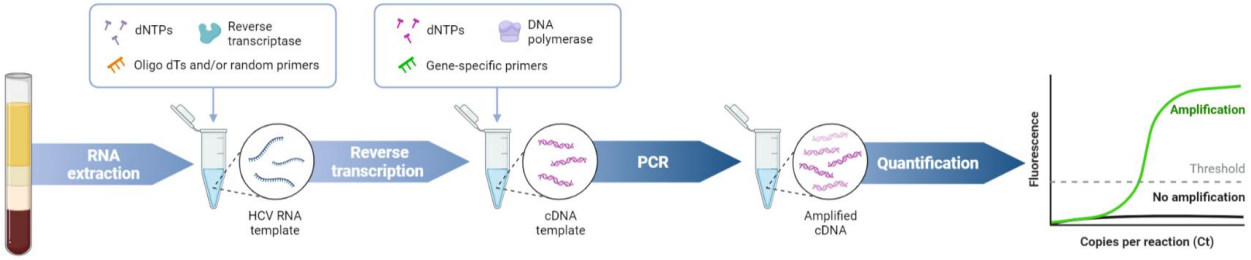

Figure 2. Depiction of real-time RT-PCR. Viral RNA extracted from peripheral blood is converted to cDNA via reverse transcription. cDNA then undergoes PCR amplification and quantification to determine viral load. Created with BioRender.com, accessed on 13 December 2022.

The two main real-time RT-PCR-based assays commonly utilized in HCV testing are the COBAS AmpliPrep/COBAS TaqMan (CAP/CTM) assay and the Abbott RealTime HCV test (Abbott) [73,74]. Both assays are very similar, basing their techniques on automated

sample preparation followed by real-time RT-PCR [75]. Following a 12-week course of HCV antiviral therapy, both the CAP/CTM assay and the Abbott RealTime HCV test were compared and exhibited comparable performance. CAP/CTM has an LLOD of 15 IU/mL with a sensitivity and specificity of 96% and 49%, respectively [76,77]. Abbott has an LLOD of 12 IU/mL with a sensitivity and specificity of 95% and 46%, respectively [76,77]. Version 1 (CAP/CTM v1.0) of the CAP/CTM assay was known to sporadically under-quantify HCV genotype 4 (GT4), though this has since been resolved with the release of the version 2 assay (CAP/CTM v2.0) [74]. A significant drawback of real-time RT-PCR is its cost. While a traditional PCR instrument costs approximately USD 5000, the price of a real-time RT-PCR instrument ranges between USD 15,000 and USD 90,000 [78].

4.2.2. Branched DNA Assays (bDNA)

Branched DNA (bDNA) assays employ bDNA amplifier molecules, which serve as a hybridization site for linear amplification of the target as opposed to exponential amplification [79,80]. In this assay, target-specific probes known as capture extenders (CEs) are used to capture targeted nucleic acids onto a microtiter well plate [79–82]. Then, the second set of probes, termed label extenders (LE), hybridizes to the target, allowing the bDNA amplifiers to bind [82]. Alkaline phosphatase and a chemiluminescent substrate are then added to the mixture, allowing for luminescence production to be measured as a direct proportion of starting HCV RNA [83]. The VERSANT HCV RNA 3.0 Assay (VERSANT 3.0 bDNA assay) is one such assay used for HCV detection. This assay has a reported LLOD of 615 IU/mL with a specificity of 98.2% [84]. The assay also has a high dynamic range of between 615 and 7,690,000 IU/mL [83]. It was also shown to be an improvement to the Quantiplex HCV RNA 2.0, where the latter required 100 µL of serum compared to the VERSANT 3.0 bDNA assay, which only uses 50 µL of serum [84]. In contrast to other molecular assays, bDNA incorporates an in-assay mechanism to isolate HCV RNA and does not necessitate separate RNA purification steps, which makes the workflow more streamlined. It is also less prone to contamination and limits false positive results [83–85].

4.2.3. Transcription-Mediated Amplification (TMA)

TMA is an isothermal method that imitates retroviral RNA replication. It involves the use of RNA polymerase and reverse transcriptase to amplify RNA [86]. Since RNA amplicons are more labile than PCR products, the risk for carryover contamination and false positives is quite low [87]. The TMA test used for HCV detection is the VERSANT HCV RNA TMA assay (VERSANT TMA). This is a qualitative assay offering 99.6% specificity, detecting >95% of clinical samples with HCV RNA at 50 copies/mL, including patients infected with HCV genotypes 1a, 1b, 2a, 2c, 3a, 4a, 5a, and 6a (genotype 2b showed detection of 88.4% at the same concentration) [88]. One of the main benefits of the VERSANT TMA assay is that it maintains specificity even in clinical RNA samples exposed to long storage durations, multiple freeze-dry cycles, various collection tubes, prescription drugs, and microorganisms [88]. The LLOD for genotype 1 ranges from 5.3 to 9.6 IU/mL, while for genotype 2b, it is 14.4 IU/mL [83]. The assay has proven more effective than RT-PCR for the detection of HCV RNA in liver tissue (p-value = 0.032) [89]. A drawback of the VERSANT TMA assay is its relatively high labor demand and bench space requirement compared to other qualitative assays [83]. Another TMA-based assay is the Aptima® HCV Quant Dx Assay, which has determined its LOD for plasma to be 3.9 IU/mL and 3.4 IU/mL in serum [90]. This assay removes the need for batch processing and condenses the automation of nucleic acid testing into a single step [91]. The Procleix® Ultrio Assay is an in vitro supplemental multiplex nucleic acid test (NAT) using TMA to detect HCV RNA

and a few other viral diseases like HIV and HBV [92]. However, according to the FDA, this test is not meant to be used for diagnosis but as a supplemental test [92].

