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REVIEW

Update on hepatitis B and C virus diagnosis

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Abstract

Viral hepatitis B and C virus (HBV and HCV) are responsible for the most of chronic liver disease worldwide and are transmitted by parenteral route, sexual and vertical transmission. One important measure to reduce the burden of these infections is the diagnosis of acute and chronic cases of HBV and HCV. In order to provide an effective diagnosis and monitoring of antiviral treatment, it is important to choose sensitive, rapid, inexpensive, and robust analytical methods. Primary diagnosis of HBV and HCV infection is made by using serological tests for detecting antigens and antibodies against these viruses. In order to confirm primary diagnosis, to quantify viral load, to determine genotypes and resistance mutants for antiviral treatment, qualitative and quantitative molecular tests are used. In this manuscript, we review the current serological and molecular methods for the diagnosis of hepatitis B and C.

Key words: Diagnostic methods; Genotypes; Hepatitis B virus; Molecular diagnostic techniques; Serological tests; Hepatitis C virus

Core tip: Reliable methods for diagnosing hepatitis B virus (HBV) and hepatitis C virus (HCV) infection are essential to reduce the burden of these infections. Serological and molecular assays are used to identify acute and chronic cases of infection. In this article, the current knowledge about HBV and HCV diagnosis is updated and emphased the characteristics of each techniques to be useful to most laboratory personnel.

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INTRODUCTION

Hepatitis B virus (HBV) infection is caused by a virus that

shows a diameter of 42 nm and comprises an icosahedral capsid surrounded by a lipid envelope containing hepatitis B surface antigen (HBsAg)[1]. Viral capsid is formed by core protein and carries viral genome and polymerase[2]. HBV genome is composed by a circle DNA partially double stranded and has four open reading frames regions overlaped (ORFs): PreC/C that encodes for hepatitis B e Antigen (HBeAg) and core protein (HBcAg); P for polymerase (reverse transcriptase), S for surface proteins [three structures of HBsAg, small (S), middle (M) and large (L)], and X for a transcriptional transactivator protein[3,4]. Despite HBV has a DNA genome, it shows high mutation rates, similar to those observed in RNA viruses and retroviruses[5] what could be due to viral polymerase mistakes associated with the additional step of reverse transcription, necessary for genetic material replication[6]. HBV has been classified in 10 genotypes (A to J) with between approximately 4 and 8% intergroup nucleotide divergence across the complete genome and genotypes A-D, F, H, and I are classified further into subgenotypes[4,7].

In 1989, hepatitis C virus (HCV) was described after extensive research conducted to find the cause of post-transfusion hepatitis[8]. In 1991, Choo *et al*[9] were able to identify complete HCV genomic sequence and thereafter, it was possible to conceive hybridization probes and primers to amplify viral genome by polymerase chain reaction (PCR)[10]. HCV particle is constituted by a spherical envelope which involves an icosahedral capsid. It possess a RNA genome with 9.5 kb and an ORF responsible for encoding viral polyprotein, constituted by structural (core, glycoproteins E1 and E2 and protein P7) and nonstructural (NS2, NS3, NS4a, NS4b, NS5a, NS5b-RNA polymerase) proteins. HCV presents high genome variability due to low proofreading of viral RNA polymerase. This variability allows virus classification into 7 genotypes (1 to 7), based on their genomic features[11].

Worldwide HBV and HCV infection are the principal etiological agents of chronic liver disease[12-14]. Both viruses are transmitted by parenteral route, sexual and vertical transmission is more common for HBV infection compared to HCV[15,16]. Approximately 240 million people are HBV chronic patients while 150 million individuals present HCV infection in the world[13,14].

HBV endemicity is classified as high, intermediate or low according HBsAg prevalence[12,17]. South East Asia, sub-Saharan Africa, China, Indonesia and Nigeria are considered high endemicity regions since HBV chronic infection can be present in more than 8% of the population[17]. Developed countries in Western Europe and North America are classified as low endemicity areas since chronic infection rates range from 0.5% to 2.0% of population. South America and Central countries, Eastern and Southern Europe and South West Asia are considered intermediate areas since HBV prevalence rates between 2% and 7% of population[12,17].

HCV global prevalence is 1.6% corresponding to 115 millions of infected individuals and viremia prevalence is 1.1% corresponding to 80 million of cases[18]. Although HCV infection presents global distribution, different prevalences are observed according geographic area[18,19]. High anti-HCV prevalence is observed in Central Asia (5.4%), Eastern Europe (3.3%), the Midwest of North Africa region (3.1%) and Central and Western Sub-Saharan Africa (4.2% and 5.3%, respectively). Intermediate prevalence rates are found in southern sub-Saharan Africa (1.3%), Central Europe (1.3%), Australia (1.4%), Latin America (1%-1.25%). Low prevalence are observed in Oceania (0.1%), Caribbean (0.8%) and Western Europe (0.9%)[18]. Highest rates of chronic infection can be found in Egypt (22%), Pakistan (4.8%) and China (3.2%) where the main infection mode is the reuse of contaminated needles[13,14,20].

One important measure to diminish the burden of HBV and HCV infection is the diagnosis of acute and chronic cases. In this article, we describe the current serological and molecular methods for HBV and HCV diagnosis.

SEROLOGICAL ASSAYS FOR HBV DIAGNOSIS

Takahashi *et al*[1] described the Australia antigen in 1965 and after this was named HBsAg, while the Dane particle (complete hepatitis B virion) was subsequently identified in 1970. After these discoveries, the identification of HBV antigens and antibodies allowed: (1) clarify the natural history of the disease; (2) evaluate the clinical phases of infection; (3) monitor antiviral treatment; (4) identify infected individuals; and (5) monitor the efficacy of immunization[21-23].

HBsAg is the first serological marker to appear indicating active infection. Chronic infection is characterized by the persistence of this marker for more than 6 mo[24]. Recently, HBsAg quantification has become important to monitor polyethylene glycol interferon treatment[25] since this assay could be used along to HBV DNA test to define the clinical phase of HBV infection and to evaluate the therapy[26]. To date, two chemiluminescent microparticle immunoassay (CMIA) (Architect HBsAg QT and Elecsys HBsAg Ⅱ, Elecsys) could be used for HBsAg quantification showing good agreement[27].

Anti-HBs is a neutralizing antibody, and its presence indicates immunity to HBV infection[28,29]. The simultaneous presence of anti-HBs and HBsAg has been documented in HBsAg positive patients[30,31], probably due to the incapacibility of antibodies to neutralize the circulating virions. In this situation, these people are classified as carriers of HBV infection.

HBeAg marker indicates viral replication and risk of transmission of infection, and seroconversion of HBeAg to anti-HBe is associated with remission of liver disease[32]. However, some anti-HBe reactive subjects continue to have active viral replication and hepatic disease caused by mutations in the pre-core and core

Table 1 Clinical significance of hepatitis B virus serological markers

Marker	Clinical significance
HBsAg	First marker to appear in course of infection Appears one to 3 wk before the onset of symptoms The permanence of this marker for more than 24 wk indicates chronicity
Anti-HBc IgM	Marker of recent infection marker Appears with the onset of symptoms and persists up to 32 wk after infection
Anti-HBc IgG	This marker did not indicate immunity and it is not elicited by vaccination This presence indicates prior contact with the virus
HBeAg	It appears before the onset of symptoms and indicates viral replication independent of disease phase (acute or chronic) This presence indicates high infectivity
Anti-HBe	It appears after the disappearance of HBeAg Their presence suggests reduction or absence of viral replication, except when infection is due to HBV strains with pre-core mutant (not producing the protein "e")
Anti-HBs	It appears one to 3 mo after HBV vaccination or after recovery of HBV acute infection and indicates immunity to HBV infection

HBV: Hepatitis B virus; HBeAg: Hepatitis Be Antigen; HBsAg: Hepatitis B surface antigen; Anti-HBe: Antibodies against HBeAg; Anti-HBs: Antibodies against HBsAg.

region in the HBV genome, which reduces the production of HBeAg[24].

