Diagnostic Modalities of Hepatitis C Infection

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Abstract

Hepatitis c virus (HCV) is one of the most prevalent blood-borne human pathogens. About 3% of the total world population are HCV infected. According to World Health Organization (WHO), annually there are about 3-4 million new cases of infection. HCV as an extremely prevalent cause of chronic hepatitis which is in turn an underlying condition preceding the progress to cirrhosis and hepatocellular carcinoma (HCC). The aim of HCV diagnosis is ultimately prevention of further viral transmission through identification of infected cases who can furtherly share in spreading viral infection. The usual diagnostic scheme of viral infections includes clinical markers, biochemical markers and virological markers. Most of acute HCV infections are not presented with clinical symptoms and the acute infectious period passes asymptomatic, so clinical markers cannot be relied upon for HCV diagnosis. Regarding biochemical markers which can be reflected as the changes of levels of liver enzymes, mainly the alanine aminotransferase enzyme (ALT), it cannot be relied upon as well, as in many cases of HCV infection ALT level remain normal in the same time when HCV RNA can be detected, which is a reflection of HCV viremia and active infection. The most reliable in diagnosis is the use of virological markers which can be detected by two general methodologies, direct and indirect methods. Indirect methods are the tests where anti HCV antibodies are detected, where IgM indicates current infection and IgG indicates current or past infection. Whereas direct methods are the detection of the virus itself by isolation, detection of the viral immunological fingerprint, namely viral antigens, and the detection of viral molecular fingerprint, namely viral RNA.

Keywords: Hepatitis C, Diagnosis

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Introduction:

Hepatitis c virus (HCV) is one of the most prevalent blood-borne human pathogens. About 3% of the total world population are HCV infected. According to World Health Organization (WHO), annually there are about 3-4 million new cases of infection. HCV as an extremely prevalent cause of chronic hepatitis which is in turn an underlying condition preceding the progress to cirrhosis and hepatocellular carcinoma (HCC). **(1).**

The main route of HCV transmission in western hemisphere developed countries is intravenous drug abuse, while infection control breeching invasive procedures and poorly decontaminated items are the main route of transmission in developing countries. HCV has been classified into seven genotypes (gt 1-7) and numerous subtypes, whose prevalence and rate of infection vary geographically among different countries. For example, North America, Western Europe and North and Australia's have the lowest HCV prevalence, while Asian and African countries have significantly higher infection prevalence. Among these countries Egypt recorded the highest virus prevalence, where reports state that about 22% of Egyptian population are HCV infected. Parenteral-antibilharzial treatment campaigns before 1985 were most incriminated as an iatrogenic route of transmission.HCV was first identified as a non-A non-B hepatitis virus in 1989. HCV was placed as a member of Flaviviridae virus family, Hepacivirus genus together with the GBV-B virus, formerly named as hepatitis G virus which is a known rodent and bat hepatitis virus **(2).**

Viral structure

HCV particles are 56-65 nm in diameter **containing** a single stranded positive polarity RNA genome surrounded by an icosahedral protective shell named nucleocapsid. The viral core, which is about 45 nm in diameter, is outlayered by viral envelope glycoprotein spikes which are about 6 nm. Spikes are formed of heterodimers of E1 and E2 glycoproteins **(3).**

After a person gets HCV infection and passes through the initial infectious stage which could commonly be asymptomatic, the infected patient progresses into one of two fates, either spontaneous clearance of the virus or chronicity. Many viral and host factors determine which outcome would be reached. Viral particles are continuously produced in a high rate of 10^{10} to 10^{12} copies per day. HCV infection not only favors persistence by the high viral load production, but also during the viral replication process -like other RNA viruses- which lack the proof reading character yielding multiple genetic variants adding more to the immune pressure **(4).**

Role of Immune Response in Outcome of Early Infection:

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Host Factors Associated with Viral Clearance:

The exact nature of spontaneous viral clearance in some individuals and not the others is only partly understood. Some of the reported factors associated with clearance are listed below:

1- Younger Age:

It is noticed that the chronicity rate of HCV infection acquired in children is lower than that of the older age groups. Only 55% out of 67 children included in a study, developed chronicity. All of the included cases acquired HCV through blood transfusion. Comparably, when 919 HCV infected patients -with ages starting from 17 years and older- were prospectively evaluated for clinical progress, a significant association was found between spontaneous viral clearance and patient age below 45 years (6).