4.3. Non-Invasive and Point-of-Care Testing

4.3.1. Dried Blood Tests

Dried blood testing originated a century ago after Christian Bang studied glucose levels from dried blood spots (DBS) eluates [93]. DBS has four inherent advantages: (1) less blood volume is required compared to conventional venipuncture, (2) blood collection is not complex and expensive, (3) bacterial infection risk is minimal, and (4) DBS is stabilized for longer time periods without any significant damage to the sample [93]. The process of obtaining blood for DBS is quite simple and does not require trained personnel. The technician punctures the subject's finger via a contact-activated lancet. Capillary blood is then collected on filter paper (Figure 3) [93]. The blood is dried, after which the card can be transported to centralized testing labs [93]. In the labs, technicians punch out the sample from the card and incubate it with a buffer to elute the sample [93]. The sample is then used for downstream analysis for HCV markers, including the anti-HCV Ab and the HCV C antigen [93,94]. The process (Figure 3) is promising for at-home self-collection, which makes HCV testing accessible to a broader population with limited access.

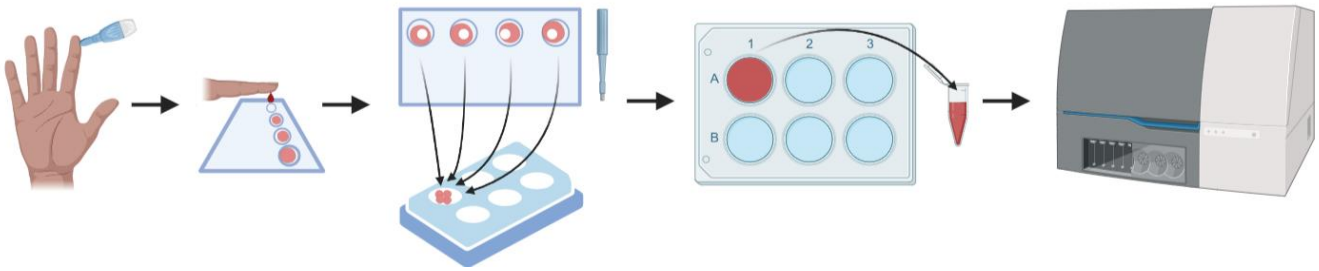

Figure 3. Depiction of dried blood spot (DBS)-based testing. Capillary blood is acquired via fingerstick and blotted onto a filter paper-based DBS card. The DBS card can be transported to a centralized lab facility for infectious disease screening. The filter paper with the sample is then incubated with a buffer. The resulting supernatant can be used to analyze viral proteins, genetic material, and Abs. Created with BioRender.com, accessed on 13 December 2022.

Biondi et al. determined the effect of freezing temperatures on DBS sensitivity [94]. The sensitivity of the HCV C antigen assay was 94% (−80 °C), 94% (4 °C), 91% (21 °C), 93% (37 °C), and 93% (37 °C/4 °C), while the sensitivity of the anti-HCV Ab tests was 100% (−80 °C), 100% (4 °C), 100% (21 °C), 98.6% (37 °C), and 98.6% (37 °C/4 °C) [94]. The study showed that the temperature change had little effect on the ability to test for HCV [94]. Their study also demonstrated that the sensitivity of DBS was 94%, which was higher than that reported in other studies [94].

Public health officials in Scotland implemented more DBS sampling as part of their community drug services to diagnose individuals infected with HCV. Following the implementation of DBS, there was a 3-fold increase in testing and a 12-fold increase in positive results. This demonstrates that DBS can detect more cases, particularly in high-risk populations [95]. DBS testing should be considered an alternative to other HCV testing methods as it is cheaper, simpler to run, and does not require trained personnel [94]. However, DBS is limited by the lack of standardized testing protocols. Temperature, blood drying time, and other related conditions often vary between testing sites. Furthermore, the assay used can make a difference in both sensitivity and specificity.

4.3.2. Point-of-Care NAAT Testing

With the importance of rapid HCV RNA detection in the diagnosis and treatment of acute and chronic infections, the development of a POC molecular diagnostic test offers great value. The first such test developed for HCV detection is the Xpert HCV Viral Load test (a real-time RT-PCR) designed in 2015 and manufactured by Cepheid [96]. This test combines all steps of real-time RT-PCR (sample preparation, DNA/RNA extraction, amplification, and detection of targets) into a single cartridge leveraging microfluidics, allowing it to fit on a small benchtop [97]. This assay has an LLOD of 10 IU/mL (similar to that of the Abbott test) with a sensitivity and specificity of 94.4% and 100%, respectively. A new version, called the Xpert HCV Viral Load Finger-Stick assay (Xpert HCV VL FS), which does not use venipuncture, has reported even higher levels of sensitivity and specificity [98]. The Xpert HCV test and its associated GeneXpert Express System have recently been approved by the FDA for operations under a CLIA (Clinical Laboratory Improvement Amendments) Certificate Waiver [99]. However, reported drawbacks to this assay have been associated with the cost of both the assay kit and the GeneXpert instrument needed for its use [96]. There are other similar assays, including the Genedrive HCV ID (98.6% sensitivity, 100% specificity), which received prequalification by WHO in 2020, and the TrueNAT HCV RNA assay, which had reported sensitivity and specificity of 95% and 99%, respectively [96,100]. Sharma et al. successfully developed an automated reverse-transcription loop-mediated isothermal amplification (RT-LAMP) test designed for HCV detection. The system they created comprises a disposable microfluid chip that is automated, a reusable magnetic actuation platform, and a compact surface heater [101–103]. The plasma samples were processed in multiple chambers within the microfluidic chip. SYBR green dye is employed to enable the visual detection of the amplification product without the need for specialized equipment [101]. Human plasma samples spiked with HCV virions were employed to evaluate the microfluid chip's performance. The observed limit of detection (LOD) was determined to be 500 virions/mL, achieved within a timeframe of 45 min [101]. Although developing a fully automated setup for rapid HCV detection using RT-LAMP is a promising advancement, there are still areas for improvement, particularly in terms of automation. Currently, the system requires a plasma sample to be prepared before testing, which can be time-consuming and may limit its practicality in point-of-care settings. To further streamline and increase the practical use of this technology in POC settings, efforts should focus on incorporating whole blood isolates into the system. Comprehensive data about key characteristics of the various diagnostic techniques discussed that have been clinically tested and conventionally used in managing HCV testing are summarized in Table 1.