The HBcAg is intracellular and for this reason is not detected in the serum of infected individuals. Antibodies against core protein (anti-HBc) appear shortly after HBsAg in acute infection and persist after acute phase indicating previous exposure. The IgM anti-HBc antibody is the first detected during acute infection, approximately 1 mo after the onset of HBsAg and disappears after 6 mo of infection. Anti-HBc IgG remains detectable in patients cured of hepatitis B and among chronic cases of HBV infection[28,33].

Isolated Anti-HBc can be found in three situations: (1) during the window period of acute phase when the anti-HBc is predominantly IgM; (2) years after acute infection had finished and anti-HBs has diminished to undetectable levels; and (3) when the titer of HBsAg has decreased thereunder the detection level after many years of chronic HBV infection. In order to evaluate the presence of isolated anti-HBc, serum samples should be retested for anti-HBc, HBsAg, anti-HBe, and anti-HBs. If the sample still had an isolated anti-HBc positive result, this sample should be tested for IgM anti-HBc in order to eliminate the possibility of recent HBV infection. HBV DNA testing should be done in chronic liver disease patients to eliminate low-level chronic HBV infection[34,35]. The clinical significance of serum markers detected during the course of infection with HBV is disclosed in Table 1.

Specific serologic assays to detect HBV markers were developed around the 70s[36]. Various serological techniques could be employed, such as radioimmunoassay (RIA), enzyme immunoassay (EIA), Electrochemiluminescence immunoassay (ECLIA) and chemiluminescence immunoassay (CLIA), micro-particle enzyme immunoassay (MEIA), CMIA[36,37].

RIA was the first technique used for HBV diagnosis where one of reactants is conjugated to radioisotopes[38,39]. This method present good sensitivity but high cost and risk to operator. In 1971, Engvall *et al*[40] described a technique named EIA that is similar to RIA where enzymes are attached to one of the reactants in an immunoassay to allow detection through the development of color after the addition of a suitable substrate/chromogen. The colored product is monitored visually or by spectrophotometer where the amount of the substance measured is related to color intensity. The advantages of this technique include highly reproducible results, automation, and low cost[41]. An evaluation of 70 HBsAg kits showed that 17 HBsAg EIA kits present high analytical sensitivity 4 IU/mL, but reduced sensitivity for HBsAg was observed in samples containing genotypes/subtypes *D/ayw3*, *E/ayw4*, *F/adw4* and *S* gene mutants[42].

MEIA is a method that uses very small microparticles in liquid suspension as solid-phase support. Particles are coated with related molecules specific for the measured material. MEIA is executed in less time than other immunological methods due to the presence of active surface area of microparticles what rises the kinetics study and decreases the incubation time. ECLIA uses molecules as conjugated, generate chemiluminescence like, luminol derivatives, nifropenil oxalate derivates or rutenium tri-bipridyl with tripropylamine. The electrochemiluminescence occurs when is applied an electric current in an electrode platinum, creating an electric field that making all materials respond. This reaction hydrolyzes the chemiluminescent substrate, producing an unstable product which after stabilization generates emission of light photons (amplified) what is measured by a photomultiplier[43].

Kim *et al*[44] compared a radioimmunoassay, an ECLIA (Modular E170 analyzer, Roche Diagnostics, Mannheim, Germany) and CMIA (Architect i2000 analyzer, Abbott Diagnostics, Abbott Park, IL, United States) for HBV markers detection showing concordance rates among the three analyzers of 100%, 91.6%, 94.6%, and 82.2% for HBsAg, anti-HBs, HBeAg, and anti-HBe, respectively. High difference results among three immunoassays analyzers (Abbott AxSYM, Roche Modular Analytics E170, and Abbott Architect i2000) for HBV markers detection was observed in samples with low level of serum HBV markers[45]. Xu *et al*[46] also showed that most weak positive results, determined by ECLIA, were negative determined by ELISA.

Huzly *et al*[47] compared the performance of six different automated immunoassays (one MEIA and five chemiluminescence assays) and three EIAs for anti-HBs quantification. The assay specificity ranged between 96.8% and 100% and sensitivity ranged between 93.5% and 100%. There was no difference between anti-HBc-

positive and -negative individuals and, hemolysis or lipemia did not seem to influence the measurement. However, classical EIAs tend to detect lower anti-HBs levels than the automated systems.

EIA is the most widely technique used for detection of HBV serological markers. Although EIAs are sensitive and specific, they are time consuming, need for sophisticated equipment and trained technicians, continuous supply of electricity, and long turnaround time hampering the execution of these assays in field settings and during the household surveys[48]. Due to this situation, rapid point-of-care tests (RPOCTs) were developed for HBV diagnosis. These assays use particle agglutination, immunochromatography, immunodot or immunofiltration. The device contains a solid support (cellulose or nylon membranes, latex microparticles or plastic cards) where viral antigens or antibodies are fixed and results can be read in up to 10 min[49]. Rapid assays offer advantages of simplicity, low need for instrumentation, minimum training to execute the assay and performance at room temperature.

Since 1990s, several RPOCTs for HBsAg detection have been developed. The sensitivity of these assays varies from 43.5% to 100% while specificity varies from 95.8% to 100%[50-54]. A recent systematic review showed that the performance of rapid tests for HBsAg detection was higher in developed countries compared to developing countries what could be due to minimal heterogeneity observed in first than in later. RPOCTs presented analytical sensitivity of 4 IU/mL, but the performance of these assays is poor in seroconversion panels and among individuals infected by several HBV mutants. Thus these tests are not indicated in situations with low concentrations of HBsAg such as in healthy blood donors, general populations, patients recovering from acute HBV infection and those on antiviral therapy[49]. RPOCTs for detection of anti-HBc, anti-HBs, HBeAg, anti-HBe markers have demonstrated sensitivities of 85.5%, 64.2%, 80% and 82.8% and specificities higher than 95% for those assays[55,56].

Actually, the evolution of development of nanoscience and nanotechnology has increased the development of immunosensors for HBV diagnosis. Immunosensors are solid-state affinity ligand-based biosensing apparatus that combine immunochemical reactions to proper transducers. Generally, an immunosensor comprises of a sensing element and a transducer. The sensing element is composed by means of the immobilization of antigens or antibodies, and the binding event is transformed into a measurable signal by the transducer[57]. The goal of immunosensor is to produce a signal proportional to the concentration of analyte[58-60]. Wang and collaborators[61] developed a gold nanorod based localized surface plasmon resonance biosensor that quantify HBsAg until 0.01 IU/mL. This limit of detection is about 40 times lower than the limit of detection of the EIA method. Other immunosensors for HBsAg detection uses magnetic nanoparticle and three dimensional carbon nanotube-conducting polymer network detecting 0.001 to 0.015 ng/mL of HBsAg[62,63].

For HBV antigen or antibodies detection, it is necessary blood sample collection by venipuncture in order to obtain sera or plasma samples. However, venipuncture is difficult in some individuals like drug users, haemodialysis, obese and elder individuals. In addition, the transport of these samples from remote areas to laboratories could be difficult. This situation has led to development of methods for HBV diagnosis in alternative fluids, like saliva or dried blood spot (DBS) samples[64,65].

EIA for HBsAg detection among saliva samples present sensitivities of 74.29% to 95.24% and specificities of 89.88% to 100%[66-70]. Assays for anti-HBs, total anti-HBc in saliva samples showed low sensitivity (< 15%) while assay for detecting IgM anti-HBc assay demonstrated sensitivity and specificity of 100%[71].

