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Diagnosis and monitoring of chronic viral hepatitis: serologic and molecular markers

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1. ABSTRACT

Chronic Hepatitis B (HBV) and Hepatitis C (HCV) virus infections are global health problems which may cause cirrhosis and even hepatocellular carcinoma. Hepatitis D virus (HDV) though a satellite virus of HBV, can also cause chronic infection. Serologic and molecular tools are needed for the diagnosis, monitoring and therapeutic management of chronic viral hepatitis associated with HBV, HDV and HCV. In HBV infection several serological markers are available for diagnosis and staging; while molecular assays are important for pretreatment evaluation, assessing drug response and identification of mutants. The endpoint of chronic HCV and HDV treatment is the sustained virological response, defined by an undetectable HCV/HDV RNA in serum with a sensitive assay 6 months after completion of treatment. HCV genotype and quantitative HCV RNA testing plays an important role in determining treatment duration, doses and also assess the likelihood of treatment response. Thus, virological assays are important in the diagnosis and management of individuals infected with chronic viral hepatitis.

2. INTRODUCTION

As approximately 500 million people worldwide are affected with chronic viral hepatitis (CH), accurate diagnosis is essential for its proper management (1, 2). More than 1.5 million people die from Hepatitis B Virus (HBV) and/or Hepatitis C Virus (HCV) related chronic liver diseases, such as decompensation of cirrhosis, end-stage liver diseases and hepatocellular carcinoma (HCC). One of the most common cancers is HCC, which has an estimated prevalence of >500,000 cases worldwide per year (3). The use of virological tools is important in the management of HBV/HCV infection- for diagnosis of infection, monitoring of CH patients and most importantly in the treatment decisions and in assessing the virological response to antiviral therapy. In this review, an overview of the chronic Hepatitis B and C infections and their diagnoses including serological tests, molecular diagnostic markers, and how these tests contribute to optimal management of CH is discussed. In addition chronic hepatitis D virus (HDV), although a satellite virus of HBV, can also cause CH and has been included.

3. HEPATITIS B VIRUS

A world-wide cause for concern, HBV has an estimated 400 million chronic carriers (1). It is the smallest known human enveloped DNA virus, containing a 3.2-kb, partially double-stranded, relaxed circular genome, which encodes four overlapping open reading frames (ORF). The ORF preS/S codes for envelop proteins including hepatitis B surface antigen (HBsAg), the marker for HBV infection; ORF preC/C, codes for the viral capsid component (core antigen or HBcAg) and a non-structural protein that, after posttranslational modification, is secreted and forms the "e" antigen (HBeAg); ORF P, codes for the viral polymerase (with DNA polymerase, reverse transcriptase and RNaseH activity), and ORF X, codes for a multifunctional transactivator for both the virus and the host (4). HBV is classified into eight genotypes A-H based on an inter-group divergence of 8% or more in the complete nucleotide sequence (5). HBV genotypes have distinct geographical distributions and appear to correlate with clinical outcomes of chronic HBV infection and response to treatment (6, 7).

3.1. Natural history of HBV infection

Remarkable progress has been made in our understanding of the natural history of chronic HBV infection. A dynamic interaction between viral replication and host immune response is the basis of the pathogenesis of liver disease. Knowledge of the HBV genome and replication cycle has led to the understanding of HBV genotypes and molecular variants, which contribute to the heterogeneity in outcome of chronic HBV infection. Most HBV infections are spontaneously resolved in immunocompetent adults (90-95%), whereas they become chronic in most neonates and infants (20-90%). Chronic carriers are at an enormous risk of developing complications such as liver cirrhosis and HCC (8). They may be in one of the four phases of infection: immune tolerance, immune clearance [hepatitis B e antigen (HBeAg)-positive chronic hepatitis B], inactive carrier state, and HBeAg-negative chronic hepatitis B (HBeAg-ve CHB); however, not all patients go through each of the four phases (9). Occult HBV infection (OBI) is defined as the presence of HBV DNA in serum/liver in the absence of HBsAg. Data from woodchuck model as well from human indicate that exposure to low doses of virus results in primary HBV infection, while recovery after HBV infection causes secondary occult infection as a reservoir from which full blown hepatitis can arise (10,11). However, in both the cases the virus replicates at a low level in the lymphatic system. Immunosuppression caused by chemotherapy or immunomodulatory agent, or immunodeficiency due to HIV infection or hematological malignancies can induce reactive occult infection (11, 12). This is especially important during screening of blood donors, as donated blood from individuals with occult infection can transmit HBV infection, especially in immunocompromised patients. Accumulating evidences suggest that occult HBV is associated with chronic liver disease, in patients with unexplained liver disease (hepatocellular carcinoma and cryptogenic cirrhosis), in HBV-seropositive individuals with resolved or recovered chronic infection (spontaneously or after interferon therapy), and even in HBV-seronegative patients without evidence of liver disease, such as hemodialysis patients (13,14,15,16,17,18).

