

# EMERGING MOLECULAR APPROACHES AND THEIR SIGNIFICANCE IN THE DIAGNOSIS AND MANAGEMENT OF HEPATITIS C VIRUS INFECTION

Sumera Naz<sup>1</sup>, Attya Bhatti<sup>1</sup>, Mohammad Shoaib Khan<sup>2</sup>

<sup>1</sup>PMRC Central Research Center, National Institute of Health, Islamabad and

<sup>2</sup>Department of Biochemistry Bannu Medical College, Bannu, Pakistan

## ABSTRACT

Molecular biology based assays are invaluable tools for the diagnosis and management of chronic viral hepatitis. They can be used to test donor's blood, to diagnose active infection, guide treatment decisions, assess the virological response to therapy and establish the prognosis. For hepatitis C infection, these include serological assays for anti-HCV antibody detection and molecular assays that detect and quantify HCV-RNA and determine the HCV genotype.

This article reviews the current molecular biology based techniques and assays and their practical use in the management of hepatitis C virus infection.

**Key words:** Hepatitis C, Molecular Biology Assay, Genotype.

## INTRODUCTION

Infection with hepatitis C virus (HCV) has been identified as the major cause of post-transfusion non-A, non-B hepatitis.<sup>1</sup> It has reached epidemic proportions. Worldwide, more than one million new cases of HCV infection are reported annually.<sup>2</sup> In Pakistan we have an enormous share of this chronic illness. The exact sero-prevalence rate in Pakistan is not known, however in various studies it has been reported to be 3-7%.<sup>3</sup> Khan et al (2003) reported 40.8% positive for anti HCV antibodies among chronic liver disease patients in Hazara district of Pakistan.<sup>4</sup> In another study 4.51% prevalence of anti HCV antibody among out door patients was reported in DHQ hospital Buner, NWFP, Pakistan.<sup>5</sup>

It is the major cause of chronic liver disease (CLD). The early diagnosis of HCV infection is crucial to prevent further transmission in high-risk groups and to allow clinicians to make a rapid decision about treatment, which has been proven to have a high degree of efficacy for acute hepatitis C.

HCV has a linear genome approximately 10 kb in length consisting of positive sense single-stranded RNA (ssRNA).<sup>6</sup> Consistent with related members of the family *Flaviviridae*, HCV demonstrates a high degree of sequence variation throughout its genome; sequence analysis of multiple strains of HCV has demonstrated that the nucleotide sequence can differ by as much as 30%.

In Pakistan, some studies have confirmed that the most prevalent genotype is 3 which is most responsive to Interferon based regimes.<sup>7</sup>

Diagnosis of acute hepatitis C largely relies on classical serological methods. However, the value of such antibody detection assays is limited during the early stages of HCV infection, first, because specific antibodies develop slowly and second, because immunocompromised patients may fail to develop a strong and rapid specific immune response against HCV.

## LABORATORY ASSAYS FOR THE DIAGNOSIS AND MANAGEMENT OF HCV INFECTION

These include:

1. Serological tests to detect HCV antibodies.
2. Molecular tests to detect and quantify HCV RNA.
3. Genotyping techniques.<sup>8</sup>

### 1. SEROLOGICAL ASSAYS

- A. Detection of anti-HCV antibodies by Enzyme linked Immunosorbent Assay (ELISA)
- B. Recombinant Immunoblot assay (RIBA)

### A. Detection of anti-HCV antibody by ELISA

The detection of antibodies to HCV (anti-HCV) has become the most practical means of diagnosing both past and present infection; three gen-

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erations of sero-diagnostic anti-HCV antigen tests have been developed, with each new generation providing incremental improvements in the sensitivity.

The first-generation anti-HCV test that was commercially available and widely used was an ELISA that incorporated the c100-3 epitope from the nonstructural NS4 region.