Using DBS samples along to immunoassays, sensitivities varies from 78.6% to 98% for HBsAg detection, 90.5% to 97.1% for anti-HBc detection and 74.2% to 97.5% for anti-HBs detection. In all of these studies specificities varies from 88.6% to 100%[72-76]. HBV markers could be detected in DBS samples using EIA until 60 d of storage in room temperature[74]. HBV prevalence has been evaluated using DBS in specific groups at risk for infection, such as drug users, and endemic areas[77-79].

Serological diagnostic tests for HBV have improved in sensitivity, time, need for trained personnel and cost over time. In laboratories with low infrastructure or in field situations, rapid tests could be useful since they can be storage at room temperature, results are available in few minutes and they do not need trained technicians. While in laboratories presenting good infrastructure and with high-demand of results, automated ELISA ECLIAs, MEIAs, CLIAs and CMIAs can be used due to its high sensitivity although trained technicians, sophisticated equipment, and continuous supply of electricity are necessary. The main characteristics, advantages, disadvantages and specific applications of HBV serological assays are disclosed in Table 2.

MOLECULAR METHODS FOR HBV DIAGNOSIS

HBV DNA is detectable at the beginning of infection (1 mo after exposure to HBV) and increases to get a peak approximately 3 mo after the exposure to HBV reaching usually more than 10^8 copies/mL and then progressively decreases in chronic infection or disappears at the resolution of infection. In many chronic patients, when HBeAg seroconversion occurs, low levels of viral load persists (< 10^4 copies/mL)[80].

HBV DNA qualitative and quantitative methods are useful to: (1) diagnose HBV replication in chronic infections; (2) evaluate the prognosis of the disease and follow the risk of progression to cirrhosis and hepatocellular carcinoma; (3) define the beginning of

Table 2 Advantages and disadvantages characteristics of hepatitis B virus serological assays

Technique	Advantages	Disadvantage	Commercial assays	Ref.
RIA	High sensitivity	High cost Risk to operator	IRMA kit (North Institute of Biological Technology, Beijing, China)	[38,39,44]
EIA	Automation High reproducible results Low cost	Time consuming, need sophisticated equipment and trained technicians, continuous supply of electricity, not suitable for field settings	ETI-AB-AUK-3 (DiaSorin) Enzygnost anti-HBs (Dade Behring) Monolisa anti-HBs (Bio-Rad Laboratories)	[41,42,47,48]
MEIA	High sensitivity Faster than other immunological methods	Sophisticated equipment, trained technicians, continuous supply of electricity	Abbott Axsym AUSAB assay	[47]
ECLIA	High sensitivity Results available in few minutes Automation	High cost, sophisticated equipment, trained technicians, continuous supply of electricity	Modular E170 analyzer, Roche Diagnostics	[43,44,46]
CLIA	High sensitivity and specificity Automation	High cost, sophisticated equipment, trained technicians, continuous supply of electricity	Advia Centaur anti-HBs Vitros anti-HBs on the Vitros ECI Immunodiagnostic system (Ortho Clinical Diagnostics) Roche Elecsys anti-HBs on the Modular System (Roche Diagnostics) Liaison anti-HBs on the Liaison system (DiaSorin) Abbott Architect anti-HBs assay on the Architect i2000 system (Abbott)	[47]
CMIA	High sensitivity and specificity Automation	High cost, sophisticated equipment, trained technicians, continuous supply of electricity	Architect i2000 analyzer (Abbott Diagnostics)	[44]
Rapid point-of-care tests	Simplicity, do not need sophisticated equipaments, minimum training to execute the assay, storage and performance at room temperature, results can be read in up to 10 min	Poor performance in seroconversion panels and among individuals infected by several HBV mutants	Determine™ HBsAg (Abbott Laboratories) Virucheck® HBsAg (Orchid Biomedical Systems) Cypress HBsAg (Cypress Diagnostics) Hexagon® HBsAg (Human) Cortez Rapidtest (Cortez Diagnostics) VIKIA HBsAg (Biomériuex) Quick Profile™ (Lumiquick) DRW-HBsAg v2.0 (Diagnostics for the Real WorldTM) AMRAD ICT Binax Advanced quality™ one step test (Intec Products)	[49-56]

HBV: Hepatitis B virus; RIA: Radioimmunoassay; EIA: Enzyme immunoassay; MEIA: Micro-particle enzyme immunoassay; ECLIA: Electro-chemiluminescence immunoassay; CLIA: Chemiluminescence immunoassay; CMIA: Chemiluminescent microparticle immunoassay; HBsAg: Epatitis B surface antigen; Anti-HBs: Antibodies against HBsAg.

antiviral treatment; and (4) monitor antiviral treatment and to identify resistance to nucleoside/nucleotide analogues drugs[81,82].

Molecular assays with high-sensitivity are clearly important for the diagnosis of chronic hepatitis B without HBeAg detection in serum (core promoter, precore stop mutation), and occult HBV, where viral loads can be quite low. The principle of methods to detect and quantify HBV DNA is the signal amplification such as branched DNA technology (bDNA) and hybrid capture or target amplification such as PCR[83-85].

In therapeutic monitoring of HBV, a more sensitive assay with a lower limit of detection of 10 IU/mL is recommended for early detection of viral reactivation. In addition, the assay employed should equally quantify all HBV genotypes[86]. A commercial PCR method to quantify HBV DNA is COBAS Amplicor HBV Monitor test (Roche Molecular Systems, Pleasanton, California, United States) that measure HBV DNA in serum samples, being considered reproducible, with a high degree of accuracy and precision in both intra-assay and inter-assay comparison[87]. This assay presented a limit of detection of 200 HBV DNA copies/mL and has the disadvantage of requiring dilution of samples with high viral load, which is not a practical solution for all laboratories[88].

Methods of signal amplification, like bDNA, allow the direct quantification of HBV DNA in human serum or plasma. In this method, sample DNA is caught by a set of capture nucleotide probes fixed on a microtitulation well. Then, another set of target probes hybridize with both the target DNA and capture probes, and this complex is hybridized with multiple copies of alkaline phosphatase. After incubation with a chemiluminescent substrate, the luminometer measures the results as relative light units and the amount of HBV DNA in each sample is determined by comparison to a standard curve. VERSANT HBV 3.0 Assay (Siemens Healthcare, United States) is a commercial method that uses bDNA technology and presented a detection limit of 2000 HBV DNA IU/mL[83,89] (Table 3).

Cobas Amplicor HBV Monitor assay (PCR technology) is more sensitive than Quantiplex HBV DNA; Bayer

Table 3 Performance characteristics of commercial methods for hepatitis B virus and hepatitis C virus detection and quantification

Assay (manufacture)	Method	Measurable range (IU/mL)	Limit of detection IU/mL (using WHO HBV standard)	Conversion factor (IU/mL to copies/mL)
HBV				
Cobas ampliPrep/Cobas TaqMan HBV test v2.0 (Roche Diagnostics, California, United States)	Semi-automated qPCR	20-1.7 × 10^7	20	5.82
Cobas TaqMan HBV test (for use with high pure system)	Semi-automated real time PCR	29-1.1 × 10^7	6	5.82
Abbott real time HBV (Abbott Diagnostic, Chicago, United States)	Automated real time PCR	10-1 × 10^9	10	3.41
Versant HBV 3.0 assay (Siemens Healthcare, United States)	Branched DNA	2000-1 × 10^8	2000	5.6
HCV qualitative				
Cobas Amplicor HCV v2.0 (Roche)	Semiautomated RT-PCR	50	50	-
Versant HCV RNA qualitative assay (TMA)	Transcription-mediated amplification	< 9.6	5-9.6	-
Quantitative				
Versant HCV RNA 3.0 (Siemens)	Branched DNA	7.7 × 10^6	615	5.2
Cobas Amplicor Monitor HCV v2.0 (Roche)	Semiautomated RT-PCR	5 × 10^5	600	2.7
Real time HCV (Abbott)	Real time PCR	1 × 10^7	10	3.8
Cobas AmpliPrep/Cobas TaqMan (Roche)	Automated real time PCR	6.9 × 10^7	43	3
High Pure/Cobas TaqMan (Roche)	Semiautomated real time PCR	3.9 × 10^8	25	3

WHO: World Health Organization; HBV: Hepatitis B virus; HCV: Hepatitis C virus; PCR: Polymerase chain reaction; RT-PCR: Reverse transcriptase-PCR; qPCR: Quantitative PCR.