2- Female Gender:

Female patients who acquired HCV from contaminated anti-D immunoglobulin, were retrospectively assessed for the chronicity rate. Where the study stated that only 55% of more than 704 females went through chronicity. Which is near to the lowest range of chronicity rates reported in the general population. Furthermore, other prospective studies that included acutely infected HCV patients who were evaluated for clinical progress, reported that the female gender is significantly associated with spontaneous viral clearance (5).

3- Race:

In the "NHANES" study, the general chronicity rate was estimated to be 74%. This rate was yet higher up to 98% when the results were restricted to the group of African American males. Similarly, a prospective study that included 1,667 intravenous drug users reported a chronicity rate of 91% in African American patients compared to a chronicity rate of 64% in other races (6).

4- Symptomatic Acute Infection:

Many studies reported that HCV infected patients that passed through a symptomatic acute phase, principally patients who manifested with jaundice, had significantly lower chronicity rates than those who went through an asymptomatic initial infection phase. This could be explained by the fact that a symptomatic acute infection is mainly due to a stronger immune reaction which may favor clearance of infection (7).

5- Absence of HIV Coinfection:

Intravenous drug users included in a prospective study were tested for virological status. This study reported that patients who were co-infected with HCV and HIV had a higher percentage of chronicity than those who were infected with HCV only. Early HCV infected patients were

included in another study which evaluated viral interactions. In this study it was reported that HIV coinfection directly caused weaker T lymphocyte response against HCV and as a result a higher chronicity rate of HCV up to 95%. Not just HIV coinfection can cause higher chronicity rates of HCV, but also HIV related CD4 count can have a role. A prospective study that evaluated viral clearance versus chronicity among 9191 patients stated that a lower CD4 counts were associated with higher chronicity rates and lower probability of HCV clearance **(8).**

6- IL28B CC Genotype:

One of the reported gene polymorphisms associated with HCV clearance is the IL28B gene single nucleotide polymorphism (SNP) "rs12979860". HCV infected patients who carry the CC allele of IL28B gene would be expected to achieve HCV spontaneous clearance more than other patients carrying the CT or TT alleles. For assessing this correlation, 1,008 HCV infected patients were enrolled in a study, where 53% of the patients carrying the CC allele of IL28B gene achieved spontaneous viral clearance, while only 23% of patients carrying the TT allele could do **(9).**

Variable Outcomes of Chronic Infection

Average data suggest that after the HCV infected patient reaches the state of chronic infection, liver pathology follows a relatively slow course over the first two decades. Over the third and fourth decades of infection, liver pathology critically deteriorates following the full development of cirrhosis reaching the decompensated liver state and development of hepatocellular carcinoma. About 20% to 30% of chronic HCV patients reach the cirrhotic liver state. After developing cirrhosis, the cirrhotic patient will have a 1-4% estimated risk per year of HCC development, and about 2-5% estimated risk per year of reaching end stage liver disease (ESLD) **(10).**

The development of liver fibrosis and cirrhosis is the starting step of deteriorating liver pathology. The estimated risk of progression to cirrhosis per year differs from one patient to another, for example, it's much higher with patients who acquired the infection in a relatively older age. Moreover, as the duration of infection increases, the annual risk of developing cirrhosis increases as well, but the association between both variables is not linear. Many factors can determine the risk of cirrhosis development. However, there are no prognostic criteria that could be applied in the early infectious phase to predict the clinical outcome **(11).**