Understanding the dynamic nature of chronic HBV infection is essential in the management of HBV carriers. Antiviral therapy can prevent progression of HBV-related liver diseases, particularly among patients with sustained response. In patients with higher risk factors of HCC, such as high serum DNA load, HBeAg status, serum aminotransferase, HBV genotypes, and pre-core or core promoter mutants; long-term monitoring and optimal timing of antiviral therapy will help to prevent progression of HBV-related liver diseases to later stages**.**

4. VIROLOGICAL TOOLS FOR MONITORING HBV INFECTION

4.1. HBV serological markers

The diagnosis and monitoring of HBV infections is based on the use of a variety of virological markers. Interpretation of serological tests of HBV infection is complex due to the presence of six HBV antigens and antibodies in the serum, HBsAg, anti-HBs antibodies (antiHBs), HBe antigen (HBeAg), antiHBe antibodies (antiHBe), antiHBc antibodies (including total anti-HBc antibodies and anti-HBc IgM) and HBV DNA (18). During acute infection symptoms develop at the immunoactive phase, when it can be diagnosed by testing HBsAg and antiHBc IgM. In acute infection HBsAg can appear 3-5 weeks prior to clinical symptoms and disappears 2-6 months after infection in those patients who recover from it. There is currently no role for molecular testing in the diagnosis of acute hepatitis B other than in the detection of asymptomatic patients during pre-transfusion screening of blood products. Recovery from hepatitis B is diagnosed by the presence of antiHBs and total antiHBc. AntiHBs is a neutralizing antibody and confers a long term protection against HBV infection. In addition antiHBs is the only serological marker in persons who respond successfully to HBV vaccination (Table 1).

AntiHBc is not a neutralizing antibody and is detectable throughout a patient's life even after recovery from infection. In acute hepatitis antiHBc as the only serological pattern can be found in the window period, after disappearance of HBsAg, prior to development of antiHBs, and also several years after hepatitis B infection. However, recent studies suggest that isolated antiHBc can also suggest OBI (17). Testing for OBI has also been advocated for a variety of settings, including cryptogenic liver disease, immunosuppressed patients with HBV risk factors and is generally detected by nucleic acid techniques (NAT).

However, diagnosis may be complicated by the presence of genetic variants of HBV. Some mutations in the antigenic 'a' determinant region of surface antigen have been described, which lead to failure of detection of HBsAg (19); most commonly detected mutants are G145R, and K141R. Recently commercial HBsAg detection kits have been developed that can detect even variant HBsAg.

Table 1. Serological markers in stages of HBV infection

Antigens		Antibodies against				Interpretation
s	**e**	**e**	**c IgM**	**c IgG**	**s**	
+	+	-	+	-	-	Acute hepatitis B
+	+	-	-	+	-	Persistent carrier state
-	-	+	+/-	+	+	Convalescence
-	-	-	-	+	+	Recovery
-	-	-	+	-	-	Infection with HBV without detectable HBsAg
-	-	-	-	+	-	Recovery with loss of detectable anti-HBs
-	-	-	-	-	+	Immunization without infection, or recovery from infection with loss of detectable anti-HBc

The marker for chronic hepatitis B is the presence of HBsAg in serum for more than 6 months. Presence of total antiHBc (both IgG and IgM) differentiates chronic HBV infection from acute. In chronic HBV infection, two phenotypes can be observed, namely HBeAg-positive and HBeAg-negative. HBeAg in serum is a marker of high viral replication and of the risk of HBV transmission. Loss of HBeAg in serum and emergence of antiHBe (termed HBeAg seroconversion) is usually associated with remission of liver disease with reduced HBV DNA and normalized serum aminotransferase (ALT) levels (9). However, several antiHBe positive patients continue to have active HBV replication and liver diseases associated with the development of mutation in precore/core promoter region, which stops or reduces HBeAg production. The most frequent mutation is G1896A in the precore region resulting in a stop codon in the precore sequence, which stops HBe synthesis. The second group of polymorphisms is located within the core promoter region, A1762T and G1764A, which reduces HBeAg production, still associated with severity of liver disease (20). Patients with active CHB, either HBeAg positive/HBeAg negative have high HBV DNA and increased serum aminotransferase level in the blood. The seroconversion phase may result in an HBeAg-negative chronic hepatitis B or in an inactive carrier state characterized by normal aminotransferase levels and undetectable or low-level (<2000 IU/ml) HBV DNA and persistently normal aminotransferase levels. The estimated annual incidence of spontaneous HBeAg sero-clearance in chronically infected patients is 5–15% (21).