Diagnostics (bDNA technology) to detect HBV DNA among HBeAg negative samples and useful for monitoring the viral load during treatment in chronic HBV infection[90]. On the other hand, Cobas Amplicor HBV Monitor presented poor agreement to Digene Hybrid Capture 2 (HC2; Digene Corporation, Gaithersburg, Md.)[88].

Currently, real time PCR has become the standard technique of choice to detect and quantify HBV DNA in clinical practice due to its capacity of detecting low viral loads (10-15 IU/mL) and having a broad dynamic range (upper range of quantification of 7-8 Log10 IU/mL) as shown in Table 2. Moreover, they do not carry over contamination and can be fully automate[91].

At this time, several real time PCR assays are commercially available, such as, Da-an real-time HBV DNA assay (Da-an Gene Co. Ltd, Sun Yat-Sen University, Guangdong, China), COBAS TaqMan HBV test (Roche Molecular Diagnostics, Pleasonton, CA, United States), Abbott RealTime HBV assay (Abbott Molecular, Des Plaines, IL, United States), Artus RealART HBV LC PCR kit (QIAGEN, Hamburg, Germany), AdvanSure HBV real-time PCR assay (LG Life Sciences, Ltd., Seoul, South Korea). Cobas Taqman HBV (Roche) can be used along to automated sample preparation, Cobas AmpliPrep system (CAP-CTM, Roche Molecular System, Pleasanton, CA)[92] and the Abbott HBV (Abbott Diagnostic, Chicago, IL) uses m2000rt amplification platform along to m2000sp device for sample preparation[93]. Characteristics of quantitative methods for HBV DNA are described on Table 3.

Kim *et al*[94] showed good correlations among Abbott RealTime HBV Quantification Kit, the COBAS TaqMan HBV Test, and the VERSANT bDNA 3.0 assay, AdvanSure HBV real-time PCR assay detecting HBV genotypes A-F and without cross reactivity with high HCV RNA levels or high protein concentrations. Qiu *et al*[95] demonstrated good correlation among Abbott and Da-an assay for HBV DNA quantification. Morris *et al*[93] observed good correlation among Cobas Taqman HBV Test and Abbott RealTime HBV for HBV DNA quantification, although Abbott assay presented wide dynamic range without additional dilution or repeating of HBV high titers. In house real time PCR molecular beacon based was builder up by Paraskevis *et al*[96] showing good correlation to COBAS TaqMan HBV test.

HBV DNA detection and quantification are made using serum samples, but alternative fluids, like saliva, DBS samples have been studied as potential fluids for HBV molecular diagnosis[64,75,97-100]. Kidd-Ljunggren *et al*[98] demonstrated that concentration of HBV DNA in saliva is 1000-fold lower than in serum, while Heiberg *et al*[99] could quantify HBV DNA using Cobas TaqMan HBV Test (Roche Diagnostics) in saliva samples. Mohamed *et al*[75] demonstrated sensitivity of 98% and specificity of 100% for the detection of HBV DNA using DBS and performed HBV genotyping and mutation detection among those samples with total concordance between the 10 paired DBS and plasma samples. Jardi *et al*[101] also revealed no decrease in HBV DNA levels or integrity among DBS storage for 7 d at room temperature and 21 d at -20 ℃.

Besides that, the detection of HBV DNA in plasma or serum is also important to determine the occult hepatitis B defined by detectable HBV DNA in peripheral blood or liver in the absence of HBsAg. Tests for occult

HBV detection are recommended in the following situations: (1) in cryptogenic liver disease, particularly when individual presented anti-HBc in serum; (2) prior to immunosuppression, due to the potential for hepatitis flares; and (3) in solid organ transplant donors whose only anti-HBc is detected in serum, due to the potential for transmission[83].

Evidences are increasingly suggesting that the HBV genotyping is important for designing appropriate antiviral treatment and determining HBV disease progression. The disease progress faster in individuals infected by HBV genotype A than genotype D, subjects infected with genotype C progressed to end stage liver disease earlier than those infected by genotype B and higher mortality rates are observed in individuals infected with genotype F than those infected with genotype A or D[102].

HBV genotyping can be determined using several methods: Reverse hybridization, restriction fragment-length polymorphism (RFLP), genotype-specific PCR assays, sequence analysis, microarray (DNAchip), real time PCR and fluorescence polarization assay[102,103]. Among them, PCR-RFLP is widely used to genotype HBV since it is inexpensive and simple. Nevertheless, this technique is poor accurate to identify some genotypes[104]. A commercial method to genotype HBV is the INNO-LIPA® HBV Genotyping (Fujirebio Europe, Tokyo, Japan) based on reverse hybridization that shows high sensitivity and a detection limit of 700 copies of HBV[105,106], but is relatively cost. INNO-LIPA can be completely automated if using the systems Auto-LiPA48 and AutoBlot 3000H and the LiRAS® for LiPA HBV to reading and interpretation of strips. INNO-LIPA® HBV Genotyping and direct sequencing presented the best results when genotyping methods of HBV were compared[107].

To identify amino acid substitutions associated to antiviral resistance to treatment, direct sequence analysis and reverse hybridization methods are used. nowadays, early detection of HBV substitutions conferring resistance to nucleoside/nucleotide analogues could be useful to modify therapy in order to avoid HBV reactivation and hepatitis flare[108]. For this purpose, commercial assays are available, such as the Trugene HBV Genotyping Kit (Siemens Medical Solutions Diagnostics), which is based on direct sequence analysis of a portion of the reverse transcriptase domain of the HBV polymerase gene[109] and can also determine HBV genotype satisfactory[110].

However, despite commercial methods can be used for mutational analysis in HBV DNA sequence, direct DNA sequencing is still the standard method cause yields accurate genotype assignments and also the method of choice for patients infected with recombinant genotypes and drug resistance mutations[110,111].

In the past years, the diversity of HBV quasispecies and minority drug resistance mutations were estimated through cloning of individual amplicons followed by Sanger sequencing. However, the "next generation" ultradeep-sequencing allows direct sequencing of the mixed population sample and relative quantification of individual mutations with extremely high coverage over a relatively short time frame[111-114]. The platforms of ultradeep sequencing include: 454 Sequencing (Roche Diagnostics), Illumina Sequencing (Illumina/Solexa) and Pyromark Sequencing (QIAGEN), SOLiD (Applied Biosystems/Life Technologies), Ion torrent (Life Technologies)[115]. All of them present the principle of sequencing by synthesis, that involves sequencing of a single strand of DNA through synthesis of the complementary strand, one base at a time, and the detection of the individual nucleotide incorporated at each step. Fluorescence or light is only emitted when the nucleotide solution complements the first unpaired base in the template DNA strand[116]. Because these signals are obtained by synthesizing new copies of DNA template, the results can be used for extremely reliable investigation of viral mutations[114].