Diagnosis of HCV infection

The aim of HCV diagnosis is ultimately prevention of further viral transmission through identification of infected cases who can furtherly share in spreading viral infection. The usual diagnostic scheme of viral infections includes clinical markers, biochemical markers and virological markers. Most of acute HCV infections are not presented with clinical symptoms and the acute infectious period passes asymptomatic, so clinical markers cannot be relied upon for HCV diagnosis. Regarding biochemical markers which can be reflected as the changes of levels of liver

enzymes, mainly the alanine aminotransferase enzyme (ALT), it cannot be relied upon as well, as in many cases of HCV infection ALT level remain normal in the same time when HCV RNA can be detected, which is a reflection of HCV viremia and active infection **(12)**.

The most reliable in diagnosis is the use of virological markers which can be detected by two general methodologies, direct and indirect methods. Indirect methods are the tests where anti HCV antibodies are detected, where IgM indicates current infection and IgG indicates current or past infection. Whereas direct methods are the detection of the virus itself by isolation, detection of the viral immunological fingerprint, namely viral antigens, and the detection of viral molecular fingerprint, namely viral RNA. Viral cultivation and isolation is not a simple methodology that could be used in diagnosis. Moreover, the mere reliance on IgM detection as an indicator of acute infection is not practical, as it was found that IgM can be detected in both acute and chronic HCV cases with prevalence of 50-93% and 50-70% respectively. Instead, total anti-HCV antibodies are detected in the tests used **(13)**.

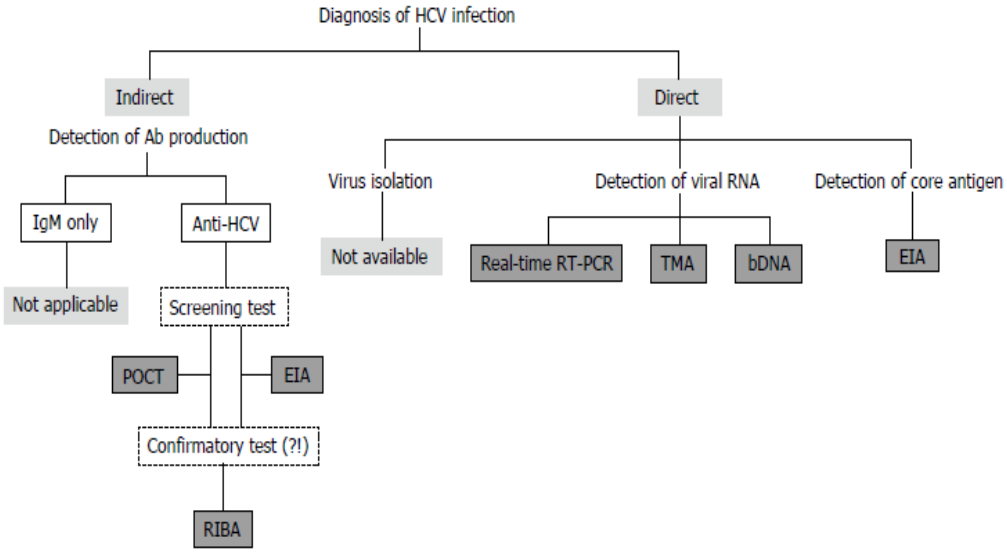

Figure (1): HCV diagnosis methods (14).

Abbreviations:

POCT: point of care tests

EIA: enzyme assay

RIBA: recombinant immunoblot assay

RT-PCR: reverse transcriptase polymerase chain reaction

TMA: transcription mediated amplification

bDNA: branched DNA

Indirect tests: Anti-HCV antibody detection:

A- Screening tests

1- Enzyme immune assay (EIA):

The commonly used EIA is the 3rd generation EIA which uses several viral antigens for capturing anti-HCV antibodies, antigens used include, viral core conserved antigen, non-structural proteins, namely, NS3, NS4 and NS5. An automated version of this test is available that helps large scale testing to be less labor intensive. This test is a reliable screening test with a sensitivity of 98.9% and a 100% specificity. Limitations to the use of this test includes, reactivity with maternal antibodies which makes it unsuitable for use in infants less than 18 months of age **(15)**.