Table 1. Data for current HCV diagnostic tests along with their key features and shortcomings.

Name	Method	Sample	Target	LOD	Time	Sensitivity	Specificity	Status	Strengths	Limitations	Reference
Vitros Anti-HCV, Ortho Clinical Diagnostics	CLIA	Serum/Plasma	Anti-HCV/Antigen; Core, NS3, NS4, NS5			100.00%	99.85%	FDA approved	High throughput; automated processing; good sensitivity and specificity	Longer processing time compared to other CLIA methods; requires specialized instruments	[104]
ARCHITECT HCV Ag/Ab Combo Assay	CMIA	Serum/Plasma	Anti-HCV/Antigen			93.40%	98.80%	Unknown	Combines antigen and antibody detection; reduces diagnostic window period; high throughput	Requires specialized equipment; higher cost compared to antibody-only tests	[61,105]
Alinity s Anti-HCV	CMIA	Serum/Plasma	Anti-HCV			100.00%	99.92%	FDA approved	High throughput; suitable for blood donor screening	Not intended for diagnostic confirmation; requires specialized equipment	[106,107]
Alinity s Anti-HCV II assay	CMIA	Serum/Plasma	Anti-HCV			100.00%	99.96%	FDA approved	High sensitivity and specificity, automated, high throughput,	Not intended for diagnostic confirmation; requires specialized equipment	[108]
Abbot Architect Anti-HCV	CMIA	Serum/Plasma	Anti-HCV			93.40%	98.92%	FDA approved	Can be used as a screening assay because of its high sensitivity, high throughput, and short turnaround time	Prone to false positives	[61,109]
Abbott PRISM HCV	ChLIA	Serum/Plasma	Anti-HCV			100.00%	99.89%	FDA approved	Qualitative detection of antibodies to HCV, suitable to donor screening	Not intended for diagnostic confirmation; requires specialized equipment	[110]
Abbott Anti-HCV 3.0	ELISA	Serum/Plasma	Anti-HCV/ Antigen; Core, NS3, NS4, NS5				99.70%	FDA approved	Widely validated, sensitive as RNA detection techniques	Expensive, requires specialized equipment for processing	[50,111]
ORTHO EIA-3.0	ELISA	Serum/Plasma	Anti-HCV/Antigen; Core, NS3, NS4, NS5				99.90%	FDA approved	Established method with high sensitivity; suitable for blood donor screening	Requires specific equipment; requires confirmatory testing	[50,112]
Murex Anti-HCV 4.0	ELISA	Serum/Plasma	Anti-HCV			100.00%	99.80%	WHO prequalified	High sensitivity and specificity; detects all major HCV genotypes; suitable for high-throughput settings	Requires laboratory infrastructure; cannot detect acute infections during the window period	[113–115]
OraQuick HCV Rapid Antibody Test	Lateral Flow Assay	Fingerstick and Venipuncture Whole Blood/ Serum/Plasma/ Oral Fluid	Anti-HCV	20 IU/mL	20–40 min	Serum: 100.00%/Oral Fluid: 97.80%	100.00%	FDA approved	Rapid results, suitable for point of care for mass screening, clinical performance of the OraQuick HCV Test is comparable to that of laboratory-based tests with both serum and oral fluid, simple, non-instrumented	Higher chance of false-negative results	[54,116,117]