For the past twenty years fluorescence based quantitative PCR (qPCR) chemistries have revolutionized nucleic acid diagnostics and become the gold standard for viral load quantification. These methods are high sensitivity and could be used to evaluate the prognosis and risk of progression, to define the beginning of antiviral treatment and to monitor antiviral treatment but the major disadvantages is the high cost for commercial assays. Methods to determine HBV genotypes and mutations, like nucleotide sequencing have been developed and recently the arouse of third generation sequencing methods promises to reduce the lengthy manual handling times associated with current ultradeep-sequencing approaches, decreasing the generation of raw data, but increasing both throughput and read length in every facet of medical research.

The main characteristics, advantages, disadvantages and specific applications of HBV molecular assays are disclosed in Table 4.

SEROLOGIC TESTS FOR HCV

The hepatitis C diagnosis is performed through the detection of antibodies anti-HCV in blood samples where a non-reactive test result indicates the absence of HCV infection. A positive result for anti-HCV detection or a suspected case of HCV exposure should be followed by HCV RNA test. Serological diagnosis for HCV infection are based on the detection of direct antibodies against viral antigens (non-structural and structural) in human serum or plasma. As the same as cited for HBV serological diagnosis and immunoassays (like EIA or ECLIA) are generally used for anti-HCV detection. EIA has the advantages of high sensitivity, fast processing, high reliability, ease of automation and relatively low cost[57,117]. This method has undergone some changes over time, seeking to improve their diagnostic capability and therefore increased sensitivity and specificity of the assay. Until now, three generations of EIA for detecting anti-HCV using recombinant proteins or synthetic peptides have been developed[57,117,118].

Table 4 Main characteristics of hepatitis B virus and hepatitis C molecular techniques

HBV and HCV molecular diagnosis	Application	Method	Advantages	Disadvantages	Ref.
HBV DNA qualitative methods	Diagnose infection HBV occult cases identification Screening on blood donors	PCR	Low cost; high sensitivity	It only determines the presence or absence of HBV DNA	[81,82,85]
HBV DNA quantitative methods	Evaluate the prognosis and risk of progression Define the beginning of antiviral treatment Monitor antiviral treatment	bDNA	Wide dynamic range	Low sensitivity to detect low HBV DNA levels	[81,82]
		Hybrid capture	More sensitive than bDNA; less labor-intensive	Low sensitivity to detect low HBV DNA levels; individual probes are required	[83]
		Real time PCR	Capacity of detecting low viral loads; broad dynamic range; do not carry over contamination; can be fully automated	High cost	[85,91]
HBV DNA genotyping methods	Determination of HBV genotype	RFLP	Easily done; low cost; simple, rapid and suitable for large number of samples	Low sensitivity for typing samples with low HBV DNA levels; poor accurate to determine some genotypes	[85,104]
		Genotype specific PCR assays	Automated systems; high sensitivity; easy to perform; suitable for detecting mixed genotype infections	High cost	[107]
		Sequence analysis	Identification of patients infected with recombinant genotypes	Technically demanded; time consuming	[107]
HBV DNA aminoacid substitution identification	Identify antiviral resistance to treatment	Direct DNA sequencing	Accurate	Technically demanded; time consuming; necessity of cloning for identification of mixed population	[107,110,111]
		Commercial methods	Sequencing of mixed population, relative quantification of individual mutations with extremely high coverage	Differences between the statistical and biological/clinical relevance of HBV mutation maximal sequence read length and PCR amplification bias	[114]
HCV RNA qualitative methods	To confirm chronic hepatitis C in patients with positive HCV antibodies To identify virological response during, at the end or after antiviral therapy To screen blood donations for evidence of infection with HCV	RT-PCR	High sensitivity Equal sensitivity for all genotypes	It only determines the presence or absence of HCV RNA	[121,168]
		Transcription-mediated amplification	High sensitivity; amplifies viral RNA; more sensitivity for detection of genotype 1	It only determines the presence or absence of HCV RNA	[121,168]
HCV RNA quantitative methods	To guide treatment decisions; To evaluate the prognosis; To monitor the antiviral efficacy of treatment	bDNA	Wide range of detection of HCV independent of HCV genotype (615 IU/mL to 8 million IU/mL)	Low sensitivity to detect samples presenting low HCV RNA levels	[172]
		qRT-PCR	Capacity of detecting low viral loads; broad dynamic range; not carry over contamination; can be fully automated	High cost	[170-173]
HCV RNA genotyping methods	HCV genotyping is mandatory for double antiviral treatment (interferon and ribavirin), since patients infected with genotypes 1 or 4 are treated for longer times than patients infected by genotypes 2 and 3	RFLP	Easily done; low cost; simple, rapid and suitable for large number of samples	Low sensitivity for typing samples with low HCV RNA levels; Poor accurate to determine some genotypes	[186,187]
		Probes	Easily done; low cost; useful to detect HCV genotypes and subtypes based on region 5'UTR and core and has a low limit of detection	Identify only subtypes 1a and 1b; discrepant results among subtypes when compared to sequence analysis of NS5B region	[180-184]

		qPCR	Can be fully automated avoiding contamination; determines the viral genotype and subtypes 1a, 1b, 2a, 2b, 3, 4, 5 and 6	High cost	[186,187]
		Direct sequencing	Gold standard; identification of patients infected with recombinant genotypes	Technically demanded; time consuming	[120,182]
HCV RNA aminoacid substitution identification	Identify antiviral resistance to treatment	Direct Sequencing	Identification of antiviral resistance in majority population	Technically demanded; time consuming; necessity of cloning for identification of quasispecies	[188,189,193]
		Deep Sequencing	Identification on resistant variants predominate in the HCV population; powerful tool for obtaining more profound insight into the dynamics of variants in the HCV quasispecies	Need for in-depth knowledge to analyze the results	[112,113,195,196]

PCR: Polymerase chain reaction; HCV: Hepatitis C virus; HBV: Hepatitis B virus; RFLP: Restriction fragment-length polymorphism; RT-PCR: Reverse transcriptase-PCR; qPCR: Quantitative PCR; RFLP: Restriction fragment-length polymorphism; bDNA: Branched DNA technology.

The first generation HCV EIA, which is no longer used, was created using recombinant protein derived from the NS4 region (C100-3 the polypeptide), with a sensitivity of 70%-80% and a poor specificity. The C-100 antibodies are developed approximately 16 wk after HCV infection[57,119-122].

The second generation HCV EIA have included recombinant/synthetic antigens from non-structural NS3 and NS4 (c33c and C100-3) and core (c22-3) regions improving sensitivity to about 95% and diminishing the number of false-positive results. Anti-HCV can be detected nearly 10 wk after HCV infection[57,119,121,122].

Third generation HCV EIA was developed using recombinant antigens from the core region, NS3, NS4 and NS5 regions of the viral genome. These assays allowed anti-HCV detection nearly 4 to 6 wk after infection with specificity and sensitivity greater than 99%[57,121,123-125].

Up to now, there is no reliable and simple diagnostic marker currently available to detect early HCV infection. The avidity of an antibody may be an early and reliable marker of recent viral infection, since antibodies of low avidity are usually indicative of recent infection. Commercial immunoassays for anti-HCV antibody detection have been optimized to evaluate avidity in serum and DBS samples and "in-house" anti-HCV IgG avidity assay has been developed using seroconversion panels and serum samples from chronically infected individuals[126-129].

Serum samples are necessary to investigate the presence of anti-HCV using EIA, but some studies have been conducted using alternative fluids like saliva and DBS. Anti-HCV assays using saliva and DBS samples showed sensitivity and specificity higher than 90% and DBS samples can be stored for a period of 117 d, at room temperature. Then, these samples could be useful tool to increase the access of diagnosis in remote areas or individuals with poor venous access[130-136].