4.2. HBV DNA detection and quantification

The presence of HBV DNA in serum/plasma is a useful marker of HBV replication. HBV DNA detection and quantification is important for assessing the state of infection, assessing the risk of progression towards cirrhosis and HCC; identifying patients who need antiviral therapy, determine response to therapy and identify emergence of drug resistant mutants, thus should be available for all chronically infected individuals (21,22). HBV DNA is detectable within a few days after infection. It generally increases to reach a peak during acute hepatitis, and then progressively decreases and disappears when the infection resolves. In the patients is progressing towards chronic HBsAg carriage, HBV DNA levels are not stable over time and depending on the infection phase, may fluctuate with time. The immunotolerance phase is characterized by high levels of HBV DNA and the immuno-elimination phase is characterized by generally lower, often fluctuating HBV DNA levels; whereas in the inactive carrier phase viral replication is at very low or undetectable level, depending on the sensitivity of the assay. Finally, the reactivation phases are generally associated with high levels of replication. Initially HBV DNA tests were solid phase hybridization based and had limited utility because of their high lower limit of detection (10^5- 10^6 copies/ml). Presently several commercial assays are available, which utilize signal or target amplification assays. In recent time, HBV DNA assays based on real-time PCR technology are available which allows a wider dynamic range of assay and are more sensitive than ordinary PCR. So real time PCR is now replacing the classical techniques in most virology laboratories. International unit (IU) for HBV DNA has been introduced which should be the unit of all assays.

4.3. HBV genotypes

In addition to classification into 8 genotypes based on 8% or more divergence of complete genome sequence, HBV is further divided into subgenotypes based on more than 4% intragenotypic divergence associated with distinct geographic and ethnic distributions. While Genotypes A and D are common in Europe, Mediterranean region, North America, sub-Saharan Africa and the Middle East, Genotypes B and C are prevalent in south-east Asia (6). Genotype E is restricted to Central and West Africa; however, an increasing trend in its prevalence is apparent in Europe. Genotype F is prevalent in Central and South America as well as Alaska and Genotype G is found in Europe and the United States. Genotype H is restricted to Central America and sometimes co-infected with genotype G. On the other hand, in countries with high migration rate, a variety of genotypes are detected, like all of the known genotypes can be found in Europe and North America. Furthermore, increased global migration is changing the pattern of genotype distribution (23). Patients with genotype A or B infection have a better response to interferon based therapy than patients with genotype C or D infection (6). Although it has limited application at present, accumulating evidence suggests that HBV genotypes influence the natural course of liver disease, progression to cirrhosis, risk of HCC and response to interferon therapy, but it does not affect nucleoside or nucleotide therapy (22,24,25).

4.4. HBV mutants

HBV mutants have been reported that have implications in diagnosis, patho-genesis and therapy of HBV infection (27). Among them polymerase gene mutants are associated with development of drug resistant mutants, and precore and core promoter mutants that abolish or reduce the production of hepatitis B e antigen can lead to

progressive liver disease. Core promoter mutants are associated with an increased risk of HCC while surface gene mutants impair diagnosis and cause active & passive immunization failure (27). Assays that detect mutants are direct sequencing, RFLP, real-time PCR with specific probe, commercial reverse hybridization assays, multiple clone sequencing of PCR amplicon etc (28). Among them direct sequencing can detect unknown mutants in addition to the known one but it cannot detect mutants that constitute less than 20% of the total viral population. RFLP and other assays can detect mutants that represent 5% of total viral population but these assays can detect only the known mutants (29).

4.5. HBV cccDNA

During HBV replication viral DNA forms covalently closed circular DNA (cccDNA) which accumulates in the episomal form in the cell nuclei and acts as a template for HBV transcription. Removal of cccDNA is a major challenge to current therapy (30). Monitoring of cccDNA needs liver biopsy, however, serum HBsAg level correlates well with intrahepatic cccDNA level, and therefore quantification of serum HBsAg level has been indicated to be a surrogate marker of intrahepatic HBV DNA. However, more detailed investigation is required.

4.6. Management of chronic HBV

Substantial advances have been made in the treatment of chronic hepatitis B in the past decade. Approved treatments for chronic hepatitis B include 2 formulations of interferon (IFN) and 5 nucleos(t)ide analogues (NAs), Lamivudine, Tenofovir, Adefovir dipivoxil, Entecavir and Telbivudine. Although NAs are more convenient than IFN-based therapies and have fewer side effects, sustained viral suppression is usually not achieved after withdrawal of a 48-week course of NA therapy necessitating long, and in many cases, indefinite treatment with increasing risk of development of drug resistance**.**

4.6.1. Using Virological tools in Management of chronic HBV

The molecular and serological markers present valuable information in monitoring of patients. To define the presence of HBV infection, serological rather than molecular assays are needed. In the absence of HBsAg, the diagnosis of HBV infection relies on the presence of HBV DNA, this is particularly important in patients with isolated antiHBc, undergoing immune-suppression with the aim of evaluating the risk of viral reactivation. Although HBsAg is the hallmark of HBV infection, HBV DNA and ALT levels provide important prognostic information. In a chronic HBV carrier, the HBV DNA level should be systematically measured by a sensitive and accurate method. The risk of HCC is significantly correlated with the level of HBV DNA above 2 X 10^3 IU/ml. The risk of HCC development is low in the absence of detectable HBV DNA, excepting in patients with cirrhosis. HBV DNA level is also critical in therapeutic decision-making.