Table 1. *Cont.*

Name	Method	Sample	Target	LOD	Time	Sensitivity	Specificity	Status	Strengths	Limitations	Reference
OraQuick HCV Self-Test	Lateral Flow Assay	Oral Fluid	Anti-HCV		20 min	85.10%	99.80%	FDA approved	Only approved self-test, can be done at-home	high cost makes it difficult to deploy in low-resource areas, additional RNA testing required to validate results	[118]
UltraQual Multiplex PCR Assay	PCR	Serum/Plasma	RNA			93.70%	100.00%	FDA approved	High sensivitivty and specificity	Requires trained staff	[119]
Xpert HCV Viral Load test by Cepheid	RT-PCR	Plasma	RNA	10 IU/mL	2 h	94.40%	100.00%,	FDA approved	High specificity, suitable for smaller labs	Requires specialized platform to run tests	[97,98]
Xpert HCV Viral Load Finger-Stick assay (Xpert HCV VL FS)	RT-PCR	100 µL Whole Blood	RNA	>40 IU/mL	1 h	98.00%	100.00%	FDA approved	Accurately detect active infection from a finger-stick sample in 1 h allowing single-visit HCV diagnosis	Requires trained staff	[99]
TrueNAT HCV RNA assay	RT-PCR	Whole Blood/Serum/Plasma	RNA	Blood: 1153.94 IU/mL/Serum: 260.42 IU/mL/Plasma: 204.71 IU/mL	35 min	95.00%	99.00%	FDA approved	Small sample required, reusable chips, rapid results, easy to use	Not suitable for early infection detection	[120–122]
COBAS® AmpliPrep/COBAS® TaqMan® HCV Test, v2.0	RT-PCR	650 µL Serum/Plasma	RNA	15 IU/mL			100.00%	FDA approved	Fully automated, provides precise viral load measurements for effective treatment monitoring, high specificity	High cost, slow, specialized equipment and lab facilities required	[123–126]
Abbott Realtime HCV	RT-PCR	500 µL Serum/Plasma	RNA	12 IU/mL		100.00%	99.74%	FDA approved	High sensitivity and specificity; suitable for monitoring viral load during therapy	Not intended for blood donor screening; requires specialized equipment	[126–128]
Genedrive HCV ID	RT-PCR	30 µL Plasma	RNA	2362 IU/mL	90 min	98.60%	100.00%	CE-IVD	Ideal for low throughput, decentralised laboratories	Specialized equipment is needed, large sample volume required	[65,120,129–131]
MedMira Miriad rapid HBc/HIV/HCV test	Rapid Immunoassay	Blood/Serum	Anti-HCV					Awaiting FDA approval	Rapid detection of multiple infectious markers; no complex infrastructure required	Lower sensitivity in early HCV infection	[132]
VERSANT HCV RNA TMA	TMA	Serum	RNA	Genotype 1: 5.3-9.6 IU/mL/Genotype 2b: 14.4 IU/mL			99.60%	FDA approved	No nucleic acid extraction is needed, and the process can be fully automated	Laborious process and bench space requirement when compared to other qualitative assays	[84,89,133,134]
Aptima® HCV Quant Dx Assay	TMA	Serum/Plasma	RNA	4.3 IU/mL	2.5 h for first 5 samples then 5 min for every sample		98.60%	FDA approved	Detects and quantitates HCV RNA genotypes 1-6, is rapid, sensitive, and reproducible and accurately quantifies HCV RNA in serum samples from patients with chronic HCV infection	Requires expensive automation systems and is not approved for use as a screening test for the presence of HCV RNA in blood or blood products	[91,92,135]

Table 1. *Cont.*

Name	Method	Sample	Target	LOD	Time	Sensitivity	Specificity	Status	Strengths	Limitations	Reference
Procleix® Ultrio Assay	TMA	2.5 mL Serum/Plasma	RNA			99.70%	100.00%	FDA approved	High sensitivity and specificity; simultaneous detection of multiple viruses (HIV-1, HCV, HBV); suitable for high-throughput screening	Requires specialized equipment and trained personnel; not intended for diagnostic purposes	[136]
VERSANT HCV RNA 3.0 Assay	bDNA	50 µL Serum	RNA	615 IU/mL			98.20%	FDA approved	Low risk of carryover contamination, enhanced branched design leads to more specificity	Requires trained staff and is expensive	[85,137]

5. Emerging and Future Detection Methods

5.1. Isothermal Amplification Based Tests

Isothermal amplification is a molecular testing method where amplification of the nucleic acids of interest is achieved utilizing a constant temperature instead of the thermocycling required of traditional PCR [138–140]. Rapid temperature cycling requires more complex power circuitry and precise temperature controllers. Isothermal tests simplify the equipment requirements by requiring basic incubators that are smaller, lighter, and consume less power [138]. These features, along with enhanced specificity, sensitivity, and shorter analysis time, make isothermal amplification techniques a promising avenue for decentralized testing as required by WHO recommendations [7]. Isothermal amplification has evolved tremendously since its initiation in the early 1990s. Numerous diverse isothermal amplification methods have been created, providing high sensitivity and commercial accessibility [141,142]. Some of the relevant isothermal amplification methods used for HCV detection are described here [143]. These isothermal amplification methods include Rolling Circle Amplification (RCA), Nucleic Acid Sequence-Based Amplification (NASBA), Recombinant polymerase amplification (RPA), Loop-mediated isothermal amplification (LAMP), and transcription-mediated amplification (TMA).

5.1.1. Loop-Mediated Isothermal Amplification (LAMP)

The technique of loop-mediated isothermal amplification (LAMP) was initially described in 2000. The LAMP method utilizes auto-cycling strand displacement of DNA, enabling the rapid accumulation of up to 10^9 copies of the target DNA/RNA within an hour [144]. The incorporation of reverse transcriptase allows for the amplification of RNA sequences (RT-LAMP). In the LAMP method, a DNA polymerase and four primers (inner and outer primers) are utilized, designed to recognize six specific target DNA sequences. During the initial stage of LAMP, two inner primers facilitate the synthesis of a stem-loop structure from the target DNA. This stem-loop structure acts as the starting point for the subsequent amplification cycles in LAMP [144]. In the second stage of LAMP, the primers bind to the loop region of the initial product, initiating displacement DNA synthesis. This procedure produces both the initial stem-loop DNA and a fresh stem-loop DNA strand twice the original length [144,145].