The third-generation includes antigens coded by the putative core and NS3, NS4, and NS5 regions of the HCV genome. ELISA-3 is widely used to screen donor blood and despite its high specificity (99.7%), false-positive results may occur.<sup>9</sup> The average period for HCV sero-conversion after blood transfusion has been shortened with each new generation as well; 7 to 8 weeks for ELISA-3 compared with 10 weeks for ELISA-2 and 16 weeks for ELISA-1.<sup>10</sup>

Automated systems can improve workflow in the clinical laboratory such as the AxSYM (Abbott) and PRISM (Abbott)

#### **Disadvantages of anti-HCV Antibody detection by ELISA**

1. The interval between HCV infection and detection of anti-HCV may be as long as 3 months and up to 6 months in some cases, long after serum aminotransferase levels have peaked.
2. Poor sensitivity in immunocompromised patients like post liver-transplant patients and HIV positive patients.
3. An abundance of false-positives in low-prevalence populations like blood donors.<sup>11</sup>

#### **Advantages of anti-HCV Antibody detection by ELISA**

1. Ease of automation
2. Relative cost-effectiveness
3. Low variability
4. High sensitivity in screening
5. The accuracy of third-generation test is very good in high-prevalence populations. Therefore, supplemental anti-HCV tests may not be necessary in high-risk patients with a positive anti-HCV ELISA screen.<sup>12</sup>

#### **B. Recombinant Immunoblot Assay (RIBA)**

False-positive results following ELISA testing continue to be observed among low-risk blood donors. Elevated aminotransferase levels along with high-risk factors are suggestive of active infection and hepatitis, yet additional testing for antibody specificity may be required to confirm

HCV infection. A number of confirmatory and/or supplemental serodiagnostic tests are available to verify seropositive results obtained with ELISA screening tests. An HCV diagnosis can be confirmed with Recombinant immunoblot assays (RIBA). Although technically more demanding than ELISA, the RIBA identifies antibodies to individual HCV antigens and therefore has a higher specificity than ELISA.<sup>13</sup>

Recombinant immunoblot assays (RIBA) and synthetic peptide assays have been developed as supplemental tests for discriminating between true- and false- positive results for samples repeatedly reactive by ELISAs.<sup>14</sup> The second- and third-generation RIBA HCV strip immunoblot assays (SIAs) (HCV 2.0 and 3.0 SIAs, respectively) are established methods for supplemental testing of samples repeatedly reactive by HCV ELISAs. The second-generation RIBA, or RIBA-2, uses the same recombinant antigens as the ELISA-2.<sup>15</sup> Since 1992, the third-generation RIBA system (RIBA HCV 3.0 SIA) has been widely used in Europe for supplementary testing of samples.

#### **Disadvantages of anti-HCV Antibody detection by RIBA**

1. RIBAs are technically more demanding than ELISA
2. RIBA positivity is not always a true indicator of active infection by HCV because recovered patients may stay anti-HCV positive for years.

#### **Advantages of anti-HCV Antibody detection by RIBA**

1. RIBAs are simpler
2. More standardized
3. More reproducible than tests for HCV RNA, such as the branched chain DNA assay

#### **2. HCV RNA DETECTION AND QUANTIFICATION**

The discovery of the HCV genome was accomplished just a decade ago. HCV was initially recognized as non-A, non-B hepatitis (NANB) in 1974 until cloning of the etiologic agent in 1989.

Although the diagnosis of HCV is currently based on the detection of antibodies via ELISA, the technique is less sensitive in the early phases of HCV infection and cannot differentiate between active infection and disease resolution. In addition, hemodialysis patients or immunocompromised patients, such as those who are infected with HIV, produce fewer antibodies.<sup>16</sup> The presence of HCV RNA in the plasma defines active infection and it can be detected 1 to 3 weeks post-expo-

sure.<sup>17</sup> A single negative HCV RNA assay result does not exclude the possibility of active infection with a transient drop in the level of viremia below the assays limit for detection.