The aim of chronic hepatitis B therapy is to abolish or at least to reduce HBV replication significantly to prevent progression of liver disease to cirrhosis and HCC. The decision to treat chronic hepatitis B is based on the assessment of multiple parameters, including clinical, biological and histological ones. In patients with chronic hepatitis B in active phase, antiviral treatment is indicated. As apparent from American Association for the Study of Liver Diseases (AASLD) guidelines, high levels of HBV DNA and immunomediated damage of hepatocytes containing HBV characterize active HBV infection, established by elevated serum ALT levels, and/or evidence of chronic hepatitis with or without cirrhosis. Among the two main forms of HBsAg positive hepatitis, the HBeAg positive form is associated with wild type infection, with serum HBV DNA levels higher than >20,000 IU/ml and elevated ALT(above the upper limit of normal), and the HBeAg negative form is associated with core promoter and/or pre-core mutant virus (26,29,30). In the later form, either HBV DNA or ALT levels fluctuate over time. For treatment decision, HBsAg positive patients should be evaluated on at least two occasions 1 to 3 months apart by the same molecular assay with a large dynamic range. HBV DNA levels >20000 IU/ml in HBeAg positive, or >2000 IU/ml, in HBeAg negative antiHBe positive patients, indicate the need of treatment (32). Although the inactive HBV carriers with normal ALT and HBV DNA levels <20000 IU/ml have developed immune response that keeps viral replication at low level, they face the risk of reactivation as they still have cccDNA, the replication template in their hepatocytes. They need monitoring by testing of ALT/AST and HBV DNA quantification every 6 months, which is useful for detecting an increase in viral replication and eventually for reconsidering the treatment option.

Once therapy is initiated careful monitoring is needed to assess the response to therapy and the chances of development of drug resistance. In some HBeAg-positive patients, HBeAg clearance followed by seroconversion to antiHBe results with short-term therapy and ensures long-term control of viral replication. In HBeAg-negative patients, long-term antiviral suppression of viral replication is needed. The most desirable endpoint of therapy is seroconversion of HBsAg to antiHBs, which is rarely achieved. During treatment of HBV lower baseline serum viral load (<$5x10^7$ copies/ml) has been found to be associated with better virological response in HBeAg positive patients during interferon treatment (32). For treatment with nucleoside/nucleotide analogue early virological response has been shown to be an important predictor for sustained virological response and for reducing drug resistance (32).

Monitoring of treatment is based on HBV DNA quantifications and ALT determinations every 3–6 months, irrespective of HBeAg serostatus and antiviral treatment (33). Ideally, in HBeAg seroconverters, HBV DNA should be undetectable (lower limit of detection of the order 10–15 IU/ml), and ALT level should be normal (32). If this does not happen, the patient may have developed HBeAg negative CB and may need continued therapy (33). If the HBV DNA level remains detectable after 48 weeks therapy,

a second antiviral with no cross-resistance with the first one should be added.

Reactivation of disease can occur after 3-6 months of withdrawal of therapy in some cases, especially in HBeAg negative CB. HBV DNA and ALT monitoring would recognize such hepatic flares and therapy should be restarted.

Patients with past HBV infection where both antiHBc and antiHBs are detectable do not need continued monitoring. Reactivation has been described in this setting such as with chemotherapy, immunosuppressive agent and HIV infection.

In the majority of occult HBV infections, antiHBc is the only detectable serological marker. Its clinical significance is not yet established, so data to support monitoring is lacking. However, these patients should be monitored like inactive carriers, as some data reported reactivation of the disease in immunosuppressed condition.

Due to similar route of transmission HBV coinfection is common among HIV infected patients, and occult HBV infection is usually found in these cases. As HIV coinfection increases risk of cirrhosis and liver related mortality, assessment for possible liver disease is very important (38).

4.6.2. Drug resistance in HBV

Treatment of patients with CB requires long-term therapy with oral nucleoside/nucleotide analogues; however, all oral agents currently approved for the treatment of such patients are associated with some risk for development of drug resistance. This can lead to a rebound in HBV levels and, eventually, progressive liver disease. Development of resistance is usually followed by elevation of ALT/AST. In patients who have responded and have been compliant to therapy, resistance should be suspected if there is ≥1 Log10 IU/ml above nadir increase in HBV DNA in two consecutive tests conducted at 1 month interval (33, 34, 35). Amino acid substitutions known to be associated with resistance can be identified by laboratory testing. Assays are based on direct sequencing of HBV pol gene region or line probe assays. Line probe assays can only identify known mutations but, direct sequencing can also identify those mutations that are not yet recognized. The advantage of line probe is that the resistance can be identified even when in minority (36, 37). A mutational analysis can be useful to differentiate between development of resistance and non compliance to therapy as well as be useful prior to beginning of therapy to determine whether mutations to the drug of choice may occur. Patients on therapy should be monitored for HCC development every 6 months (35). HIV HBV co-infected patients often get treatment with lamivudine for HIV, so they might be already resistant to it (39). This will increase risk of developing resistance to other nucleoside/nucleotide analogues, so monitoring should be done every three months in HIV co-infected patients.