LAMP provides commercial and hospital laboratories with increased specificity, simplicity, and timeliness. The sole equipment necessary for this reaction is a heat block or a water bath maintained at a consistent temperature range of 62–65 °C (Figure 4) [144–146]. The convenience of this process allows it to be used in POC screening for targeted populations at higher risk for virus infection that often remain asymptomatic, as is the case with HCV [142]. This assay also has a high tolerance to biological substrate contamination [70]. The LAMP assays' drawbacks include the primer design complexity and their inability to perform multiplex amplification [70]. LAMP has also been used to successfully identify multiple viruses, including influenza virus, SARS-CoV-2, mumps virus, West Nile virus, hepatitis B (HBV), human immunodeficiency virus 1 (HIV-1), hepatitis A virus (HAV), and hepatitis E virus (HEV) [147–154].

5.1.2. AP-LAMP

The use of LAMP in the detection of HCV, which has traditionally been complicated by the complex secondary structure of the 5′ untranslated region (5′-UTR), was achieved through a modified method by Yang et al. in 2011 [155]. In their method, termed AP-LAMP, they utilized three additional primers: two loop primers (LF and LB) and an acceleration primer (AP) [155]. Here, the AP is particularly important to the modified reaction as it adds a starting DNA synthesis site, binding between the active sites of primers F1 and B1. SYBR

Green I cyanine dye was added to the assay to allow easier detection of the nucleic acids without specialized equipment, turning the sample from orange to green upon positive AP-LAMP reactions [155]. AP-LAMP showed a sensitivity of 84 IU/mL [155]. They also reported an average threshold time (Tt) for positive assay signal detection of 17.99 ± 0.62 min (mean ± SD) for a 5×10^5 IU RNA sample and 31.70 ± 0.43 min (mean ± SD) for a sample containing 5×10^2 IU of HCV RNA [155].

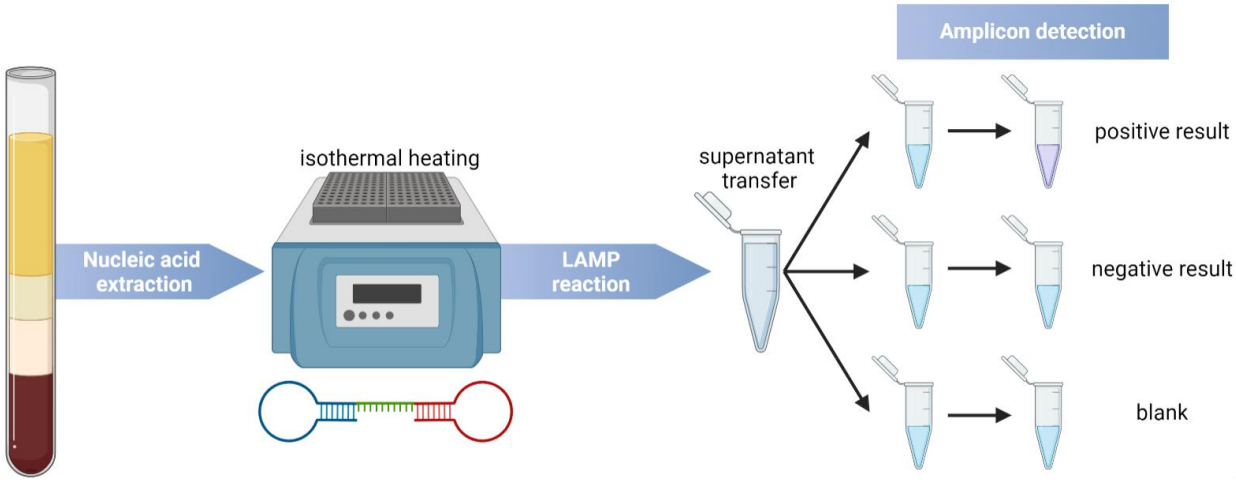

Figure 4. LAMP process. The figure illustrates the LAMP reaction that is used for the amplification of DNA. The process begins with the binding of the primers to the target DNA sequence. The primers then initiate the strand displacement amplification, where the target DNA is amplified at a constant temperature. Visual detection can be achieved by adding an appropriate reagent. Created with BioRender.com, accessed on 13 December 2022.

5.1.3. Other LAMP Assays

Additionally, LAMP assay variations have also been developed to detect HCV. In 2015, Nyan et al. published a rapid fluorogenic multiplex isothermal amplification assay allowing for naked-eye visualization of a positive test under UV light and quantitative measurability with a fluorospectrophotometer [156]. Their assay showed an impressive 97% sensitivity and 100% specificity with no viral cross-reactions observed [156]. In 2016, Nyan and Swinson published an RT-LAMP method for simultaneous HCV detection and identification of genotypes 1–6 in under 60 min [156]. Their assay had a 91.5% diagnostic sensitivity and detection rates of 50% and 100% at 50 and 10^2 IU of HCV RNA, respectively [157]. In 2018, Chen et al. published a method called BEAMing LAMP, which combines emulsion microreactors and single-molecule magnetic capture with on-bead LAMP [158]. This assay first immobilizes FIP primers onto magnetic beads and incubates with an HCV cDNA sample [158]. The cycling process is initiated once the beads are washed and resuspended in a mixture containing LAMP reagents and primers. This method demonstrates significant potential for the future diagnosis of HCV, boasting a detection limit of 300 RNA copies. However, it still faces challenges related to its intricate workflow and primer design [158].