### **Techniques for HCV RNA detection**

#### **Qualitative Assays**

- A. Reverse transcriptase PCR (RT-PCR)
- B. Transcription mediated amplification (TMA)

#### **Quantitative Assays**

- A. Branched DNA (bDNA)
- B. Quantitative PCR or Real Time PCR

Qualitative assays should be used to confirm viremia and assess the therapeutic response until quantitative assays with comparable sensitivities are available.

#### **Qualitative Assays**

##### **A. Reverse transcriptase PCR**

HCV RNA is extracted and reverse transcribed into a double stranded complementary DNA (cDNA), which is subsequently processed into a cyclic enzymatic reaction leading to the generation of a large number of detectable copies. Double-stranded DNA copies of HCV genome are synthesized in PCR-based assays.<sup>18</sup>

The direct molecular qualitative detection of HCV RNA by reverse transcription (RT) and PCR are considered the gold standard for the diagnosis of HCV infection and for assessing the antiviral response to INF therapy. PCR-based assays are able to ascertain minute amounts of HCV RNA in serum or plasma. HCV RNA detection by PCR helps to resolve weakly positive or negative ELISA results when clinical signs and/or risk factors are compatible with HCV infection. Laboratory detects HCV RNA with commercially available kits. Reverse transcriptase PCR is a target amplification method and has lower limit of detection of 50IU/ml.<sup>19</sup>

A reliable, standardized assay for HCV RNA may convey to clinicians whether a patient will likely respond to therapy and whether a virologic response has occurred; it also may improve our understanding of the relationship between viral load and the natural history of chronic HCV infection. Late serum clearance of HCV RNA was associated with a sustained virologic response, a phenomenon not typically observed in patients treated with alpha interferon monotherapy.<sup>20</sup>

##### **B. Transcription mediated amplification (TMA)**

Another qualitative methodology that may have practical application in chronic hepatitis C involves TMA. TMA is a target amplification method

and is able to detect very low levels of HCV RNA (5IU/ml) that are undetectable with RT-PCR systems.<sup>21</sup>

The method is simple, rapid, and sensitive (<50 copies/ml) for all of the major HCV genotypes. Single-stranded RNA copies are generated in TMA. Detection of amplified products is achieved by hybridizing the produced amplicons onto specific probes after the reaction in "classic" PCR or TMA techniques.<sup>22</sup>

#### **Quantitative Assays**

##### **A. Branched DNA (bDNA)**

The branched chain DNA assay (bDNA), a signal amplification technique, is highly standardized. bDNA method of RNA detection uses solid phase oligonucleotide probes that capture target RNA, followed by hybridization of branched secondary probe. The bDNA amplifier's bind to enzyme conjugated tertiary probes and after substrate is added the chemiluminescence produced is proportional to the amount of target RNA. The second generation bDNA provides a modest increase in sensitivity compared to the previous generation and minimal bias in measuring HCV RNA levels for the major HCV genotypes.<sup>23</sup> Direct detection of as few as 1,000 hepatitis viral genomes is possible.

The clinical value of bDNA assay has been the object of several performance studies. Jacob et al concluded that this method can be used to detect HCV RNA in patients who are infected with the genotypes that are most commonly encountered and offer advantages when attempting to quantify high-level viremia.<sup>24</sup>

##### **B. Quantitative PCR**

Quantitative assessment of HCV RNA levels, via signal amplification and quantitative PCR (Q-PCR), are valuable tools in the clinical management of patients before, during and after therapy.<sup>25</sup> The most promising approach is fully automated real-time PCR assays, which are faster, more sensitive than classical target amplification techniques and are not prone to carryover contamination.