Anti-HCV detection is not useful to identify current or past infection, since this marker will be in serum for life after HCV exposure[125,137]. In this situation, HCV RNA detection is generally recommended, but the cost and availability of this assay difficult the access of this method. Assays for detection of HCV core antigen (HCV Ag) or simultaneous detection of HCV Ag and anti-HCV antibodies (HCV AgAb) were developed. HCV Ag levels seems to correlate well with HCV RNA levels indicating its potential use as an inexpensive technique for diagnosing HCV acute cases[138-141]. Brandão *et al*[133] optimized commercial methods for HCV Ag/Ab in DBS samples showing specificity of 99.71% for Monolisa™ HCV AgAb ULTRA and 95.95% for Murex HCV AgAb and sensitivity of 97.5% for both assays. Larrat *et al*[142] tested Monolisa™ HCV AgAb ULTRA along to oral mucosal transudate demonstrated sensitivity of 71.7% and specificity of 94.3%. These methods were employed among haemodialysis and homosexual individuals showing good performance to early detection of hepatitis C virus infection during window period of HCV infection[143,144].

Other immunoassays for anti-HCV detection include ECLIA, CMIA, CLIA, MEIA. In these assays, HCV antigens are presented on distinct solid phases (microwell, magnetic and paramagnetic particles) and anti-HCV at clinical sample is then identified using a conjugate antibody (anti-human IgG labeled with acridinium or horseradish peroxidase) that catalyzes the oxidation of a luminol, generating light. The system measures the light signal that is normalized in relation to cut-off value [signal/cut-off (S/CO)], or given as relative lights units[145,146]. Most of these assays used antigens from core, NS3, NS4 and NS5 of HCV. Sample volume varies from 10 to 40 microliters and reaction is executed from 18 to 58 min[147].

To confirm positive or indeterminate results by immunoassays, recombinant immunoblot assays (RIBA) can be used as complementary test, especially in low prevalence settings. This test is highly specific due to the presence of recombinant proteins and synthetic peptides of envelope, NS3, NS4, NS5 regions of HCV on a membrane strip. HCV antibodies present in clinical samples should react to these proteins leading to the

appearance of cored dots at specific antigens in the strips. Interpretation may be visual or automated and a positive result is considered when two or more bands are visualized on the nitrocellulose strip, representing a specific antigen-antibody reaction. Indeterminate result is showed when only one band is visualized and negative results are obtained when no bands are observed. The major disadvantage of RIBA is the occurrence of indeterminate results, especially in those specimens with grey-zone results in the screening assays. Currently, this assay has been substituted as a confirmatory test by widely-used molecular techniques, which can additionally distinguish between active and resolved infections[121,147,148].

Nowadays there have been emergent needs for developing highly accurate, rapid and cheap analytical tools. To achieve this goal, many attempts have been focused on the development of rapid point-of-care testing (POCT) such as lateral flow tests. These techniques are based on immunochromatographic assay, lateral flow tests (LFT) or test strips. The advantages of POCT are the time for execution, simplicity and cost-effective[149]. The principle of LFT is similar to ELISA, and the base substrate is nitrocellulose membrane in which the solid phase is immobilized capture binding protein, usually an antibody or antigen. Labels such as latex, colloidal gold, carbon, and recently up-converting phosphorus technology have been employed in LFT development[150,151]. POCT is useful for HCV detection in field situations, particularly among hard-to-reach, high-risk populations, such as drug users or individuals living in remote areas[152-154]. A good performance of POCTs compared to EIA or PCR results were observed with sensitivities and specificities above 90%[152-156]. A recent metanalysis compared seven POCT and OraQuick had the highest test sensitivity and specificity and showed better performance than a third generation enzyme immunoassay in seroconversion panels[156]. This assay could be used along to serum, whole blood and saliva samples what could increase the access of diagnosis in emergency situations[151,154].

Biosensor technology has emerged as alternative technique with low detection limit, higher selectivity and sensitivity, and faster responses for anti-HCV detection. Biosensor employs specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles, or whole cells to detect chemical compounds, usually by means of electrical, thermal, or optical signals. The surface of bioreceptor contains a biological recognition element that interacts to analyte; then the transducer converts the recognition event into a measurable signal; finally, the output from the transducer is amplified, processed and displayed. The concentration of the analyte is proportional to the intensity of signal[57]. The main advantages of biosensors are offering a quantitative test for detection in cases with about 100 copies of hepatitis virus, in addition to automation, multiplexing analysis and throughput. Some biosensors available for anti-HCV detection include surface plasmon resonance, piezoelectric biosensors, microcantilever based biosensors, electrochemical biosensor and apta-sensor[121,149].

EIA is widely used for anti-HCV detection, and has the advantage of high sensitivity, fast processing, high reliability, ease of automation and relatively low cost[57,117], but this technique did not identify if the infection is current or past, and therefore requires confirmation of the results by a more specific method, such as HCV RNA[125,137]. Other immunoassays for anti-HCV detection as MEIA, CMIA, ECLIA and CLIA have the advantage of being performed in less time, with less incubation period and easily automatable. While RIBA can be used as a complementary test to confirm positive or indeterminate by immunoassays. The advantage is that this test is highly specific, due to the presence of recombinant proteins and synthetic peptide regions of the envelope, NS3, NS4, NS5 of HCV. However, its drawback is the occurrence of indeterminate results. Therefore, this assay has been replaced by molecular testing to confirm results, so distinguishing active infection cured[48,121,147].

Every day more, is continuing need to develop highly accurate, rapid and inexpensive analytical tools. Therefore, the immunochromatographic techniques, such as rapid diagnostic tests have been developed and widely used. Present as the short runtime advantages, simplicity and low cost[149] as well as being useful for detecting HCV in difficult access populations, high risk, such as injecting drug users or people living in remote areas[152-154]. The biosensor technology has emerged as an alternative technique with low anti-HCV detection limit, higher sensitivity and selectivity, and faster for the detection of anti-HCV responses. The main advantages of biosensors are automation, multiplexing analysis and fast processing[121,149], but these assays are not widespread and present high costs. Information regarding different aspects of HCV assays is available on Table 5.

MOLECULAR TECHNIQUES FOR DIAGNOSING HCV INFECTION

In hepatitis C infection, the molecular virological techniques play a key role both in diagnosis and in monitoring of HCV antiviral treatment. Due to the difficulty to grow HCV in cellular culture, molecular techniques were used to identify this virus, making HCV one of the first pathogens to be identified solely by molecular diagnostics[8].

The hepatitis C viral genome can be detected in blood, by nucleic acid testing (NAT). The presence of HCV RNA is a marker for HCV viremia and is detected only in persons who are currently infected. Patients with anti-HCV detected should be evaluated for the presence of HCV RNA in their blood[157]. In this regard, the detection and quantification is useful in clinical practice to: (1) diagnose chronic HCV infection; (2) guide treatment decisions (identify patients who need antiviral therapy and offer them the most adapted genotype treatment);

Table 5 Main Characteristics of serological assays for hepatitis C virus diagnosis