5.2. Surface Plasmon Resonance Based Testing

Surface plasmon resonance (SPR) is a highly sensitive and label-free detection technique utilized to measure biomolecular interactions in real-time (Figure 5) [159]. Since its development in the 1990s, there have been multiple advancements in SPR-type technology. SPR finds several biomedical applications, including the analysis of molecular interactions, investigations into conformational changes, mutation detection, and the primary screening of potentially druggable molecules [159]. Raman spectroscopy (RS) is an example of a technology similar to SPR that has been employed in the detection and diagnosis of infectious

diseases. RS relies on the inelastic scattering of light photons following interaction with molecular bonds within a given sample. This provides valuable information regarding the molecular composition of a sample; however, RS is not without its own shortcomings. Differentiating the spectra of pathological samples from normal ones with the naked eye is challenging due to the complex nature of Raman spectra [160]. This is where other technological developments may come into play in the future when considering increasing the utility of this method.

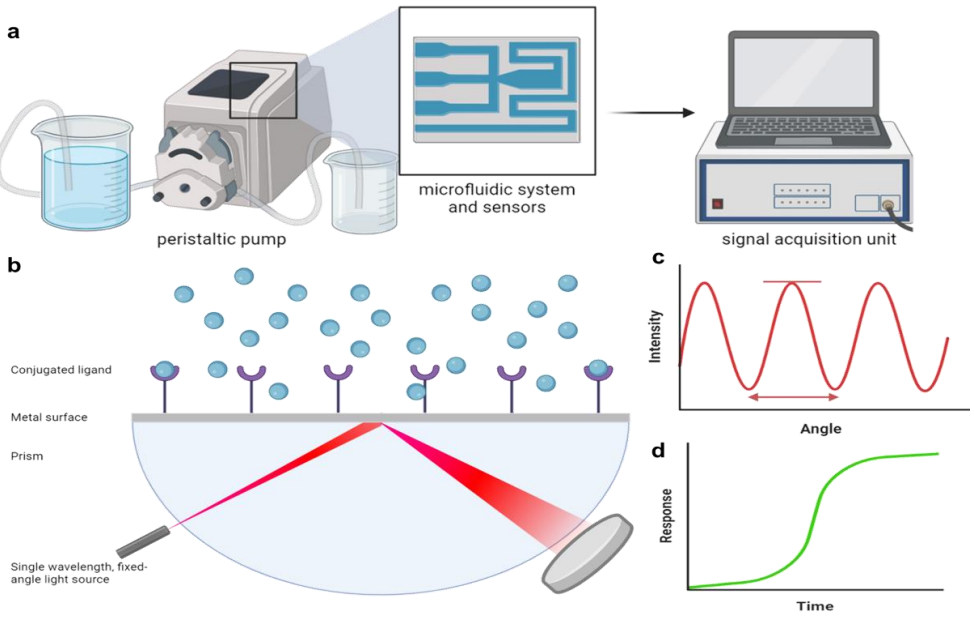

Figure 5. (**a**) Schematic of SPR sensor integrated with microfluidics and other peripherals. (**b**) Prototypical SPR biosensor. Analyte (blue) binds ligand (purple), causing a change in the refractive index of the surrounding solution. (**c,d**) Differences in single-wavelength light reflection, absorbance, and refraction are detected using surface plasmon resonance. Created with BioRender.com, accessed on 13 December 2022.

Another specific example of an SPR-type technology that has been used in the study of HCV is surface-enhanced Raman spectroscopy (SERS). This technique increases the detection signal in comparison with Raman spectroscopy by detecting absorbed particles on a metal surface. Noble metal nanoparticles (NPs) are required to enable high detection sensitivity. These NPs should be of the proper shape and size for the SERS platform [161]. The metallic nanostructures amplify the Raman scattering signals, which has been shown to increase sensitivity by 10–400 fold [162]. Additionally, using different Raman-active labels, Shi et al. have shown multiplex detection. This is important in HCV diagnostics because it offers comprehensive antigen detection and simultaneously tests for HBV and HIV coinfections [163]. The ability to minimize the autofluorescence of the sample helps to enhance the clarity of the specific signals, improving the sensitivity of the assay since background fluorescence can obscure specific signals.

In one study, SERS was used for the detection of HCV from human serum samples. SERS resulted in a peak detected at 785 cm^{-1} (ring breathing modes in the RNA base/uracil). This SERS signal is related to RNA present in the virus, as uracil is present only in the RNA (not DNA) [164]. This feature is of utmost importance, given that HCV is an RNA virus. This study observed that the primary distinguishing SERS characteristics are absent in the control (uninfected) sample while being present in the HCV-positive samples [164]. Therefore, the spectral features mentioned with SERS in the HCV RNA could be used for

diagnostic purposes and further employed for qualitative and quantitative analysis of HCV from patient blood samples [165].

Researchers have developed another method utilizing polypyrrole-peptide chips and surface plasmon imaging (Figure 5) [166]. One peptide was derived from ovalbumin, while 11 peptides were derived from the HCV virus, encompassing both structural and non-structural proteins [166]. These peptides were functionalized and immobilized on the chip's gold surface (as 300 and 500 µm spots) (Figure 5b). A p-polarized LED beam was used to illuminate the gold sensor chip, and the light reflected is recorded by a CCD camera, enabling simultaneous analysis of binding events on all spots [166]. This method allowed the detection of antibodies even at low concentrations in serum [166]. This technique has shown promise to be highly specific in studying and screening biological samples in label-free, real-time conditions [166].