Another limitation is the window phase between the initial infection and the detectability of antibodies in EIA, which is reported to be about 40 days. This window phase could be overcome with the development of 4th generation EIA **(www.microgenbioproducts.com)** which detects antibodies much earlier than 3rd generation EIA. In the 4th generation EIA, capture antigens include, 2 different epitopes derived from viral core protein, nonstructural proteins namely, NS3, NS4A, NS4B, as well as the NS5A regions **(16)**.

2- Point of care tests:

These are screening tests used without the need to laboratory skills and specially in resource limited localities. These tests show high sensitivities and specificities. The currently used test depends on the principal of lateral flow assay. OraQuick test **(OraSure Technologies, Bethlehem, PA)** was approved by the Food and Drug Administration (FDA) in 2010 for rapid detection of anti-HCV antibodies in blood obtained by fingerstick or any other method. The capture antigens are recombinant core protein, NS3 and NS4 fixed to a nitrocellulose membrane, the labeling substance is protein A with colloidal gold. Positive results appear within 20 to 40 minutes in the form of 2 colored bands in the areas of test and control **(17)**.

B- Confirmatory tests:

Patients with positive results on screening tests are confirmed by recombinant immunoblot assays (RIBA). This test has excellent specificity; it detects antibodies to different HCV antigens as separate bands on the test strip. For accommodating large scale of testing, this test principal was developed in an automated version the INNO-LIA™ HCV Score **(Fujirebio Europe, previously Innogenetics)**. In this test, capture antigens include, recombinant E2 hypervariable region peptide, NS3, NS4A, NS4B and NS5A. Recently due to the high sensitivity and specificity of EIAs, RIBA is not used as a confirmatory test anymore, instead confirmation is done by HCV RNA detection **(12)**.

Direct tests:

A- Detection of viral RNA:

Presence of a detectable HCV RNA in serum is an indicator of viremia. This detection could be reported just qualitatively or could be quantified according to WHO standardization in terms of HCV RNA international units. Nucleic acid amplification tests (NATs) include three categories according to the methodology used, target amplification, signal amplification and probe amplification. Two of them are mainly used in practice for HCV diagnosis, first of them is the target amplification, target amplification category includes two different methodologies, the thermal cyclic methodology or reverse transcriptase PCR (RT-PCR), and the isothermal

methodology or transcription mediated amplification (TMA). The second category is the signal amplification used in branched DNA (bDNA) methodology **(18)**.

1- Qualitative HCV RNA detection:

This includes the previously mentioned methodologies RT-PCR and TMA. Both methodologies start by extracting viral RNA and its processing by reverse transcriptase enzyme to transform it into "complementary DNA (cDNA)". The cDNA is then introduced into the thermal cyclic enzymatic processing for amplification in RT-PCR methodology, in this methodology the amplicon consists of amplified number of double stranded DNA. In case of isothermal amplification in TMA, the amplicon consists of single stranded RNA. The amplicons could be tracked in the post amplification phase by using labelled probes complementary to 5'UTR region which is conserved among all HCV genotypes **(19)**.

2- Quantitative HCV RNA detection:

Quantification could be achieved throughout the process of amplification, either target amplification in real time RT-PCR or TMA, or throughout the amplification of signal in bDNA. The most commonly used method is the real time RT-PCR **(15)**.

In the present time, real time PCR technique is not only used to quantitatively detect the presence of viral RNA for the purpose of diagnosis, but it's also used in the monitoring phase throughout the treatment process to achieve the target "sustained virological response (SVR)" **(20)**.

B- Detection of viral core antigen:

This is a method that could be used as a cheap alternative of NATs that require highly skilled personnel and costly laboratory technicalities. The principal of using this method developed after finding that the quantitative level of viral core antigen in terms of fmol/L could be easily correlated with standardized IUs of viral RNA **(12)**.