5. HEPATITIS C VIRUS

Chronic HCV infection and its consequences are estimated to be attributable to $2.5x10^5$ to $3.5x\ 10^5$ deaths annually. The worldwide reservoir of chronically infected persons is estimated at 170 million (40). More than 50% individuals at highest risk for HCV become infected but it remains undetected, leading to spread of the infection (41). HCV is an enveloped RNA virus with a genome of approximately 9500 nucleotides, belonging to the family *Flaviviridae,* genus *Hepacivirus*. The single positive-stranded RNA genome contains an ORF that encodes a non-functional polyprotein of approximately 3000 amino acids in length (42). This non-functional polyprotein is cleaved and after co- and post-translational modification by cellular and viral proteases yields a number of structural and non-structural proteins (43). HCV strains are classified into six major genotypes and a large number of subtypes; with distinct geographical distributions (43). HCV was the first pathogenic human virus identified purely by molecular methods. As it is difficult to culture the virus, molecular virological techniques play a key role in diagnosis and monitoring of treatment, which is based on the use of virological markers, including total anti-HCV antibodies, HCV RNA and HCV genotype (43).

5.1. Natural history of HCV infection

Most patients develop asymptomatic infection but will often have high-level viremia and elevated ALT levels in the acute infection period. HCV RNA can be detected in circulation, 1 to 3 weeks after infection, approximately 1 month before the appearance of antibodies. Among the individuals initially exposed to HCV, 15% to 40% generally clear the infection within 6 months (44, 45). The remaining 85% to 60% of patients with detectable HCV RNA for more than 6 months will progress to chronic HCV infection, and are at risk for the development of extrahepatic manifestations, compensated and decompensated cirrhosis, and HCC (46). However, some chronically infected patients have persistently normal ALT (47). Thus, in addition to ALT levels and a positive HCV serology, detection of HCV RNA is essential for establishing chronic HCV infection.

6. VIROLOGIC TOOLS FOR MONITORING CHRONIC HCV INFECTION

6.1. HCV serological markers

At present, 3rd generation Enzyme immunoassays (EIAs) that detect antibodies directed against various HCV epitopes, are the most common method for detection of anti-HCV antibodies. Recombinant antigens are used to capture circulating antibodies in the wells of microtitre plates, microbeads etc. The captured antibodies are identified by anti-antibodies labelled with an enzyme that catalyses the reaction, which turns the substrate into a coloured compound. Presence of antigens or antibodies in the sample is detected from the optical density (OD) ratio of the reaction (sample OD/internal control OD). Although this ELISA is significantly sensitive, a major drawback of this assay is that it fails to differentiate between past and present infection. Anti-HCV IgM has been reported in 50–93% of patients with acute hepatitis C and 50–70% of patients with chronic hepatitis C (48). Therefore, antiHCV IgM is not a reliable marker for acute infection. However, serial measurements of the anti-HCV IgM titres based on at

least three determinations from the 5th to the 15th day from the onset of the symptoms have been reported to identify patients with acute hepatitis C (49).

HCV core antigen assay detects HCV infection. Evaluations in transfusion settings showed that it detects HCV infection between 40 and 50 days earlier than the third generation HCV antibody screening assays (50,51), similar to NAT. HCV core antigen levels closely track HCV RNA dynamics, and allow clinical monitoring of a patient's therapy, independent of HCV genotype; however, it is mainly in the samples with HCV RNA levels above 20,000 IU/ml. The lower sensitivity of HCV core antigen detection in comparison to NAT, makes the HCV core antigen assay not a practical tool for the determination of the end of treatment response and sustained viral response. But it could be useful for the determination of early viral response in the pegylated interferon-alpha and ribavirin treated patients infected with HCV genotype 1, which has 100% negative predictive value in contrast to 80–100% negative predictive value of EVR determined by HCV RNA quantification (52). The HCV core antigen can be used as a marker of HCV replication in anti-HCV positive individuals in resource poor settings when NAT is too expensive and/or in the settings that are not equipped to perform HCV RNA testing (53).

6.2. HCV RNA detection and quantification

Detection of viral RNA in peripheral blood is the hallmark of HCV diagnosis both in HCV antibody positive and in HCV antibody negative patients with unexplained ALT elevations. HCV RNA can be detected a week after contaminated blood transfusion or experimental infection of chimpanzees (54). Qualitative nucleic acid detection assays are used to confirm viremia (especially low-level viremia). In addition, HCV RNA detection is indicated in acute hepatitis before seroconversion; seronegative patients with immune deficiency; indeterminate serological test results; monitoring patients under therapy and investigation of HCV infection of new born.