Serological assays		Antigen (region of the genome)	Assay/manufacturer	Sensitivity	Specificity	Ref.
EIA	1st generation EIA	c100-3 (NS3-NS4)	HCV-Ac-EIE Salck	70%-80%	50%-70%	[118,119]
	2nd generation EIA	c100-3 (NS3-NS4), c33-c (NS3), c22-3 (core)	ORTHO HCV ELISA test system	92%-95%		[118,119]
	3rd generation EIA	c100-3 (NS3-NS4), c33-c (NS3), c22-3 (core), NS5	ORTHO HCV 3.0 ELISA (Ortho)/ETI-AB-HCVK Sorin	95%-99%	99%	[57,118]
	4th generation EIA		Monolisa™ HCV AgAb ULTRA (BioRad)/HCV Murex AgAb (Abbott)	100%	99.5%	[117,133,144]
MEIA		HCr43 (Fusion core e NS3), c200 (NS3 - NS4), c100-3 (NS3-NS4), NS5	AxSYM® HCV 3.0 (Abbott)	100%	99.8%	[117,118]
ECLIA		Core, NS3 and NS4 proteins	Elecsys anti-HCV assay (Roche)	100%	99.7%	[117,118]
CLIA		[c22-3 (core), c200 (NS3 - NS4) and NS5]	ARCHITECT i4000 anti-HCV assay (Abbott); VITROS Eci anti-HCV assay (Ortho)	99.5%	98.2%	[145]
CMIA		HCr43 (core - NS3), c100-3 (NS3-NS4)	ARCHITECT® anti-HCV (Abbott)	99.1%	99.6%	[146]
RIBA	RIBA-1	5-1-1 (NS4) e c100-3 (NS3-NS4)		NP	NP	[118]
	RIBA-2	5-1-1 (NS4), c100-3 (NS3-NS4), c33-c (NS3), c22-3 (core)	Chiron RIBA-2.0 RIBA-2	NP	NP	[148]
	RIBA-3	c100-3 (NS3-NS4), c33-c (NS3), c22-3 (core), NS5	Chiron RIBA HCV 3.0 SIA	NP	NP	[118]
Biosensor technology		Core protein (p22 fusion protein), NS3, NS4 and NS5	mBio Diagnostics® company	NP	NP	[57,137]

NP: Not presented; EIA: Enzyme immunoassay; MEIA: Micro-particle enzyme immunoassay; ECLIA: Electrochemiluminescence immunoassay; CLIA: Chemiluminescence immunoassay; CMIA: Chemiluminescent microparticle immunoassay; RIBA: Recombinant immunoblot assays; HCV: Hepatitis C virus; ELISA: Enzyme-linked immunosorbent assay.

(3) monitor the antiviral efficacy of treatment; (4) identify amino acid substitutions responsible for direct acting antiviral drug (DAA) resistance; (5) to confirm the presence of HCV viremia in patients who are seronegative (anti-HCV non reactive) but immunocompromised such as HIV infected individuals; and (6) in babies who are born to HCV positive mothers- once antibody testing in babies can give false positive results up to 18 mo of age[121,158].

Approximately 1 mo before the appearance of total anti-HCV antibodies HCV RNA can be already detected 1-3 wk after infection[81]. Molecular methods are useful to identify the stage of infection. A negative NAT result with positive serological test is usually indicative of a resolved infection or spontaneous resolution[159], but low-level of viremia may occur during chronic infection, for these reasons a second NAT should be performed 6 to 12 mo later. A positive HCV NAT result indicates active infection independent of antibody test results. NAT are used before, during, and after antiviral treatment to indicate whether HCV is present or not, and to determine when and whether treatment should be stopped or continued[160]. In acute infections, the NAT result will become positive within 1 to 3 wk, several weeks earlier than serological tests, as in occupational exposures[120]. This way, the serological window present in HCV infection can be resolved using qualitative and quantitative nucleic acid testing, whereas these techniques have a wide dynamic range of detection, which is well chosen according to the clinical needs (upper range of quantification: 7-8 log10 IU/mL)[161].

HCV detection and quantification is made using sera samples, but saliva, DBS and platelets have been studied as alternative samples[162-165]. The detection of HCV RNA in these samples could be useful to increase the access of molecular diagnosis for HCV infection and to evaluate antiviral response in some groups, like haemodialysis, children, drug users. A systematic review demonstrated good correlation among HCV RNA quantification from DBS and whole plasma[166], but low levels of HCV among saliva and platelets compared to sera samples[162,163].

Qualitative HCV assays comprises viral RNA isolation, complementary DNA (cDNA) production, PCR amplification and detection of PCR amplicons[121]. A large amount of commercial and in-house PCR for HCV uses primers for amplification of 5' untranslated region (UTR) since this region has above 90% of sequence identity among distinct HCV genotypes, where several fragments are almost undistinguishable among distinct strains[121]. The 5'UTR is the first region to be transcribed and has secondary and tertiary structures that are largely conserved[167]. Furthermore, core and 3'UTR are also used in PCR for detection of HCV[121].

Qualitative NAT are used as the first diagnosis of a suspected acute infection, to confirm chronic HCV infection in patients with antibodies anti-HCV positive, confirmation of virological response during, at the end or after antiviral therapy and to screen blood donations for evidence of infection with HCV[120,168]. These tests usually utilize conventional reverse transcriptase-PCR (RT-PCR) or transcription-mediated amplification (TMA).

In RT-PCR-based assays, HCV RNA is the source for production of a single-stranded complementary cDNA by reverse transcriptase. DNA polymerase amplifies cDNA into multiple double-stranded DNA copies. In TMA assay, viral RNA is isolated from clinical sample and two enzymes (T7 RNA polymerase and reverse transcriptase) amplify this RNA. Hybridization protection assay detects these amplicons by probe hybridization in which only hybridized probes stay chemiluminescent and are detected in a luminometer[168].

Qualitative RT-PCR assays should detect 50 HCV RNA IU/mL or less with the same sensitivity for all genotypes. With the advent of more qPCR that has a lower limit of detection to as low as 30 copies/mL, qualitative assays were replaced especially in diagnostic laboratories[120,169]. These qualitative tests are still very common due to its higher sensitivity, but the main inconvenience is that it only verifies the absence or presence of HCV RNA[121,168].

Some qualitative commercial assays include Cobas Amplicor HCV version 2.0 (Roche Molecular Diagnostics, Pleasonton, CA, United States) and Versant HCV RNA qualitative assay (Siemens Healthcare Diagnostics, Deerfield, IL, United States). Cobas Amplicor HCV is performed according to standard RT-PCR and 50 IU/mL is the limit of detection for all HCV genotypes, while Versant HCV qualitative assay employs TMA and presents analytical sensitivity of 10 IU/mL for most genotypes and 5.3 IU/mL for genotype 1[121].

HCV RNA quantification can be accomplished by target or signal amplification, respectively quantitative RT-PCR (qRT-PCR) and branched deoxyribonucleic acid (bDNA) technology. Due to its good sensitivity (99%) and specificity (98%-99%), the classical techniques for viral genome detection and quantification are progressively being replaced by quantitative PCR[120,169]. When these tests are used to monitor viral load during treatment, it is critical to use the same assay before and during therapy[120,169].

Several tests are commercially available for HCV quantification and those assays employ competitive PCR (Cobas Amplicor HCV Monitor), bDNA technique (Versant HCV RNA), real time PCR (COBAS TaqMan assay and Abbott Real Time HCV test). To quantify HCV by Cobas Amplicor HCV Monitor 2.0, the target and internal standard is amplified in a single reaction tube. The initial quantity of HCV RNA is obtained by comparing the final amounts of both templates. The dynamic range of the Amplicor™ HCV 2.0 monitor assay is 500 to approximately 500000 IU/mL with a specificity of almost 100%, independent of the HCV genotype[170,171]. The Versant HCV quantitative test (Siemens Healthcare Diagnostics) is based on signal amplification by bDNA and present a range of detection of 615 IU/mL to 8 million IU/mL independent of HCV genotype[172]. Martins *et al*[173] compared qualitative (in-house RT-nested PCR and COBAS AMPLICOR HCV Test v2.0 and TMA) and quantitative (COBAS AMPLICOR HCV Monitor Test v2.0 and bDNA) techniques for HCV quantification and detection, and TMA presented the highest rate (87.8%) of HCV detection among qualitative tests being the most sensitive for HCV RNA detection over the early and late phases of HCV infection.