5.3. Microfluidics

Microfluidics relies on two main factors: micrometric size and the handling of nanoliter quantities of fluids [167]. Microfluidic chip devices provide precise fluid control, a high surface-to-volume ratio, low sample requirements, and high integration with functional components. Several microfluidic-based HCV testing methods have been developed, including microfluidic DNA/RNA amplification, rapid immunoassay systems, and sensors. The immunoassay system comprises lateral flow assays (LFAs) and immuno-filtration assays (IFAs) that detect specific antigens or antibodies in samples. LFAs detect the target in a liquid sample that migrates laterally across a test strip via capillary action. The detection is made when the target analyte binds with the labeled antibodies, providing qualitative results in the form of a visible colored line [168]. Guo et al. developed a single-line flow assay platform that is based on orthogonal emissive upconversion nanoparticles. Their single-line platform combines the test and control lines on traditional LFAs, where one emission is used as a reporting signal and the other as a calibrating signal [169]. Since the diffusion distance of the sample is limited on the chip, this method reduces the size of the strip or allows the testing of multiple targets in a sample [169]. Tang et al. demonstrated a signal enhancement technique for LFAs by integrating a sponge shunt to decrease the fluid flow rate. Their idea was to allow an extended time for the analyte to bind to immobilized antibodies, resulting in a higher number of detectable complexes. They demonstrated a 10-fold increase in sensitivity over unmodified LFA by detecting down to 10^3 copies/mL of HBV DNA [170]. This technique highlights the potential for point-of-care application in various disease diagnostics, including HCV [170].

IFA, on the other hand, is known as a flow-through assay, in which a porous filtration membrane is used where specific antigens are immobilized so that specific HCV antibody binding can take place along with filtration [167]. IFAs are constricted in their clinical diagnostic capabilities and are qualitative or semi-qualitative. Zhang et al. devised an immunofluorescence assay (IFA) using quantum dots (QDs) as fluorescent labels, which were coated with polyethylene glycol (PEG) and glutathione. This IFA was designed specifically for quantitatively detecting C-reactive proteins, a commonly utilized diagnostic indicator for acute viral and bacterial infections [167].

Furthermore, microfluidic paper-based analytic devices (µPAD) use paper as an emerging substrate material, which is less expensive [171]. Its intrinsic capillary force has the ability to move liquids; as a result, it eliminates the need for external power sources [171]. Nitrocellulose is a patterned structure at 100 °C to create multiple test zones [171]. The antigen or antibody is diluted to specific concentrations using phosphate-buffered saline (PBS) with a pH of 7.4 [171]. Then, 0.5 µL of the solutions is pipetted onto each detection zone of the patterned paper [171]. The paper is left to air dry for 30 min, and then it is subjected to

blocking in a solution of PBS containing 5% BSA and 0.05% Tween-20 for 60 min [171]. A total of 0.3 µL of positive and negative control and diluted patient serum are pipetted into the blocked detection zone for 1 min to detect anti-HCV. Subsequently, the detection zones are washed [171]. The patient's serum is diluted (1:50) in PBS containing 10% BSA [171]. The patterned paper is subsequently exposed to a solution containing HRP-labeled anti-human IgG (diluted at a ratio of 1:50,000) for 2 min. After the incubation period, the paper is carefully washed. A total of 250 µL of mixed chemiluminescence substrate is poured into the patterned paper after separating enzyme-labeled antibodies [171]. A minute later, the majority of the liquid is aspirated out, followed by detection [171]. The modulus micro-plate multimode reader detects the chemiluminescence of the commercial ELISA kit [171].

A novel lab-on-disk (LOD) system utilizing LAMP was specifically developed to enable the concurrent detection of cytomegalovirus (CMV), hepatitis B (HBV), and (HCV) infections [172]. This system offers a highly sensitive and specific one-step nucleic acid amplification process in the microfluidic-based LOD system [172]. The LAMP-based LOD test results are determined by observing turbidity using the SPR lamp detection system [172]. Chang et al. developed a fully automated LAMP-based LOD system that enables rapid diagnostics in less than one hour. This system is capable of detecting hepatitis B virus (HBV), cytomegalovirus (CMV), and hepatitis C virus (HCV) infections [172]. The Chang et al. system has key advantages: it requires less sample volume and time for sample processing than conventional LAMP [172]. These features can make it a POC technology used in resource-limited areas. However, the test takes longer to report results than an ideal POC test, so future research should focus on reducing the time to obtain results.

The advantage of using the microfluid technique is that it can work with a small sample size, which helps in reducing the overall cost [173]. Other advantages are in the potential applications in the POC setting [173]. Sensing or sample pretreatment modules used by microfluidic systems significantly increase efficiency and decrease the chances of cross-contamination [173].

5.4. Electrochemical-Based Sensing

Electrochemical sensing has been used to detect viruses and other pathogens. This technique utilizes conducting and semiconducting materials as an electrode. The binding between the biorecognition element and the pathogen produces chemical energy, which is then converted to electrical energy [174]. The different methods utilizing electrochemical energy for pathogen detection can be categorized as amperometric, conductometric, ion-charge/field effect, impedimetric, and potentiometric [175]. Electrochemical genosensor technology used to detect HCV facilitates obtaining results with higher sensitivity. However, it is essential to note that replication of cDNA requires PCR [176].

Another type of electrochemical-based sensing that has been used in the study of HCV is electrochemical immunosensors. In a recent study, a biosensor was created employing graphitized mesoporous carbon-methylene blue as an electrode modification material. At the same time, a secondary antibody layer was formed using horseradish peroxidase-DNA-coated carboxyl multi-wall carbon nanotubes [177]. The immunosensor exhibited excellent performance, demonstrating a low detection limit of 0.01 pg/mL [177]. The future of this technology appears promising.