The presence of HCV RNA can be detected by nucleic acid tests using a combination of amplification and detection techniques. Qualitative detection assays are based on the principle of target amplification using either “classic” polymerase chain reaction (PCR) or “transcription-mediated amplification” (TMA) (55). Extracted HCV RNA is reverse transcribed into a single-stranded complementary DNA (cDNA), which is subsequently amplified by polymerase chain reaction which generates a large number of copies that can be readily detected. Double-stranded DNA copies of HCV genome are synthesized in PCR-based assays, whereas single-stranded RNA copies are generated in TMA. Hybridization of the amplified products onto specific probes after the reaction in “classic” PCR or TMA techniques detects the product (55). Sensitivity of the test used is important while evaluating the result. Qualitative detection assays must detect all HCV genotypes with equal sensitivity and have lower detection limit of 50 HCV RNA IU/mL or less. One of the most important problems is false positivity due to contamination, especially due to carry-over of amplicon. Accumulation of experience and knowledge in laboratories and standardization of tests have increased the number of laboratories with accurate results in recent multi-center studies (over 90%) (55, 56). HCV RNA assays based on real-time PCR are now used in clinical virology laboratories for RNA detection and quantification.

Quantification of viral genome in serum or plasma is a valuable tool to assess prognosis of chronic HCV infection, to identify individuals who need antiviral therapy and monitor response to therapy. Quantitative reverse transcription–PCR, real-time PCR, and bDNA are the commercially available assays which are widely used for evaluation of HCV viral load (57). In “real-time” PCR, each round of amplification leads to the emission of a fluorescent signal and the number of signals per cycle is proportional to the amount of HCV RNA in the starting sample (58,59,60). The bDNA method differs from reverse transcription–PCR tests in that the detection signal is amplified rather than target RNA. HCV RNA levels can be precisely quantified by Real-time PCR over a linear range (10 IU/mL to 100 million IU/mL). The assay is faster and more cost-effective than the other techniques. Thus, HCV RNA assays based on real-time PCR has already replaced other testing methods in many clinical laboratories.

HCV RNA quantification techniques often use different units. World Health Organization international standard has now been introduced to make results of different assays interconvertible. The international unit has been defined such as 800 000 IU/mL corresponds to 2 million copies/mL. Nonetheless, there are differences in calibration of the assays relative to the primary WHO HCV RNA standard, which lead to slight differences between the results given in the same samples by different assays in spite of the use of international units. Therefore, use of the same assay throughout the treatment course of any given patient is recommended (61).

6.3. HCV genotypes

Based on less than 72% identity at the nucleotide level HCV is classified into 6 major genotypes, (numbered 1 through 6), and within these genotypes, multiple subtypes with 75% to 86% nucleotide identities may occur. HCV genotypes are associated with different susceptibility levels to interferon (IFN) based therapies (62) and help in deciding the duration of therapy, as well as determination of the dosage of ribavirin. Patients infected with HCV genotype 1 have a much lower response rate (40% to 50%) than patients with genotype 2 or 3 (75% to 80%). Furthermore, for genotypes 2 and 3, rates of sustained virological response were equivalent with 6 months versus 12 months of therapy (63). Therefore, patients genotype testing is done before initiating therapy.

The reference method for HCV genotype determination is direct sequencing of the NS5B or E1 regions of HCV genome by means of “in-house” techniques, followed by sequence alignment with prototype sequences and phylogenetic analysis (64). However, it only identifies viral variants representing at least 20–25% of the circulating viral populations. Reverse hybridization of PCR amplicons to membrane-bound probes is more sensitive than direct sequence analysis to detect minor variants

representing as few as 5% of the entire viral population (65). Several commercial tests are available for assigning HCV genotype. Most assays target the highly conserved 5′ noncoding region (5′NCR) of the HCV genome, but spurious mutations within the 5′NCR result in misclassification of HCV genotypes in 10% to 25% of cases. As genotype is used for therapeutic decision-making, the difference has significant implications for treatment decisions and outcomes.

The HCV genotype can also be determined by commercial ELISA using antibodies directed to genotype-specific HCV epitopes (66). This assay allows identification of the six HCV genotypes (2) but does not discriminate among the subtypes.

Besides genotype and subtype another level of variability, which is found within a given HCV infected patient, is termed as the quasispecies population. Quasispecies are populations of different but closely related genomes found within individual patients that differ from each other by a few percent at the nucleotide level. These variants are in competition with each other, and the most fit one or a few sequences are dominant at any given time (67,68). Data have suggested that Interferon alpha exerts a selective pressure on HCV quasispecies (68,69). Thus, these molecular polymorphisms are clinically relevant and are one of the major factors in determining the outcome of the Interferon therapy.

NAT has been introduced to blood transfusion centers to shorten the preseroconversion window by 59 days down to 10–30 days and reduce virus transmission (70). Nucleic acid testing of blood donations for HCV RNA dramatically reduced the incidence of posttransfusion hepatitis C, with the risk of HCV acquisition dropping from 1 per 276 000 donations to 1 per 2 million donations in USA.

Plasma or serum pool samples are screened by NAT which can be performed with several amplification techniques such as RT-PCR, NASBA, TMA, and ligase chain reaction. A multiplex TMA test for screening of HCV and HIV in pooled plasma samples has also been developed (70).