A reliable, standardized assay for HCV RNA can convey:

1. Level of HCV replication
2. Whether a patient will likely respond to INF therapy
3. Whether a virological response has occurred or not
4. Promote a better comprehension of the relationship between viral load and the natural history of chronic HCV infection.<sup>26</sup>

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### 3. GENOTYPING

HCV is a single-stranded RNA virus of about 9,500 bp, which is assumed to cause chronic hepatitis in more than 90% of HCV-infected individuals. It has been classified into at least six major genotypes and a number of more closely related subtypes.<sup>27</sup> A consensus nomenclature for HCV genotypes has been proposed, in which the six main HCV types identified so far are numbered in the order of their discovery, i.e. 1 to 6. Within each type, subtypes have been identified by lower-case letters, which are also given in order of discovery.<sup>28</sup> The term genotype is used generically to refer to subtypes, types or both. The subtype of the infecting HCV strain seems to influence the clinical course of the infection as well as the outcome of therapy with alpha interferon.

Genotyping studies are significant for:

1. The determination of the presence or absence of HCV infection.
2. The viral transmission studies.
3. Epidemiological investigations.
4. Evaluating the progression of disease and the likelihood of developing hepatocellular carcinoma.
5. Determination of associations between viral genotype and interferon responsiveness.

**Quasispecies:** Within any given individual, HCV exists as a heterogeneous mixture of closely related viruses called quasispecies. In contrast to HCV genotypes, which vary by 31-35% of bases over the entire length of the genome, quasispecies vary from each other by 1-9% of bases.<sup>29</sup> The quasispecies nature of HCV has several potentially significant biological consequences. They are likely an important factor in the inability of acutely infected individuals to clear infection. Additionally, mutations in the viral populations likely contribute to drug "resistance" during INF treatment and to the ineffectiveness of isolate-specific vaccines.

Use of the term genotype to describe quasispecies variants is not appropriate.

#### Genotyping techniques

A. Molecular biology based techniques

B. Serology based techniques

#### A. Molecular biology based techniques

Several different parts of the HCV genome, namely, the 5' non-coding region,<sup>30</sup> the core region, the envelope region, the NS-3 region, the

NS-4 region, and the NS-5 region<sup>31</sup> have been shown to be appropriate for use in the grouping of HCV isolates into different types by nucleotide sequencing. Tests like PCR with genotype-specific primers, restriction fragment length polymorphism assay hybridization techniques have been developed for determination of HCV genotypes.<sup>32</sup>

Molecular biology-based genotyping methods have been widely used for research purposes. However, they are time consuming and expensive, require specific molecular biology equipment and experience, and are not adapted to large-scale HCV genotype determination.

#### B. Serological determination of the HCV genotype

The HCV genotype can be determined by seeking for antibodies directed to genotype-specific HCV epitopes with a competitive EIA. The currently available assays identify the type (1 to 6) discriminate among the subtypes and provides interpretable results in approximately 90% of chronically infected immunocompetent patients.<sup>33</sup> Mixed serological reactivities can be observed that could be related to mixed infection although cross-reactivity or recovery from one genotype infection and persistence of viremia with another genotype cannot be ruled out.

### CONCLUSION

The clinical detection of HCV infection has evolved rapidly over the last 10 years, and today many of the latest diagnostic tools, including quantification of HCV-RNA levels, may prove invaluable in guiding therapeutic intervention and/or therapy customized for individual patients.

Quantification of HCV-RNA levels, determination of HCV genotypes, and measurement of HCV quasispecies have been employed to characterize the natural history of HCV disease, and have opened up new areas for active investigation.

Increasing the precision of HCV diagnosis may have important implications in optimizing therapeutic intervention and in providing patients with realistic expectation about their prognoses. Detecting and quantifying quasispecies can account for why a particular patient is unable to clear an HCV infection or has become interferon-resistant as well as explain why an isolate-specific vaccine is not effective. The genetic, geographic, and etiologic variations in HCV are observable via genotyping and serotyping. Genotyping also has important implications in the design of hepatitis-related vaccines and biotherapeutic agents.

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**Address for Correspondance:**

Sumera Abid  
Medical Technologist  
PMRC, Public Health Division  
Islamabad, Pakistan