Electrochemical sensors have even been integrated with microfluidics for the detection of viral RNA from HCV. A 2016 study showed that a DNA-grafted multiwall carbon nanotube (MWCNT) biosensor in a microfluidic device with enhanced flow protocol demonstrated promising sensitivity. For HCV, this study demonstrated an enhanced dynamic range from one per second to six per second compared to macroscale systems [178].

5.5. CRISPR

Clustered regularly interspaced short palindromic repeats (CRISPR) systems can potentially be extended into diagnosing HCV. CRISPR is a gene-editing technology that has many applications in addition to diagnostics. CRISPR has two components: an endonuclease, most commonly CAS9, and a guide RNA that matches the gene target [179]. The endonuclease causes a break in the DNA genome, allowing it to be modified in many ways. Some uses include modifying genes that underlie genetic disorders, treatment of HIV, and engineering somatic stem cells as novel therapeutic approaches to treat malignancies [179].

Recently CRISPR has been used to detect HCV and also to inhibit the virus. Weiss et al. developed the ability to target HCV within eukaryotic cells using the endonuclease from Francisella novicida [180]. They also used FnCas9 proteins to inhibit viral protein production during HCV infection within the infected cells [180]. Furthermore, Jain et al. used CRISPR-Cas12a complexes to detect HCV [181]. In addition, they also developed a platform with crRNAs and optimized conditions to detect various clinically significant nucleic acid targets in the femtomolar range [181]. CRISPR is an emerging technology being used to detect viral infection; however, additional advances will be needed before it can be developed into a routinely utilized technology to detect HCV RNA. The characteristics of the various diagnostic techniques discussed above that show promising use in HCV testing in the future are highlighted (Table 2).

Table 2. Summary of future diagnostic techniques for HCV, their strengths and limitations.

	Strengths	Limitations	References
LAMP	Isothermal technique does not require expensive bulky thermocyclers, making it suitable for low-resource settings. Tests for viral nucleic acid, which makes it more sensitive than immunoassays.	Is known to have higher false positive rates. Utilizes 4–6 primers, which makes primer designing complex.	[138,144,156]
Surface Plasmon Resonance	Real-time quantification with high sensitivity. Precise measurement of binding kinetics. High multiplexing capacity. Label-free method.	Requires instruments that are not currently point-of-care friendly. Performance can be affected by non-specific binding. Needs extensive sample preparation for effective analysis.	[159,165,182]
Microfluidics	Minimizes reagent and sample volumes, reducing costs. Facilitates the combination of multiple analytical steps on a single chip. Enables high-throughput analysis with faster turnaround times.	Developing and manufacturing microfluidic devices can be intricate and expensive. Susceptible to blockages and issues with sample flow control.	[173,183]
Electrochemical Sensors	Capable of detecting low concentrations of analytes. Potential for compact, point-of-care devices. Generally lower production costs compared to optical systems. Label-free.	Electrochemical signals can be affected by impurities. Electrode surfaces may degrade over time, impacting performance. Requires regular calibration for accurate results.	[177,178,184]
CRISPR	Utilizes CRISPR-Cas systems for precise target recognition, offering high specificity. Potential to be point-of-care friendly.	Is still under development, with limited widespread clinical validation. Pending comprehensive regulatory endorsements for clinical use.	[185,186]

6. Future Directions and Conclusions

In conclusion, HCV is a viral infection that is associated with significant mortality and morbidity globally. HCV prevalence is estimated to be around 50 million people. Early detection of HCV infection through testing is crucial in preventing long-term complications such as cirrhosis, hepatocellular carcinoma, and decompensated liver disease. Despite the limitations of testing methods, early detection of HCV remains a crucial step in the clinical management of the infection and facilitates the initiation of antiviral therapy.

The most common testing methods, as stated earlier, are PCR and EIA. The most widely used approach for testing is an EIA to test for the presence of HCV Abs, which indicates exposure. If the EIA is positive, a PCR test is subsequently performed to confirm active viral infection. EIA can produce false-negative results if conducted too soon after exposure to the virus and the inability to differentiate between those with active infections, those who have spontaneously cleared the virus, or those who have been cured through antiviral therapy. PCR, on the other hand, is more expensive and requires specialized laboratory equipment and trained personnel, which hinders its ability to be used in resource-limited areas. Additionally, the two-step testing approach is not feasible since it results in incomplete testing when patients do not return for confirmatory molecular testing. There is a dire need for single-step sample collection and dried blood spot testing to be adopted globally. In the long term, testing needs to be decentralized, for which technologies such as LAMP, SPR, microfluidics, electrochemical-based sensing, and CRISPR are looking to bridge the gap and cut the cost of traditional molecular tests that leverage PCR. If developed for HCV, these technologies can be used for the detection of other viral diseases.

The emerging technologies being developed are paramount in improving affordability and sensitivity. POC rapid RNA detection can detect HCV when an active infection occurs, and antibodies are yet to be produced. However, POC rapid RNA detection methods may require more extensive sample preparation, which is a significant challenge. Ideally, new technologies will facilitate the development of a low-cost automated sample preparation process that can take whole blood without extensive preprocessing and create a more amenable analyte to enable HCV detection on the same platform/device in under an hour. If successful, this would be a significant advance in HCV diagnosis, particularly in resource-limited settings, making sure that undiagnosed patients could be linked with appropriate antiviral therapy.

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