Several recent studies have challenged the conclusion that HCV is truly eradicated after a documented sustained virological response. HCV RNA have been detected in serum, peripheral blood mononuclear cells, in liver biopsy specimens after either spontaneous or treatment-induced resolution leading to speculation of a new clinical entity designated as occult hepatitis C. In general, detectable HCV replication in the absence of antibodies to HCV is exceptional with current anti-HCV EIAs. However, it is almost exclusively observed in profoundly immunosuppressed patients such as those who are HIV-positive and on haemodialysis (71,72).

6.4. Management of chronic HCV

At present the standard treatment for chronic hepatitis C is the combination of pegylated interferon (IFN) alfa and ribavirin (44). Critical clinical decisions on the start of the therapy depend on the availability of information provided by the virological tools in addition to other factors like severity of liver disease, contraindication to therapy etc. Patients with low HCV RNA levels had a 15% to 39% better response rate than those with high HCV RNA levels (a high viral load is considered greater than 800 000 IU/mL and a low viral load is defined as less than 800 000 IU/mL(56). Hepatitis C virus genotype should be systematically determined before treatment, as it determines the indication, the duration of treatment, the dose of ribavirin and the virological monitoring procedure (44). Genotype 2- and 3-infected patients require 24 weeks of treatment and a low dose of ribavirin. In contrast, genotypes 1-, 4-, 5- and 6-infected patients require 48 weeks of treatment and a higher, body weight-based dose of ribavirin, (44).

6.4.1. Using Virological Tools in Management of Chronic HCV

Serological and molecular tools are needed to diagnose chronic HCV infections, guiding treatment decisions and assessing the virological responses to therapy.

To diagnose HCV infection, anti-HCV, HCV RNA and serum ALT values are commonly used, but up to 40% of viraemic patients can have a normal aminotransferase value on a single determination. Therefore, persistence of HCV RNA for more than 6 months defines chronic HCV infection. In patients with clinical and/or biological signs of chronic liver disease, chronic hepatitis C is diagnosed by the simultaneous presence of antibodies to HCV and HCV RNA.

Monitoring of HCV RNA levels is recommended to tailor treatment to the actual virological response with a sensitive assay, for which a real-time PCR assay is ideal. The endpoint of hepatitis C treatment is the “sustained virological response” (SVR), defined as testing negative for HCV RNA 6 months after cessation of therapy and is the gold standard for treatment response (44). Monitoring changes in HCV viral load after 4 and 12 weeks of therapy predicts the likelihood of sustained virological response (44, 45). Patients infected with genotype 1 HCV, whose HCV RNA levels have not declined by at least 2 logs after 12 weeks of therapy, have virtually no possibility of achieving an SVR and so, ending of therapy should be considered. If, a 2 Log10 drop in HCV RNA level has been observed at week 12, treatment should be continued. Thus, the treatment should continue until week 48 if HCV RNA is undetectable and until week 72 if HCV RNA is still detectable at week 12 (44).

In patients who have no indication for therapy, virological tests cannot be used to predict the natural course of infection or the onset of extrahepatic manifestations. The same type of quantitative HCV RNA test should be used throughout a patient’s treatment course.

6.4.2. Drug resistance in HCV

About 60% of patients with chronic hepatitis C do not clear HCV infection with the currently approved standard treatment with pegylated alpha interferon (IFN-alpha) in combination with the nucleoside analogue ribavirin, and they are considered “resistant” to therapy (63). IFN-alpha is classified as an indirect treatment, as it interacts with the host's immune response. However, the two drugs currently used in the treatment of HCV, interferon alfa (IFN-a) and ribavirin, have complex and

indirect actions, making the mechanisms of HCV resistance generally different from those of viral resistance to specific antiviral agents. Molecular mechanisms of resistance to IFN-based therapy have been proposed on the basis of *in vitro* experiments but have not been validated in patients, in whom treatment failure appears to be multifactorial (69). *In vitro*, several mechanisms to circumvent the host immune defense or to block treatment-induced antiviral activities have been described for different HCV proteins. By the introduction of direct antiviral drugs, hepatitis C therapy now is entering a new era in which the development of resistance may become the most important parameter for treatment success or failure.

7. HEPATITIS D VIRUS

Hepatitis D, caused by Hepatitis delta virus, was discovered in patients infected with HBV, responsible for some 20 million infections globally. HDV is one of the most unusual pathogens in nature, as it can only infect hepatocytes in the presence of a helper virus. HDV and HBV coinfection can be associated with complex and dynamic viral dominance patterns.

7.1. Overview of Hepatitis D Virus

HDV is a defective, negative strand RNA virus. HDV requires the coexistence of HBV to supply envelope proteins for its assembly into mature virions and is hence, a defective virus and is known as a satellite virus of HBV (73,74). In other aspects of replication, HDV is both independent of and very different from HBV. Distribution of hepatitis D virus (HDV) is very uneven, and the prevalence of HDV infection does not follow that of hepatitis B virus (HBV). The relatively low prevalence (<5%) of HDV infection in HBV carriers in the Far East and in homosexual men is unexplained and there is still a poor understanding of the host and viral factors that determine the transmission of HDV (75). However, transmission of HDV can occur either via simultaneous infection with HBV (coinfection) or via infection of an individual previously infected with HBV (superinfection). About 5% of the 350 million HBV carriers are co infected or superinfected with HDV (76) HDV is spread in the same way as HBV, mainly through parenteral exposure. The virus is highly endemic in Mediterranean countries, the Middle East, Central Africa, and northern parts of South America (77). In western countries, there is a high prevalence of HDV infection in intravenous drug addicts. (78).