Real time PCR technology allows the direct monitoring of the PCR process due to the detection and amplification of the target nucleic acid at the same time. In order to detect and amplify DNA at the same time, a probe (oligonucleotides containing a quenching molecule and a fluorescent reporter molecule) binds to target cDNA between the two PCR primers and are degraded or released by DNA polymerase during DNA synthesis. When probe is degraded, it occurs the separation of reporter and quencher molecules, which leads to emission of an increased fluorescence signal from the reporter. The quantity of RNA in the starting (first cycle) sample is proportional to the fluorescence signal. Quantification in absolute numbers is obtained by the comparison of kinetics of the target amplification with the amplification kinetics of an internal control of a defined initial concentration[168].

Cobas Taqman HCV present limit of detection and quantification of approximately 15 IU/mL among all HCV genotypes, and a linear amplification range of HCV RNA from approximately 15 to 10000000 IU/mL[174]. Abbott RealTime HCV assay has a lower detection limit of nearly 10 IU/mL, a specificity higher than 99.5% and a linear amplification range from 12 to 10000000 IU/mL independent of the hepatitis C genotype[175-177].

Currently, there are leastwise seven genotypes and more than 80 subtypes of HCV[178,179]. Methods for HCV genotyping are direct sequence analysis, real time PCR, RFLP, and reverse hybridization technology. HCV genotyping is mandatory for double antiviral treatment (interferon and ribavirin), since patients infected with genotypes 1 or 4 are treated for longer times than patients infected by genotypes 2 and 3[180]. Nevertheless, with the accessibility of new and highly effective antiviral therapies, HCV genotyping will not be important in the future.

The first commercial assays for HCV genotyping evaluated exclusively the 5'UTR, which has a high ratio of misclassification particularly on the subtype level. Nowadays commercial tests analyze coding regions, especially non-structural protein NS5B and core protein.

TRUGENE-SIEMENS HCV 5'NC Genotyping Kit (Siemens Medical Solutions Diagnostics, Tarrytown, NY, United States) is useful to detect HCV genotypes and subtypes based on region 5'UTR and has a lower limit of detection of 5000 IU/mL[181,182]. Versant HCV Genotype 2.0 Assay (LiPA) uses oligonucleotide probes specific for the 5'UTR and core regions of the six HCV genotypes and has lower limit of detection of 3700 IU/mL[183]. LIPA is efficient for HCV genotyping, but some divergent results were observed when compared to sequence analysis of the NS5B region at the subtype level (sensitivity of 95.2% for subtype 1b and 96.1% for subtype 1a)[184].

Real time PCR using TaqMan technology as Abbott Real time PCR HCV Assay (Abbott Diagnostics Europe,

Wiesbaden, Germany) determines the viral genotype of the samples for HCV genotypes 1a, 1b, 2a, 2b, 3, 4, 5 and 6 and has limit of detection of 6053 IU/mL[185]. On the other hand, in house methods like RFLP presented a limit of detection using the Probit test ranging from 51 to 3300 IU/mL[161]. RFLP is a useful technique for HCV genotyping in different groups since presents low cost compared to commercial methods[186,187].

The direct sequencing (Sanger sequencing) is the gold standard for determining HCV genotypes and subtypes HCV. Nucleotide sequencing involves sequencing of one or more genes in the HCV genome (mainly the 5'UTR, core, E1, NS3 and NS5) and comparing these sequences to the established genotypes by computer analysis[120]. This provides the most complete information on the variations of the sequences analyzed. Furthermore, it is the most useful method for the study of viral genetic variability[182].

The sequencing technique for HCV genotyping consists of PCR amplification of part of the viral genome, especially these regions: The 5'NC, NS5B and core regions. These regions present diversity for the discrimination of viral genotypes and subtypes and are sufficiently conserved for the development of reliable primers[188]. In addition to genotype determination, direct sequencing is also used for molecular epidemiological studies and to DAA resistance mutations[189-193]. In DAA treatment, the replication is intensively inhibited in drug-sensitive viral population, and the resistant variants gradually predominate in the HCV population[194]. This way, in a near future, the most appropriate treatment for HCV patients will be based on the analysis of the nucleotide or amino acid sequence[195].

Actually, deep sequencing technologies is a promising approach to characterizing viral diversity, they have the ability to generate high throughput screening that provide exceptional resolution for studying the underlying genetic diversity of complex viral populations[112,113,196]. Currently Illumina deep sequencing technology (Illumina Inc. San Diego, CA) and PacBio sequencing technologies (Pacific Biosciences of California, Inc.) are the newest platforms in the market. In HCV studies, deep sequencing technologies are powerful tools for obtaining more profound insight into the dynamics of variants in the HCV quasispecies of human serum. It allows sequencing the complete genome in a short time and is able to generate much more information on the viral genome sequences in internal organs[195].

Due to the rate of 10^{-3} mutations per nucleotide, HCV results in high-circulating quasispecies in infected patients. Recently, it was observed that approximately 15.6% of samples from Pakistan did not match any genotype[197]. Ultra deep sequencing could be useful for identification of these genotypes what is important to determine the pattern of double antiviral therapy (interferon and ribavirin) that is a standard therapy in Pakistan and other countries with few resources.

HCV quantitative methods, like qPCR are useful to monitor antiviral therapy and could detect low viral loads with broad dynamic range. These assays are fully automated and reduced contamination. However, commercial assays present high cost compared to in house qualitative methods. For HCV genotyping, the method most useful is nucleotide sequencing, principally ultra deep sequencing that could identify resistant variants predominate in the HCV population and give information about dynamics of HCV quasispecies. However, it is necessary in-depth knowledge to analyze the results.

The main characteristics, advantages, disadvantages and specific applications of HCV molecular assays are disclosed in Table 4.

CONCLUSION

In this review, we attempted to give information regarding HCV and HBV serological and molecular methods available at clinical and research areas. Most of review articles regarding HBV and HCV diagnosis are relatively old[28,120,148,198] or discuss only one aspect of viral hepatitis diagnosis, for example, rapid tests for HBV[24], rapid tests for HCV[156], serological methods for HBV[49], serological methods for HCV[117], molecular methods for HBV[83] or molecular methods for HCV[120]. In the present review, both serological and molecular methods for HBV and HCV diagnosis were included and new methods such as biosensors and ultra deep sequencing were discussed giving new and updated information about this theme.

Diagnosis of HBV and HCV infection is a key tool to identify acute and chronic cases of infection in order to define preventive measures and to initiate antiviral treatment. Nowadays HBV vaccination and antiviral therapies for HBV and HCV infection have arisen drug resistant, vaccine and diagnosis escape variants that complicate diagnosis and treatment. In this situation, effective diagnosis presenting high sensitivity and specificity is crucial.

Each detection method presents advantages and limitations. EIAs are the most important serological assays used for HBV and HCV detection due to its simplicity, automation and convenience. Nevertheless, they can be time-consuming and expensive and rapid assays have been developed in order to overcome these disadvantages. Molecular techniques are useful to diagnose chronic infection; to identify HBV occult cases; to evaluate the prognosis of disease; to help in treatment decisions and monitor the antiviral treatment efficacy; and to identify resistance mutants to antiviral treatment. Molecular methods present higher specificity and sensitivity and larger dynamic range of detection compared to other diagnostic assays like serological assays. Nevertheless, these methods are relatively expensive and require special instruments and specialized techniques. The choice of each method should be done according advantages and disadvantages and the purpose of diagnosis.

In the near future, biosensors and biochips seems to be useful technologies for serological diagnosis of

HBV and HCV, principally due to real-time diagnosis and early intervention to reduce the burden of diseases. Alternative specimens and rapid assays can also be extremely useful for remote areas, low resource settings and health services with limited laboratory infrastructure. In respect to molecular HBV and HCV assays, digital PCR promises to resolve some of the deficiencies of qPCR by transforming the analog, exponential nature of PCR into a digital and linear signal. For DNA sequencing, ultra deep sequencing will be helpful for analysis of HBV and HCV mutants in order to study the dynamics of viral variants.

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