Chemiluminescent assay is used to detect the viral core antigen with the advantage of being automated to adapt for large scale detection **(Abbott Laboratories)**. The test is 100% specific and a sensitivity level up to 3 fmol/L of the core antigen which is equivalent to 1000 IU/mL of HCV RNA **(12)**.

When comparing core antigen lower detection limit that is equivalent to 1000 IU/mL of HCV RNA, the lower detection limit of quantitative RNA tests is down to 5-15 IU/mL. Putting into consideration that 90% of positive cases when quantified with NATs usually show RNA level of 10,000 IU/mL or more which is included in the detection limits of viral core antigen tests **(21)**.

Core antigen detection could sensitively detect positive cases and therefore, could replace NATs when the patient is expected to show positivity regarding viral RNA, this is applicable in confirmation after a positive antibody test by EIA. Depending on these data, commercially available coupled tests have been developed that could detect both viral antibodies and viral core antigen simultaneously. On the other hand, the detection limit of core antigen does not make it a suitable alternative to NATs in monitoring SVR following treatment. It also cannot be used as a screening method for donated blood **(22)**

Genotyping:

Viral genotyping is not only done for epidemiological and research purposes, but also after the era of DAAs, viral genotype became an important parameter in the choice of antiviral treatment plan **(15)**.

Serological genotyping:

Different HCV genotypes is represented by the presence of different epitopes specific to each genotype. This could be commercially used in the setup of a serological method for genotyping. These specific epitopes could be used as capture antigens to detect the presence of complementary antibodies in competitive EIA **(23)**.

The commercially available genotyping kit "Murex HCV serotyping 1-6 HC02" **(Abbott Laboratories, North Chicago, Illinois)** can detect the main 6 genotypes, but cannot detect the subtypes. It's suitable for use with immunocompetent individuals only **(24)**.

Molecular genotyping:

Genome sequencing is the most reliable method for genotyping, but it's confined to certain laboratories. The most genotype related genomic parts used as targets for sequencing are, core, E1 and NS5B regions. An alternative method used for molecular typing starts with the traditional RT-PCR, then the amplicon is targeted for the process of reverse hybridization to labeled genotype specific probes fixed to a nitrocellulose membrane. Then the bands representing the hybridization pattern are read either by visualization or by the help of a scanner. The genomic area targeted for this genotyping technique is the 5'UTR. Assay is called "Linear Array HCV Genotyping Test **(Roche Molecular Systems)**" **(25)**.

Beside Roche's test, Siemens developed another reverse hybridization based methodology that not only targets 5'UTR, but also targets the core region, "The Versant HCV genotype 2.0 assay" **(Siemens)**, whose results go beyond genotyping to subtyping, as it can differentiate HCV genotype 1 into 1a and 1b. Another technology developed by Abbott, is the "RealTime HCV Genotype II **(Abbott Molecular)**". Unlike the two previously mentioned systems, Abbott's system is a single step system that does not depend on conventional RT-PCR followed by reverse hybridization. Instead, its genotyping methodology includes real time RT-PCR that can perform the genotyping internally parallel to amplification by using probes specific to different HCV genotypes and subtypes. In this technique laboratory workers do not need to handle the amplicon and re-process it in the process of reverse hybridization, this decreases the risk of external contamination **(26)**.

Subtyping

The purpose behind performing subtyping is mainly epidemiological. It was not necessary for setting treatment plan in the era of ribavirin/interferon α. However, it may be rational to perform subtyping in the DAAs era, based on variation of response to different DAAs among different HCV subtypes **(27).**

Conclusion

We concluded that inherited thrombophilia is considered a cause of RPL in unexplained cases and we recommend testing for inherited thrombophilia in women with recurrent miscarriages especially those history of venous thromboembolism and performing of clinical trials regarding the effectiveness of anticoagulants in treatment of women with recurrent miscarriages and inherited thrombophilia.

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