The small single-stranded circular RNA genome of HDV, and its mechanism of replication, demonstrates an increasing number of similarities to the viroids - a large family of helper-independent subviral agents that cause pathogenesis in plants. Although HBV and other hepadnaviruses are found in a number of mammals, HDV has thus far been found only in humans and thus HDV is the only prototype of the *deltaviridae* family. It is the smallest RNA virus identified to date, contains only one ORF, which is a 1.7 kb closed circular RNA molecule capable of internal hybridization, self cleavage and self ligation (73,74). Extensive intramolecular base-pairing of the single-stranded RNA makes the rod-like genome structure (79). HDV uses the host DNA-dependent RNA polymerases to facilitate the replication of its genome and antigenome (80) through a double-rolling circle mechanism [81]. HDAg is the only protein encoded by HDV. It is a nuclear phosphoprotein that exists in two forms. A 24 k-Da small delta antigen, S-HDAg, required for replication and a 27 k-Da L-HDAg, required for virion assembly and inhibits replication. L-HDAg has additional 19 amino acids at the C terminus (73,74). In addition to the coding sequence, the HDV genome contains a viroid-like sequence in both the genome and the antigenome. A ribozyme, a self-cleaving RNA sequence, resides in the viroid-like sequence of the HDV genome that cleaves a linear form of multiple-copy length of the viral genome (75) or antigenome into monomeric units that then circularized to complete the replication cycle.

At least eight different HDV genotypes have been described and each has a characteristic geographic distribution and a distinct clinical course. HDV and HBV coinfection can be associated with complex and dynamic viral dominance patterns (77).

7.2. Management of hepatitis D

Currently, interferon-α (IFNα) is the only recommended treatment for HDV infection in humans and has been linked to improved long term outcomes. Treatment with PEG-IFN-α leads to HDV clearance in about 25% of patients as apparent from recent studies showing sustained virologic response to therapy, measured in terms of undetectable serum HDV RNA levels. Several nucleoside and nucleotide analogues used for the treatment of HBV infection are shown to be ineffective against HDV. Novel alternative treatment options are awaiting clinical development for use in hepatitis D (81)

7.2.1. Virological tools for monitoring chronic HDV infection

The traditional methods for the diagnosis of HDV infection, such as detection of serum anti-HDV antibody, IgM anti-HDV testing, are sufficient for the clinical diagnosis of delta infection (75). However, such techniques lack the sensitivity and specificity required to more accurately characterize the nature of HDV infection and to assess the efficacy of therapies. A positive result for the presence of anti-HDV antibodies, however, does not necessarily indicate active hepatitis D, HDV RNA can disappear indicating recovery from HDV infection. However, anti-HDV antibodies may persist for years, even when the patient has experienced HBsAg seroconversion or has undergone liver transplantation (82).

Recent improvements in molecular techniques, such as HDV RNA hybridization and RT-PCR, have provided increased diagnostic precision and a more thorough understanding of the natural course of HDV infection. However, there is no evidence that serum HDV RNA load correlate with any clinical marker of activity or stage of liver disease (83). These advances have enhanced the clinician's ability to accurately evaluate the stage of HDV infection, response to therapy, and occurrence of reinfection after orthotopic liver transplant (77).

8. PERSPECTIVES

Monitoring of the antiviral response is largely done by measuring the presence and levels of virus using molecular diagnostics. Several serological markers are accepted as important tool for diagnosis and staging of HBV infection. Once HBV is diagnosed, assessment of the liver damage and quantification of HBV DNA is useful to predict disease progression, drug response and development of resistance. While utility of quantification of HBsAg and HBeAg, cccDNA pool, markers for genotypic resistance to antivirals, needs further investigations. The diagnosis and management of hepatitis C depend on accurate molecular diagnostic tests. The best possible use of molecular testing across all drug regimens, including several new antiviral agents presently under evaluation in clinical trials has to be established. The adoption of new technologies and the identification of new virological or host markers could potentially provide opportunities for growth and evolution of molecular testing in chronic hepatitis. Treatment options of HDV, which produce most severe form of viral hepatitis, are limited and assessed by serum HDV RNA level. Thus Important emerging issues include the identification, utility and best use of virological tools in the management of new HBV, HDV and HCV therapies. Reducing cost of these tests should be of priority. Microchip technology promises tests capable of multiplex amplification in minute volumes for screening, quantification, genotype determination, and detection of drug sensitivity. Use of nanobiotechnologies will extend the limits of current molecular diagnostics and enable point-of care diagnostics as well as allow integration of diagnostics with therapeutics, and development of personalized medicine

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